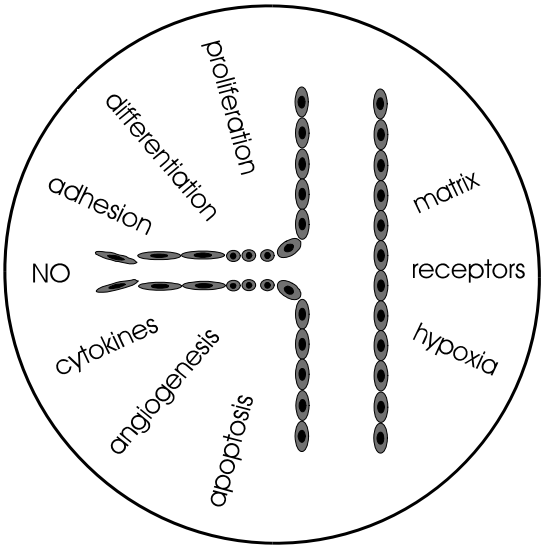
CONCLUDING REPORT
(Abschlussbericht)

for the DFG Priority Research Program 1069

Angiogenesis
Molecular Mechanisms and Functional Interactions

1999 – 2006

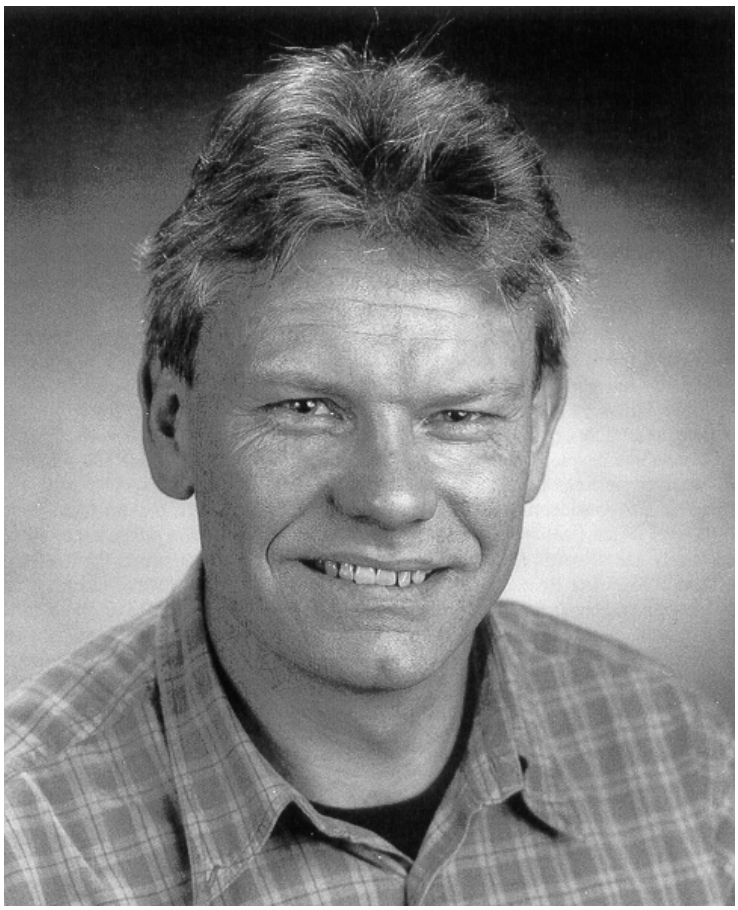


submitted by

Hellmut G. Augustin
Coordinator of the SPP1069

Dedicated to the memory of

Werner Risau



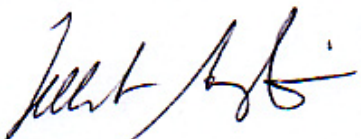
1953 – 1998

TABLE OF CONTENTS

Preface

The Schwerpunktprogramm “Angiogenese” (nationwide priority research program “Angiogenesis”) (SPP1069) has been funded by the Deutsche Forschungsgemeinschaft (German Research Council) (DFG) for a six year period from 1999 to 2005. The DFG has invested almost 10 million Euros to fund between 18 and 24 research groups throughout the 3 consecutive 2 year funding periods. Ten million Euros may not be much for a nationwide core grant for a country of more than 80 million people. Yet, it is a significant investment. This Abschlussbericht (Concluding Report) summaries the activities within the SPP1069 and formally concludes the SPP. It therefore needs to answer the critical question, if the DFG funds have been used wisely and efficiently. As such, this report will to some extend unavoidably have the character of an apple and pear counting expedition, i.e., how many papers have been published?; what is the percentage of high impact factor publications?; how many Diploma theses, Doctoral theses, and Habilitations have been written and successfully defended?; what has happened to the scientists within the SPP?; and so on. This is unavoidable as it is part of the usual routine of evaluation. Nevertheless, despite all of this formal and somewhat statistical approach to scientific assessment, the concluding evaluation essentially boils down to one key critical question: “*Was the money used to do good science?*” I think so. Or, I should say more carefully, I hope so. This is my summarizing conclusion not after counting papers and impact factors, but an assessment based on an intimate knowledge of all the people involved, be it the PIs, the postdocs, and the graduate students. All of them have with great passion, diligence, and unparalleled motivation worked towards the goals of the SPP. I have had the privilege of working with all of them as Coordinator of the SPP1069. I have taken this job over as an honorable duty after Werner Risau had asked me to do so in September 1998. Werner had taken the initiative to establish an SPP “Angiogenesis” in 1997. This first attempt failed and he tried again in 1998. The concept had just been approved by the DFG in Spring of 1998 when he learnt of his disease that led to his tragic and untimely death in December 1998. Werner had been Max-Planck-Director in Bad Nauheim for a couple of years. Beyond the scientific activities of his own lab, he had grown into national and European prominence in the field of vascular biology as an integrating personality who could bring people together. He clearly had a visionary mind recognizing early the emergence of vascular biology as a developing interdisciplinary biomedical discipline. He may have been among the first ones who early on realized the key critical role of VEGF in initiating the angiogenic cascade. Surely, the SPP1069 did not compensate for the loss of Werner Risau. Yet, The SPP1069 may have helped to keep Werner’s legacy alive. I sincerely hope that Werner would have liked what we have aimed for and accomplished within the SPP1069 in the last six years. This report is therefore dedicated to the memory of Werner Risau.

Freiburg, July 2006



(Hellmut Augustin)

Acknowledgements

A number of individuals and organizations have contributed to support the SPP1069. Sincere thanks are extended to all of them, be their support in manpower, money, intellectual support, or personal (morale, occasional comforting, mental support).

Thanks go to the Deutsche Forschungsgemeinschaft and their governing bodies for their decision to implement and support the SPP1069. Clearly, given the immaturity of the field in Germany in 1997 / 1998, the original decision to establish the SPP1069 was courageous. The PIs of the SPP1069 sincerely hope that they have lived up to the expectations of those originally in support of this decision. Particular thanks go to the Program Director of the DFG in charge of the SPP1069, Dr. Petra Hintze. Despite a busy schedule, Dr. Hintze always had open ears for the problems of the Coordinator and the PIs. It clearly is to a great extend her merit that the administrative aspects of the SPP1069 were always dealt with properly, professionally, and the least amount of bureaucracy. Thanks also go to Dr. Armin Krawisch who has helped in the DFG administration of the SPP1069 in the first funding period.

Secondly, the Reviewers of the SPP1069 (Table 1) deserve particular thanks for their input into the SPP1069. Reviewing is oftentimes a time consuming job with little reward. Unavoidably, Reviewers also need to make unpopular and negative recommendations. Nevertheless, an impressive faculty of some of the best European angiogenesis researchers has invested significant time and intellectual energy to serve on the SPP1069 Board of Reviewers. Over the years, the Board of Reviewers may to some extend even have evolved into some kind of Scientific Advisory Board rather than a boundary-forming evaluation committee. Clearly, the diligent contributions of the Reviewers have greatly contributed to the success of the SPP1069 for which the Coordinator and the PIs are very grateful.

Thirdly, thanks are extended to the financial support of the corporate sponsors of the Seeon Meeting (Table 4) as well as the Max-Planck-Society. A scientific meeting of the scope and format of the Seeon meetings can not be realized without significant financial contribution from private sources. The organizers of the Seeon meetings are thankful for this support and sincerely hope that the corporate sponsors will continue to be loyal supporters of future Seeon meetings.

Lastly and likely most importantly, warm-hearted thanks go to the secretaries of the SPP1069, Stephan Müller-Groh (now: Dr. med and Dr. jur) and Manuela Fellmann. Stephan Müller-Groh has as a student aide supported the Coordinator during the first funding period in all administrative and organizational aspects of the SPP1069. He has done pioneering work by organizing the first Seeon meeting. Manuela Fellmann, a certified foreign language correspondent, has supported the SPP1069 as professional secretary throughout the second and third funding period. Her service for the SPP1069 and the angiogenesis community at large went far beyond secretarial duties growing into a full blown, efficient, self-made scientific administrator. She has worked hard, oftentimes late hours beyond her formal duties. She has (mostly) had endless patience for the Coordinator and other not quite so simple to handle personalities she had the privilege and duty to interact with. The sincere personal thanks of the Coordinator go to Manuela Fellmann for five years of joyful cooperation and assistance under oftentimes not trivial circumstances.

Introduction

Starting situation, necessity for the establishment of an SPP, approval of the concept, and establishment of the SPP1069

Angiogenesis research has come a long way: From the concepts of modern angiogenesis research in the early nineteen seventies through the characterization of pleiotropic growth factors and the identification of the specific inducers of angiogenesis, the first anti-angiogenic tumor drug has received clinical approval in 2004. In fact, it has just taken 15 years from the identification of VEGF to the clinical implementation of a specific VEGF-neutralizing therapy which has to be considered as a major success story. Yet, this was also a bumpy road characterized by doubts about the angiogenesis-dependency of tumor growth in the early days and unrealistic hype just a couple of years ago (Jim Watson, 1998: "*...Judah [Folkman] is going to cure cancer in two years...*") which was followed by sobering results of the first phase III clinical trials. Clearly, anti-angiogenesis is not the magic cure to cancer. Yet, it is today firmly established that anti-angiogenic therapy will become part of standard tumor (and other) therapies. With the first drug having received clinical approval, rapid progress can be expected to develop reliable diagnostic and prognostic tools and techniques to identify those patients that will benefit most from an anti-angiogenic therapy. This work and the molecular identification of the mechanisms that limit current anti-angiogenic regimen will also guide the rational development of multi-targeted second generation angiogenesis-modulating compounds. At the same time, intense transcriptomic and proteomic efforts are under way, to identify and validate additional key players of the angiogenic cascade.

The rapid pace of basic and translational angiogenesis research becomes also evident when scanning Pubmed for publications that contain the term "*angiogenesis*". That number has passed the 10.000 mark in early 2001. Today, a simple Pubmed search identifies more than 25.000 angiogenesis-related publications indicating that the number of angiogenesis-related publications has more than doubled in less than 5 years (Fig. 1).

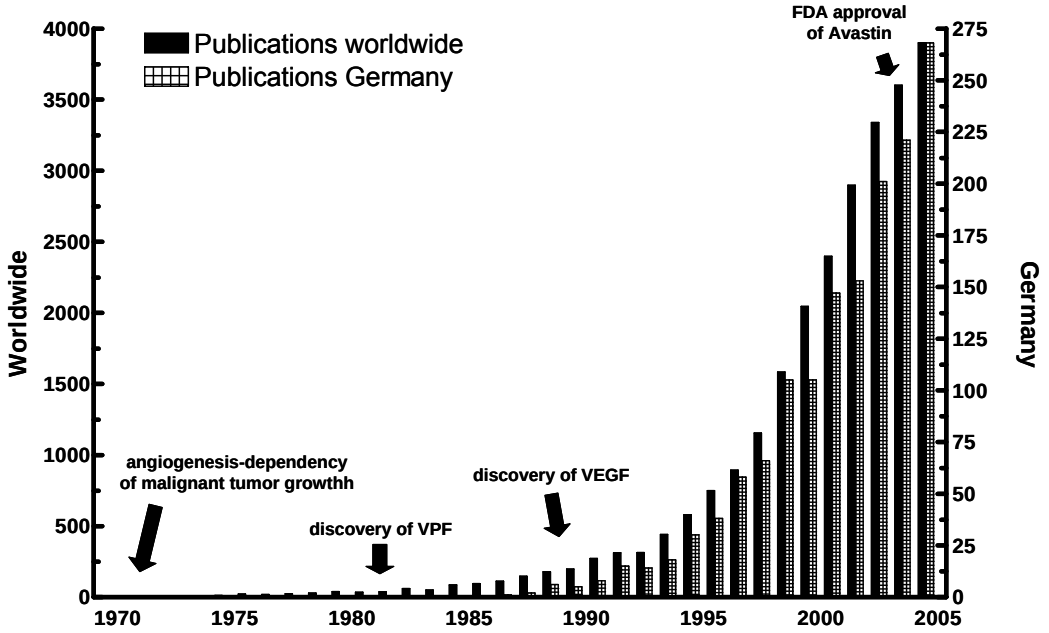


Fig. 1: Number of angiogenesis-related publications listed in PubMed. Shown is the annual number of publications worldwide (black bars, left) and publications from Germany (stripped bars, right) that contain the word "angiogenesis" in the title or the abstract. Key discoveries in the field of angiogenesis research are marked.

In view of the increasing importance of the field of angiogenesis research, the German Research Council (DFG) has launched a nationwide angiogenesis priority research grant in 1998 funding a proposal that was originally submitted by Werner Risau in 1997. In principle, the DFG sponsors two different types of priority research grants: Local priority grants (Sonderforschungsbereich, SFB) and nationwide priority grants (Schwerpunktprogramm, SPP) with SFBs being the preferred program of funding. There had been several attempts to establish local angiogenesis-related priority research programs throughout the nineteen nineties. Yet, it soon became evident that no single university within Germany would be able to gather sufficient quantitative and qualitative critical mass to successfully launch an angiogenesis SFB. It was therefore Werner Risau's vision that a nationwide program would be more suitable and appropriate to coordinate and foster angiogenesis research in Germany.

A first draft proposal for the establishment of an angiogenesis SPP was submitted to the DFG in 1997 with Werner Risau (Bad Nauheim) as Coordinator and Hellmut Augustin (Göttingen), Dieter Marmé (Freiburg), and Axel Ullrich (Munich) as members of the program committee. This proposal was not recommended for funding, but the applicants were encouraged to submit a revised proposal addressing specific Reviewers' comments and criticism. This was done in early 1998. This revised proposal was recommended for funding in a highly competitive selection process (15 positively reviewed grants out of 70 proposals). Following the approval of the SPP1069 concept, a nationwide call asked for the submission of individual grants. A total of 72 individual grants were submitted and reviewed by an international board of reviewers (Table 1). The reviewers recommended 24 grants for funding. These 24 projects inaugurated the SPP1069 in October 1998 (Fig. 2, Table 2).

Table 1: International Board of Reviewers

Reviewer, City	Reviewer for funding period
Christer Betsholtz, Gotemborg/Stockholm	2 + 3
Gerhard Christofori, Basel	2 + 3
Elisabetta Dejana, Milan	2
Michael Detmar, Boston/Zürich	1 - 2
Detlef Drenckhahn, Würzburg	1
Carl Hendrik Heldin, Uppsala	1 - 3
Victor van Hinsbergh, Leiden/Amsterdam	2 + 3
Eli Keshet, Jerusalem	1 - 2
Armin Kurtz, Regensburg	1 - 3
Stephen Smith, Cambridge/London	1 - 3
Norbert Suttorp, Berlin	1 - 3
Dietmar Vestweber, Münster	1 - 3
Marina Ziche, Florence/Siena	1 - 3

Goals and structure of the SPP1069

The focus and scope of the SPP1069 was defined in the one page open call for proposals that was published in May 1998. This read as follows:

Angiogenesis: Molecular mechanisms and functional interactions

The center of interest within this cooperative research project is the molecular mechanism of neo-angiogenesis, especially:

- *the functional analysis of expression and localisation of growth factors, receptors, adhesion molecules, integrins, inhibitors and other factors in signal transduction, during vasculo- and angiogenesis in the embryonic and the adult state, under physiological (e. g. in the ovarium) or pathophysiological (e. g. retinopathy, arthritis, wound healing, infarction, and tumors) conditions. Purely descriptive analysis should not be part of the investigation.*
- *mechanisms of neo-angiogenesis, studied in in vivo models; the investigation of influencing factors for a better understanding of the underlying biological processes and for the development of a rational therapy.*
- *mechanisms of neo-angiogenesis studied in in vitro models: proliferation, cell-cycle-control, apoptosis, cell-matrix-adhesion, cell-cell-adhesion, budding, migration, ripening, lumen development, permeability, and organ-specific differentiation of endothelial cells.*
- *biochemical and functional analysis of signal transduction and gene regulation in these processes.*
- *genetic analysis of these processes in vertebrates.*

Researchers with clinical background are requested to participate in this priority programme.

However, purely descriptive work or pharmacological trials with substances or factors in model system for angiogenesis will not be in the interest of this programme, as is the role of the vascular wall for vasodilatation, vasoconstriction, or artheriosclerosis. The investigation of inflammation of the vascular system is only of relevance to the priority programme, if it was connected with neo-angiogenesis.

As a positive side-effect the priority programme should enhance the concentration of efforts in this field in Germany.

Following the establishment of the SPP1069, projects were grouped in theme-related clusters in order to establish focussed priority areas within the SPP. With some variations throughout the three consecutive funding periods, the following theme clusters were established within the SPP (a cluster is considered a group of at least 2 related projects – most of the groups participated in more than one cluster):

- Developmental angiogenesis
- VEGF
- Ephrin ligands and Eph receptors
- Angiopoietin/Tie signaling
- Hypoxia
- Angiogenic signaling
- Vascular Differentiation
- Cardiac angiogenesis and arteriogenesis
- Tumor angiogenesis
- Lymphatic angiogenesis

These ten priority clusters covered the most important areas of ongoing angiogenesis research throughout the funding of the SPP. The emerging field of lymphangiogenesis research established a priority cluster only in the last funding period. The field of endothelial cell progenitor cell biology was not pursued as a priority cluster within the SPP1069.

Projects and Principal Investigators of the SPP1069

Unlike a local SFB, the Coordinator of an SPP has very little influence on the composition of an SPP. Instead, the composition of projects is largely determined by a highly selective reviewing process. When the SPP1069 was established in 1998, a total of 72 research proposals were submitted for review. Of these, 24 were recommended for funding (Fig. 2, Table 2). As such, the composition of an SPP is more reflective of an investigator-initiated bottom-up process rather than the more hierarchical top-down structure of a classical SFB.

The regional distribution of projects within Germany shows that the SPP1069 has truly evolved to become a nationwide research network (Fig. 2). Yet, most of the participating groups were located in the southern part of Germany which may be reflective north-south gradient within Germany in most field of ongoing biomedical research. Likewise, it is quite disappointing to note that even after more than 10 years of German unification only two projects located in the former East Germany were members of the SPP1069. Both of these groups were not original East German labs, but rather imports from the western part of the country.

The educational background of the participating Principal Investigators is highly indicative of the interdisciplinary character of the field of angiogenesis research. Approximately 50% of the PIs had a medical background, whereas the other 50% had a background in biology or biochemistry. Correspondingly, a broad array of different biomedical disciplines was engaged in the SPP1069 (Table 3). The SPP included university departments as well as non-university research institutions (Max-Planck-Society, Helmholtz-Society).

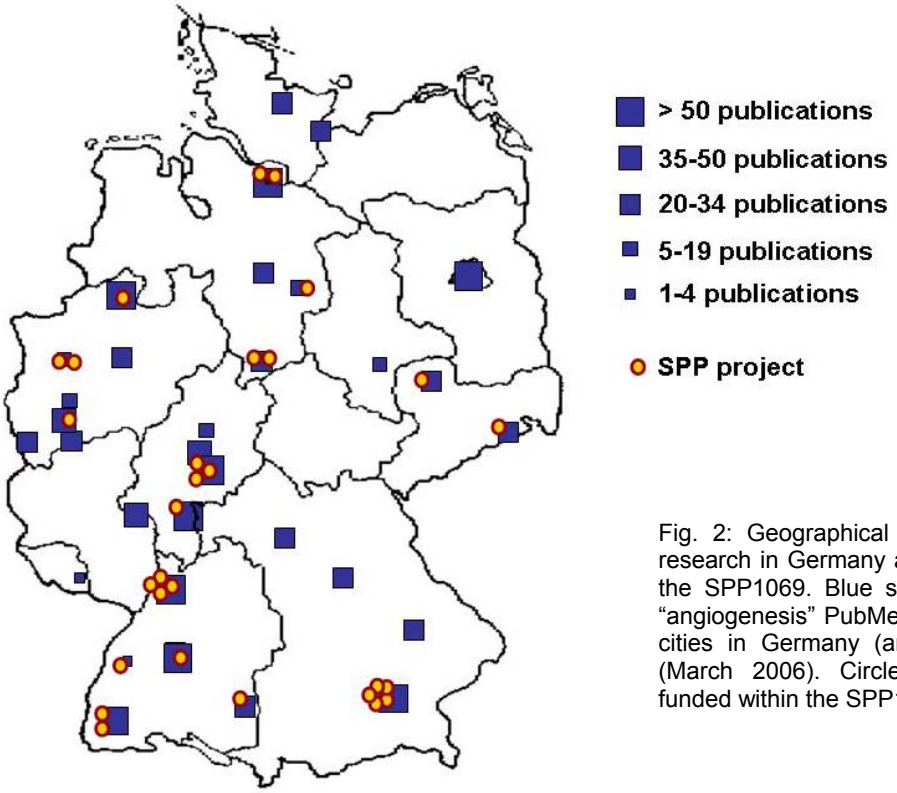


Fig. 2: Geographical distribution of angiogenesis research in Germany and the groups funded within the SPP1069. Blue squares mark the number of “angiogenesis” PubMed hits of the major university cities in Germany (angiogenesis AND city [AD]) (March 2006). Circles mark individual projects funded within the SPP1069.

Table 2: Projects and Principal Investigators of the SPP1069

Principal Investigator	Institution	Discipline	Title of Project	Member of SPP1069	Promotion of PI
Augustin, Hellmut	Univ. of Göttingen / Tumor Biology Center Freiburg	Vascular Biology	Functional analysis of endothelial cells during angiogenesis	1999-2006	Full Professor, Univ. of Heidelberg and Division Head, DKFZ Heidelberg
Bader, Bernhard	Max-Planck-Institute for Biochemistry / TU Munich-Weihenstephan	Genetics	Conditional knockout of the αv integrin gene in mice: Functional analysis of αv integrins during blood vessel formation	1999-2006	Tenured Senior Scientist, TU Munich
Boekstegers, Peter Kupatt, Christian	LMU Munich	Cardiology	Coronary angiogenesis induced by retro-infusion of angiogenic growth factors and liposomal cDNA	1999-2006	PB: Associate Professor, LMU Munich CK: Assistant Professor LMU Munich
Breier, Georg	MPI Bad Nauheim / TU Dresden	Vascular Biology	The role of vascular endothelial growth factor and of endothelial transcriptional regulators in embryonic angiogenesis	1999-2006	Associate Professor, TU Dresden
Clauss, Matthias	MPI Bad Nauheim	Vascular Biology	Identification of VEGF-induced signaling events involved in angiogenesis and vascular permeability	2001-2003	Associate Professor, Univ. of Indiana, USA
Dehio, Christoph	MPI Tübingen	Microbiology	Pathogen-encoded angiogenesis factors	1999-2001	Associate Professor, Biocenter Basel, Switzerland
Deutsch, Urban	MPI Bad Nauheim / MPI Münster / Theodor-Kocher-Institute Bern	Vascular Biology	Contribution of the Tie/Angiopoietin receptor/ ligand system to growth, morphology and function of blood vessels in adult mice	2001-2006	
Ergün, Süleyman Kilic, Nerbil	Univ. of Hamburg	Anatomy	Overexpression of CEACAM1 in vascular cells and its role in angiogenesis	2001-2003	Full Professor, University of Essen
Fiedler, Ulrike Martiny-Baron, Georg Marmé, Dieter	Tumor Biology Center Freiburg (TBCF)	Tumor Biology	Analysis of Angiopoietin function during angiogenesis	1999-2006	UF: Tenured Senior Scientist, TBCF GM: Tenured Senior Scientist, Novartis

Principal Investigator	Institution	Discipline	Title of Project	Member of SPP1069	Promotion of PI
Finkenzeller, Günter Dieter Marmé	Tumor Biology Center Freiburg (TBCF)	Tumor Biology	Identification of molecular mechanisms of oncogen-induced, Sp1 mediated activation of the VEGF promoter	1999-2001	GF: Senior Scientist, Univ. of Freiburg
Flamme, Ingo	MPI Bad Nauheim	Vascular Biology	Effect of endothelial cell specific transcription factors on the regulation of vasculogenesis and embryonic angiogenesis in vivo	1999-2001	Senior Scientist, Bayer Pharmaceuticals
Fusenig, Norbert Müller, Margareta	DKFZ Heidelberg	Tumor Biology	Role of matrix metalloproteinases in the regulation of tumor angiogenesis and invasion	2001-2003	
Hammes, Hans-Peter	Univ. of Giessen / Univ. of Heidelberg (MA)	Endocrinology	Angiopoietin-2 and its modulation in diabetic retinopathy	1999-2006	Associate Professor, University of Heidelberg
Hatzopoulos, Antonis	GSF Munich	Developmental Biology	Recruitment and activation mechanisms of embryonic endothelial progenitor cells in neovascularization	1999-2006	Associate Professor, Vanderbilt University, USA
Hawighorst, Thomas Koch, Manuel	Univ. of Göttingen and Univ. of Cologne	TH: Gynecology/ Obstetrics MK: Biochemistry	Functional analysis of the lymphatic system in normal and diseased tissue	2003-2006	MK: Associate Professor, Univ. of Cologne
Heumann, Rolf	Univ. of Bochum	Biochemistry	Selective functions and intracellular signaling mechanisms of vascular endothelial growth factor receptor VEGFR1 (Flt-1)	1999-2001	
Ito, Wulf	Univ. of Hamburg	Cardiology	Interaction of hemodynamic changes, chemo-attractants, adhesion molecules and macro-phage recruitment during collateral growth	1999-2006	Head of Reserach
Klein, Rüdiger Acker-Palmer, Amparo	MPI Martinsried	Molecular Cell Biology	Mechanism of ephrinB2 reverse signaling during angiogenesis	1999-2006	RK: Max-Planck-Director, MPI for Neurobiology
Kräling, Birgit Kurzen, Hjalmar	DKFZ Heidelberg & Univ. of Heidelberg	BK: Cell Biology HK: Dermatology	Endothelial differentiation and integrity of cell-cell-contact complexes during angiogenesis	1999-2001	

Principal Investigator	Institution	Displine	Title of Project	Member of SPP1069	Promotion of PI
Nikol, Sigrid Manegold, Johannes	LMU Munich	Cardiology	District-specific influence of $VEGF_{165}$ on arteriogenesis and angiogenesis of coronary and peripheral arteries	1999-2001	SN: Associate Professor, University of Münster
Plate, Karl Acker, Till	Univ. of Freiburg / Univ. of Erlangen / Univ. of Frankfurt	Neuropathology	The role of hypoxia inducible transcription factors (HIF) in tumor growth	1999-2006	KP: Assoc. Professor, Univ. of Erlangen KP: Full Professor, Univ. of Frankfurt
Schwarte-Waldhoff, Irmgard Schmiegel Wolff	Univ. of Bochum	Medicine	Impact of the tumor suppressor gene DPC4/Smad4 on regulators of angiogenesis	1999-2001	
Schweigerer, Lothar	Univ. of Essen / Univ. of Göttingen	Pediatrics	IL-6 as an N-myc-regulated angiogenesis inhibitor: mechanisms and implications	1999-2003	Full Professor, Univ. of Göttingen
Sleeman, Jonathan	FZ Karlsruhe	Genetics / Tumor Biology	Analysis of lymphangiogenesis during tumorigenesis, wound healing, lymphedema and filariasis	1999-2006	Tenured Senior Scientist, FZ Karlsruhe
Vajkoczy, Peter Ullrich, Axel	Univ. of Heidelberg (MA) and MPI Martinsried	PV: Neurosurgery AU: Molecular Cell Biology	Role of protein tyrosine kinases and Eph family receptors for angiogenesis, microcirculation, and growth of malignant gliomas	1999-2006	PV: Offer for Full Professorship, FU Berlin (pending)
Waltenberger, Johannes	Univ. of Ulm / Univ. of Maastricht	Cardiology	The role of VEGF in arteriogenesis	1999-2006	Full Professor, Univ. of Maastricht, Holland
Weich, Herbert	GBF Braunschweig	Molecular Biology	Soluble VEGFR-1: Overexpression and function in different animal models	1999-2006	
Wiedemann, Peter	Univ. of Leipzig	Ophthalmology	Modulation of choroidal neovascularization by age-related end products (AGE) and angiogenesis-related molecules	1999-2001	
Wilting, Jörg Schweigerer, Lothar	Univ. of Göttingen	JW: Anatomy LS: Pediatrics	Lymphangiogenesis and hemangiogenesis: Common molecular determinants?	2003-2006	JW and LS: Associate Professor University of Göttingen

Table 3: Biomedical disciplines involved in the SPP1069

Clinical departments	Preclinical departments
Cardiology	Anatomy
Dermatology	Biochemistry
Gynecology	Cell Biology
Medicine	Genetics
Neurosurgery	Microbiology
Pediatrics	Pathology
	Tumor Biology

Reviewers of the SPP

The review process of the DFG for individual grant proposals as well as for coordinated programs has historically been a national peer review process, i.e., proposals are written in German and reviewed by scientists within Germany or by German scientists in other countries. Deviating from this well established process of review, the program committee of the SPP1069 had suggested to the DFG from the very beginning to only allow English written proposals and to entertain a strict international review process. This was suggested 1.) in recognition of the fact that in principle every researcher involved in the respective field of research within Germany is eligible for funding thereby likely leaving insufficient critical reviewer mass for a qualified review, and 2.) to ensure the most authoritative review process by some of the most well renowned international scientists in the field. As a result, a high profile International Board of Reviewers of 13 prominent scientists from the field of angiogenesis research and vascular biology in general has reviewed the individual proposals that have been submitted for the three consecutive two year funding periods (Table 1). Grants were reviewed on the basis of submitted proposals. Yet, the Board of Reviewers has over the years acquired a solid personal knowledge of all the participating groups by participating in the biannual International Kloster Seeon Meeting “Angiogenesis” which has been hosted by the SPP1069.

Coordinated activities within the SPP1069

An SPP is composed of a certain number of individual grant proposals. As such, the SPP’s productivity and performance is determined by the scientific strength of the individual projects. Yet, as a coordinated program, an SPP is also expected to be more than just the sum of individual proposals. Rather, it is expected to create synergy through the exchange of reagents, tools, and ideas. This should lead to joint research projects eventually resulting in joint publications.

Clearly, no individual PI can be forced into a specific collaboration. The coordinating activities within the SPP were therefore aimed at creating an atmosphere of exchange and interaction that is conducive to bringing people together. A number of instruments were practiced within the SPP as part of the central coordinating activities to support cooperation and collaboration within the SPP.

Webpage

The SPP1069 has established the webpage www.angiogenese.de soon after its inauguration (Fig. 3). This website was supposed to serve as an information bulletin for the groups within the SPP and beyond as well as an instrument to foster awareness of the lay public about the field of angiogenesis research and the SPP itself. The website included 1.) profile pages about the individual

groups and projects within the SPP, 2.) thematically structured lists of recent (less than one month old) angiogenesis-related publications [title, authors, abstract with direct link to the full text manuscript], 3.) a list of upcoming angiogenesis-related national and international meetings, 4.) angiogenesis-related scientific job offers from within and outside the SPP, 5.) an angiogenesis jump station with a collection of the most important angiogenesis-related websites (scientific journals, databases, companies, etc.). Much effort was spent to regularly update the website in three weekly intervals to keep it timely and alive. Timeliness and relevance of the information published on the website may have contributed to the broad acceptance of the website by the angiogenesis community at large. Today, the site is accessed several thousand times per months. The site has grown and earned international visibility as evidenced by the notion that the site now receives significantly more international hits from outside Germany than from within Germany. It may be realistic to conclude that www.angiogenese.de is today the most frequently visited angiogenesis portale worldwide. It is for this reason that it has been decided to maintain www.angiogenese.de beyond the expiration of the SPP1069.

Fig. 3: The website "www.angiogenese.de" of the SPP1069.

Meetings

A number of closed and open meetings have been hosted during the last six years (Table 4). These included bi-annual consortium meetings, theme cluster-oriented subgroup meetings, a Young Investigator Meeting, as well as the bi-annual International Kloster Seeon Meeting "Angiogenesis".

Consortium meetings: Consortium meetings of all PIs and scientists working in the SPP (postdocs and graduate students) were hosted bi-annually as two day meetings (October 1999, Bad Nauheim; October 2001, Heidelberg; November 2003, Freiburg; April 2005, Göttingen). Each project was presented in a 20 to 25 minute presentation followed by a 5 to 10 minute discussion. Much time was devoted during these meetings to informal discussions.

Subgroup meetings: Theme-oriented subgroup meetings of 5 to 10 participants were hosted as ad hoc meetings throughout the duration of the SPP. These meetings were organized decentrally each by an individual PI. The SPP's secretariate provided logistical assistance.

Young Investigator Meeting: A young investigator meeting was hosted in the fourth year of the SPP. This meeting was organized by two senior graduate students (Anja Hegen, Tim Füller [Augustin lab] and Alexander Licht [Breier lab]) and was held in the Reisensburg castle near Ulm, Germany. This meeting (approx. 25 participants) was aimed at fostering exchange and networking between the graduate students and postdoctoral fellows.

Kloster Seeon Meeting "Angiogenesis": Soon after its inauguration, the SPP 1069 made plans to host biannually an open international angiogenesis meeting. The Kloster Seeon in Bavaria was selected as site of these meetings in recognition of the unique atmosphere of the monastery. It was deliberately decided to keep the meeting small with participation by invitation only rather than hosting a large-size open meeting. As such, the meetings were from the beginning supposed to have the typical "work hard – play hard" atmosphere for which the Gordon conference series is famous for.

The Seeon monastery can host up to 125 guests. Of the 125 participant slots, 50 were reserved for the members of the SPP. Another 15 slots were given to representatives of the corporate sponsors. Thirty slots were allocated for invited speakers. The remaining 30 slots were competitively given out on the basis of submitted abstract (scoring system by 5 international reviewers). It may not be surprising that the second and third Seeon meetings in 2002 and 2004 were heavily oversubscribed. As a consequence, many applications had to be rejected. As much as the organizers of the meeting would have liked to admit all applicants, the competitive nature of admission has also contributed to the reputation that this meeting series has earned in very few years.

The organizers of the Seeon meeting have aimed for the highest possible standard of science. As such, only leaders in the field were invited for presentation (Fig. 4-6). Compliance of invited speakers was very good with more than 90% of invited speakers accepting the invitation to participate in the meeting. All participants (including the invited speakers) were expected to stay for the duration of the meeting. This rule was with very few exceptions strictly applied.

The budget of the Seeon meeting has gotten close to 100.000 €. Public funds could only partially come up with the total cost required to host such a high profile meeting. The DFG has been the primary sponsor of the meeting. Likewise, the Max-Planck-society has contributed significant funds to support the Seeon meeting. Nevertheless, some 60% of the budget was provided by corporate sponsors (Table 5). The SPP1069 highly appreciates the generous support of pharmaceutical and biotech corporate sponsors. The solid corporate support of the meeting also reflects the excellent acceptance and profile that the meeting has earned in very few years.

Following the 2004 Seeon meeting, the organizers of the meeting were approached by quite a few people in the angiogenesis community suggesting to find a way to maintain the Seeon meeting series after the expiration of the SPP1069. As a consequence, 13 members of the SPP1069 have in 2005 established the *Verein für wissenschaftliche Fachtagungen in der Biomedizin e.V.* (www.vwfb.de). This Verein has been registered by the Amtsgericht Freiburg and received the status of a tax-benefited charity organization by the Finanzamt Freiburg. The VWFB e.V. will serve as the legal host of future Kloster Seeon angiogenesis meetings. It delegates the program responsibility to the meeting's Chairman and Co-Chairman which are elected by the participants of the Seeon meeting. For the 2006 Seeon angiogenesis meeting, Dr. Georg Breier (Dresden) and Dr. Christer Betsholtz (Stockholm) were elected as Chairman and Co-Chairman, respectively.

Table 4: Meetings hosted by the SPP1069

Date	Meeting	City	Participation	Comment
October 1999	Kickoff Meeting	Bad Nauheim	50	closed SPP meeting
Throughout 1999	Subgroup meetings	various locations	5-10	closed SPP meetings
September 2000	1st Int. Kloster Seeon Meeting	Kloster Seeon	125	open meeting
Throughout 2000	Subgroup meetings	various locations	5-10	closed SPP meetings
Throughout 2001	Subgroup meetings	various locations	5-10	closed SPP meetings
October 2001	Consortium Meeting	Heidelberg	50	closed SPP meeting
September 2002	2nd Int. Kloster Seeon Meeting	Kloster Seeon	125	open meeting
Throughout 2002	Subgroup meetings	various locations	5-10	closed SPP meetings
Throughout 2003	Subgroup meetings	various locations	5-10	closed SPP meetings
September 2003	Young Investigator Meeting	Reisensburg	25	closed SPP meeting
November 2003	Consortium Meeting	Freiburg	45	closed SPP meeting
September 2004	3rd Int. Kloster Seeon Meeting	Kloster Seeon	125	open meeting
Throughout 2004	Subgroup meetings	various locations	5-10	closed SPP meetings
Throughout 2005	Subgroup meetings	various locations	5-10	closed SPP meetings
April 2005	Consortium Meeting	Göttingen	35	closed SPP meeting
March 2006	Concluding meeting	Aschau, Bavaria	45	closed SPP meeting

Table 5: Corporate sponsors of the Kloster Seeon Meeting "Angiogenesis"

Sponsor, City	sponsored meeting(s)
Abbott, Worcester, USA	2002
Amgen, Thousand Oaks, USA	2000, 2002
Artemis/Exelixis, Tübingen, Germany	2000, 2002
AstracZeneca, Macclesfield, UK	2000, 2002, 2004
Aventis, Paris, France	2000, 2002, 2004
Bayer, Wuppertal, Germany	2000, 2002, 2004
Boehringer-Ingelheim, Vienna, Austria	2004
GenCell, Paris, France	2004
ImClone, New York, USA	2000, 2002, 2004
Merck Pharmaceuticals, Darmstadt, Germany	2004
Munich Biotechnology, Martinsried, Germany	2000, 2002
Novartis, Basel, Switzerland	2000, 2002, 2004
Roche Pharmaceuticals, Basel, Switzerland	2000, 2002, 2004
Schering, Berlin, Germany	2000, 2002, 2004
Sugen, San Francisco, USA	2000
Xantos Biomedicine, Martinsried, Germany	2002, 2004
Zentaris, Frankfurt, Germany	2004

Following are the group photos of the three Seeon Meetings conducted by the SPP 1069:

Fig. 4: 1st Kloster Seeon Meeting Angiogenesis, October 1-4, 2000

Invited Speakers: Kari Alitalo, Hellmut Augustin, Peter Bohlen, Georg Breier, Peter Carmeliet, Michael Detmar, Dan Dumont, Anne Eichmann, Brian Eliceiri, Napoleone Ferrara, Ingo Flamme, Jean Michel Foidart, Norbert Fusenig, Nicholas Gale, Antonis Hatzopoulos, Christopher Heeschen, Mats Hellström, Luisa Iruela-Arispe, Eli Keshet, Jan Kitajewski, Rüdiger Klein, Dieter Marmé, Karl-Heinz Plate, Sahin Rafii, Hans-Reimer Rodewald, Wolfgang Schaper, Lothar Schweigerer, Celeste Simon, Stephen Smith, Phil Thorpe, Axel Ullrich, Peter Vajkoczy, Johannes Waltenberger, Herbert Weich, Tao Zhong, Marina Ziche

Fig. 5: 2nd Kloster Seeon Meeting Angiogenesis, September 21-24, 2002

Invited Speakers: Ralf Adams, Kari Alitalo, Takayuki Asahara, Laura Benjamin, Gabriele Bergers, Christer Betsholtz, Andreas Bikvalvi, Peter Bohlen, Peter Carmeliet, Gerhard Christofori, Lena Cleasson-Welsh, Elisabetta Dejana, Napoleone Ferrara, Adrian Harris, Carl Henrik Heldin, Eli Keshet, Andras Nagy, Dario Neri, Shin Ichi Nishikawa, Micheal Pepper, Jacques Pouysségur, Marco Presta, Sahin Rafii, Peter Ratcliffe, Masabumi Shibuya, Celeste Simon, Michaela Skobe, Gavin Thurston, Victor van Hinsbergh, Seppo Ylä-Herttuala

Fig. 6: 3rd Kloster Seeon Meeting Angiogenesis, September 18-21, 2004

Invited Speakers: Ralf Adams, Kari Alitalo, David Anderson, Christer Betsholtz, Roy Bicknell, Federico Bussolino, Peter Carmeliet, Elisabetta Dejana, Anne Eichmann, Napoleone Ferrara, Judah Folkman, Guillermo García Cardeña, Tim Hla, Raghu Kalluri, Eli Keshet, Jan Kitajewski, Gou Young Koh, David Lyden, Donald Mc-Donald, Michal Neeman, Gera Neufeld, Jacques Pouysségur, Curzio Rüegg, William Sessa, Gavin Thurston, Mikka Vikkula, Brant Weinstein

List of publications originating from the SPP1069

Publication is the ultimate outcome of a scientific project. Thus, the number and quality of publications is the best indication of an individual lab's productivity. Furthermore, the degree of interaction and collaboration within a research consortium can be assessed by scanning the publication record for joint publications. The SPP1069 has been successful on both accounts. It would be tempting to list selected highlight accomplishments of the SPP 1069 of which there are quite a few. Yet, it would likely go beyond the scope of this introductory chapter to subjectively highlight individual publications. The reader is rather referred to the following list of all SPP1069 originating publications published in journals with an impact factor of >3 (Table 6). A detailed account of all publications and individual group's activities pursued within the SPP1069 can be obtained from the individual group's reports in the main part of this report. The following list also demonstrates the degree of interaction and collaboration within the SPP as evidenced by the fact that some 75% of all groups have joint publications with other groups of the consortium.

Table 6: SPP1069 publications in journals with an impact of >3 (PIs of the SPP in bold)

1999

Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, **Deutsch U**, Risau W, **Klein R:** Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis and sprouting angiogenesis. *Genes Dev* 13: 295-306, 1999.

Fotsis T, Breit S, Lutz W, Rössler J, Hatzi E, Schwab M, **Schweigerer L**: Down-regulation of endothelial cell growth inhibitors by enhanced MYCN oncogene expression in human neuroblastoma cells. *Eur J Biochem* 263: 757-764, 1999.

Herold-Mende C, Steiner HH, Andl T, Riede D, Buttler A, Reisser C, **Fusenig NE**, **Müller MM**: Expression and functional significance of vascular endothelial growth factor receptors in human tumor cells. *Lab Invest* 79: 1573-1582, 1999.

Korff T, **Augustin H**G: Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. *J Cell Sci* 112: 3249-58, 1999.

Meyer M, Lanz C, **Clauss M**, Lepple-Wienhus A, **Waltenberger J**, **Augustin H**, Ziche M, Büttner M, Rziha HJ, **Dehio C**: A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO J* 18: 363-374, 1999.

2000

Ala-aho R, Johansson N, Grenman R, **Fusenig NE**, Lopez-Otin C, Kahari VM: Inhibition of collagenase-3 (MMP-13) expression in transformed human keratinocytes by interferon-gamma is associated with activation of extracellular signal-regulated kinase-1,2 and STAT1. *Oncogene* 19: 248-257, 2000.

Arroyo AG, Taverna D, Whittaker C, Strauch UG, **Bader BL**, Rayburn H, Crowley D, Parker CM, Hynes RO: *In vivo* roles of integrins during leukocyte development and traffic: Insights from the analysis of mice chimeric for α5, αv, and α4 integrins. *J Immunol* 165: 4667-4675, 2000.

Beck H, **Acker T**, Wiessner C, Allegrini PR, **Plate KH**: Expression of angiopoietin-1, angiopoietin-2, and tie receptors after middle cerebral artery occlusion in the rat. *Am J Pathol* 157: 1473-1483, 2000.

Breit S, Rössler J, Fotsis T, **Schweigerer L**: N-myc down-regulates activin A. *Biochem Biophys Res Commun* 274: 405-409, 2000.

Breit S, Ashman K, **Wilting J**, Rössler J, Hatzi E, Fotsis T, **Schweigerer L**: The N-myc oncogene in human neuroblastoma cells: down-regulation of an angiogenesis inhibitor identified as activin A. *Cancer Res* 60: 4596-4601, 2000.

Eichler W, Kuhrt H, Hoffmann S, **Wiedemann P**, Reichenbach A: VEGF release by retinal glia depends on both oxygen and glucose supply. *Neuroreport* 11: 3533-3357, 2000.

Hornig C, Barleon B, Ahmad S, Vuorela P, Ahmed A, **Weich HA**: Release and complex formation of soluble VEGFR-1 from endothelial cells and biological fluids. *Lab Invest* 80: 443-454, 2000.

Johansson N, Ala-aho R, Uitto V, Grenman R, **Fusenig NE**, Lopez-Otin C, Kahari VM: Expression of collagenase-3 (MMP-13) and collagenase-1 (MMP-1) by transformed keratinocytes is dependent on the activity of p38 mitogen-activated protein kinase. *J Cell Sci* 113: 227-235, 2000.

Kappel A, Schlaeger TM, **Flamme I**, Orkin SH, Risau W, **Breier G**: Role of SCL/Tal-1, GATA, and ets transcription factor binding sites for the regulation of flk-1 expression during murine vascular development. *Blood* 96: 3078-3085, 2000.

Krieg M, Haas R, Brauch H, **Acker T**, **Flamme I**, **Plate KH**: Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. *Oncogene* 19: 5435-5443, 2000.

Laird AD, **Vajkoczy P**, Shawver LK, Thurnher A, Liang C, Mohammadi M, Schlessinger J, Ullrich A, Hubbard SR, Blake RA, Fong TA, Strawn LM, Sun L, Tang C, Hawtin R, Tang F, Shenoy N, Hirth KP, McMahon G, Cherrington: SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. *Cancer Res* 60: 4152-4160, 2000.

Schwarte-Waldhoff I, Volpert O, Bouck N, Sipos B, Hahn S, Klein-Scory S, Luttges J, Kloppel G, Graeven U, Eilert-Micus C, Hintelmann A, **Schmiegel W**: Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc Natl Acad Sci USA* 97: 9624-9629, 2000.

Vajkoczy P, Menger MD, Goldbrunner R, Ge S, Fong TA, Vollmar B, Schilling L, Ullrich A, Hirth KP, Tonn JC, Schmiedek P, Rempel: Targeting angiogenesis inhibits tumor infiltration and expression of the pro-invasive protein SPARC. *Int J Cancer* 87: 261-268, 2000.

Vuorela P, Helske S, Hornig C, Alitalo K, **Weich HA**, Halmesmäki E: Amniotic fluid soluble vascular endothelial growth factor receptor-1d in pre-eclampsia *Obstet Gynecol* 95: 353-357, 2000.

2001

Acker T, Beck H, **Plate KH**: Cell type specific expression of vascular endothelial growth factor and angiopoietin-1 and –2 suggests an important role of astrocytes in cerebellar vascularization. *Mech Dev* 108: 45-57, 2001.

Adams RH, Diella F, Hennig S, Helmbacher F, **Deutsch U**, **Klein R:** The cytoplasmic domain of the ligand ephrinB2 Is required for vascular morphogenesis but not cranial neural crest migration. *Cell* 104: 57-69, 2001.

Airola K, **Fusenig NE**: Differential stromal regulation of MMP-1 expression in benign and malignant keratinocytes. *J Invest Dermatol* 116: 85-92, 2001.

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, **Acker T**, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, **Plate KH**, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG: Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 7: 575-583, 2001.

Eichler W, Yafai Y, Kuhrt H, Gräter R, Hoffmann S, **Wiedemann P,** Reichenbach A: Hypoxia: modulation of endothelial cell proliferation by soluble factors released by retinal cells, *Neuroreport* 12: 4103-4108, 2001.

Fuhrmann O, Arvand M, Krull M, Hippenstiel S, Seybold J, Schmid M, **Dehio C**, Suttorp N: *Bartonella henselae* induces NF-kB-dependent upregulation of adhesion molecules in cultured human endothelial cells: possible role of outer membrane proteins as pathogenic factors. *Infect Immun* 69: 5088-5097, 2001.

Helske S, Vuorela P, Carpen O, Hornig C, **Weich HA**, Halmesmäki E: Expression of VEGF receptors 1, 2 and 3 in placentas from normal and complicated pregnancies. *Mol Hum Reprod* 7: 205-210, 2001.

Kirkin V, Mazitschek R, Krishnan J, Steffen A, **Waltenberger J**, Pepper MS, Giannis A, **Sleeman JP**: Characterization of indolinones which preferentially inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2. *Eur J Biochem* 268: 5530-5540, 2001.

Korff T, Kimmina S, Martiny-Baron G, **Augustin HG**: Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J* 15: 447-57, 2001.

Kunz M, Ibrahim S, Koczan D, Thiesen HJ, Köhler HJ, **Acker T**, **Plate KH**, Ludwig S, Rapp UR, Bröcker EB, van Muijen GN, Flory E, and Gross G: Activation of c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) is critical for hypoxia-induced apoptosis of human malignant melanoma. *Cell Growth Differ* 12: 137-145, 2001.

Neulen J, Wenzel D, Wünsch E, Weissenborn U, Büttner R, **Weich, HA**: Poor responder- high responder: the importance of soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) in ovarian stimulation protocols. *Hum Reprod* 16: 621-626, 2001.

Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Dorpe JV, Hellings P, Gorselink M, Heymans S, Theilmeier G, Dewerchin M, Laudenbach V, Vermylen P, Raat H, **Acker T**, Vleminckx V, Bosch LV, Cashman N, Fujisawa H, Drost MR, Sciot R, Bruyninckx F, Hicklin DJ, Ince C, Gressens P, Lupu F, **Plate KH**, Robberecht W, Herbert JM, Collen D, Carmeliet P: Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet* 28: 131-138, 2001.

Stratmann A, **Acker T**, Burger AM, Amann K, Risau W, **Plate KH**: Differential inhibition of tumor angiogenesis by tie2 and vascular endothelial growth factor receptor-2 dominant-negative receptor mutants. *Int J Cancer* 91: 273-282, 2001.

Uhlmann S, Friedrichs U, Eichler W, Hoffmann S, **Wiedemann P:** Direct measurement of VEGF-induced nitric oxide production by choroidal endothelial cells. *Microvasc Res* 62: 179-189, 2001.

2002

Compernolle V, Brusselmans K, **Acker T**, Hoet P, Tjwa M, Beck H, Plaisance S, Dor Y, Keshet E, Lupu F, Nemery B, Dewerchin M, Van Veldhoven P, **Plate KH**, Moons L, Collen D, Carmeliet P: Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat Med* 8: 702-710, 2002.

Eichler W, Friedrichs U, Thies A, Tratz C, **Wiedemann P:** Modulation of matrix metalloproteinase and TIMP-1 expression by cytokines in human RPE cells. *Invest Ophthalmol Vis Sci* 43: 2767-2773, 2002.

Francis SE, Goh KL, Hodivala-Dilke K, **Bader BL**, Stark M, Davidson D, RO Hynes: Central roles of alpha5beta1 integrin and fibronectin in vascular development in mouse embryos and embryoid bodies. *Arterioscler Thromb Vasc Biol* 22: 927-933, 2002.

Hammes HP, Lin J, Renner O, Shani M, Lundqvist A, Betsholtz C, Brownlee M, **Deutsch U:** Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes* 51: 3107-3112, 2002.

Hatzi E, Murphy C, Zoephel A, Ahorn H, Tontsch U, Bamberger AM, Yamauchi-Takihara K, **Schweigerer L**, Fotsis T: N-myc overexpression down-regulates leukemia inhibitory factor in neuroblastoma *Eur J Biochem* 269: 3732-3741, 2002.

Hatzi E, Murphy C, Zoephel A, Rasmussen H, Morbidelli L, Ahorn H, Kunisada K, Tontsch U, Klenk M, Yamauchi-Takihara K, Ziche M, Rofstad EK, **Schweigerer L***, Fotsis T*: N-myc oncogene overexpression down-regulates IL-6; evidence that IL-6 inhibits angiogenesis and suppresses neuroblastoma tumor growth (*equal contribution). *Oncogene* 21: 3552-3561, 2002.

McCarty JH, Monahan-Earley RA, Brown LF, Keller M, Gerhardt H, Rubin K, Shani M, Dvorak HF, Wolburg H, **Bader BL**, Dvorak AM, Hynes RO: Defective associations between blood vessels and brain parenchyma lead to cerebral hemorrhage in mice lacking alphav integrins. *Mol Cell Biol* 22: 7667-7677, 2002.

Müller MM, **Fusenig NE**: Tumor-stroma interactions directing phenotype and progression of epithelial skin tumor cells. *Differentiation* 70: 486-497, 2002.

Palmer A, Zimmer M, Erdmann KS, Eulenburg V, Porthin A, **Heumann R**, **Deutsch U**, **Klein R**: EphrinB phosphorylation and reverse signaling: regulation by Src kinases and PTP-BL phosphatase. *Mol Cell* 9: 725-737, 2002.

Toi M, Bando H, Ogawa T, Muta M, Hornig C, **Weich HA**: Significance of vascular endothelial growth factor (VEGF)/ soluble VEGF receptor-1 relationship in breast cancer. *Int J Cancer* 98: 14-18, 2002.

Vajkoczy P, Farhadi M, Gaumann A, Heidenreich R, Erber R, Wunder A, Tonn JC, Menger MD, **Breier G**: Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. *J Clin Invest* 109: 777-785, 2002.

Wai DH, Schaefer KL, Schramm A, Korsching E, Van Valen F, Ozaki T, Boecker W, **Schweigerer L**: Dockhorn-Dworniczak B, Poremba C: Expression analysis of pediatric solid tumor cell lines using oligonucleotide microarrays. *Int J Oncol* 20: 441-451, 2002.

2003

Augustin HG, **Breier G**: Angiogenesis: molecular mechanisms and functional interactions. 2nd Kloster Seeon Meeting of the German Priority Research Grant "Angiogenesis" (SPP1069). *Thromb Haemost* 89: 190-197, 2003.

Augustin HG, Reiss Y: EphB receptors and ephrinB ligands: regulators of vascular assembly and homeostasis. *Cell Tissue Res* 314: 25-31, 2003.

Autiero M*, **Waltenberger J***, Communi D*, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, **Dombrowski S**, Stanimovic D, van Hummelen P, **Dehio C**, Hicklin D, Persico G, Herbert JM, Communi D, Shibuya M, Collen D, Conway EM,Carmeliet P: Role of PlGF in *intra*- and *inter*molecular crosstalk between VEGF receptors Flt1 and Flk-1 (*equal contribution). *Nat Med* 9: 936-943, 2003.

Beck H, Voswinckel R, Wagner S, Ziegelhoeffer T, Heil M, Helisch A, Schaper W, **Acker T**, **Hatzopoulos AK**, **Plate KH**: Participation of bone marrow-derived cells in long-term repair processes following experimental stroke. *J Cerebral Blood Flow Metab* 23: 709-717, 2003.

Dehio C: Recent progress in understanding *Bartonella*-induced vascular proliferation. *Curr Opin Microbiol* 6: 61-65, 2003.

Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, **Acker T**, Rauter M, **Plate KH**, Sieweke M, **Breier G**, **Flamme I**: Cooperative interaction of hypoxia inducible factor (HIF)-2a and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *J Biol Chem* 278: 7520-7530, 2003.

Fiedler U, Krissl T, Koidl S, Weiss C, Koblizek T, **Deutsch U**, **Martiny-Baron G**, **Marmé D**, **Augustin HG**: Angiopoietin-1 and angiopoietin-2 share the same binding domains in the Tie-2 receptor involving the first Ig-like loop and the epidermal growth factor-like repeats. *J Biol Chem* 278: 1721-1727, 2003.

Füller T, Korff T, Kilian A, Dandekar G, **Augustin HG**: Forward EphB4 signaling in endothelial cells controls cellular repulsion and segregation from ephrinB2 positive cells. *J Cell Sci* 116: 2461-2470, 2003.

Gong H, Pöttgen C, Stüben G, Havers W, Stuschke M, **Schweigerer L**: Arginine deiminase and other antiangiogenic agents inhibit unfavourable neuroblastoma growth: potentiation by irradiation. *Int J Cancer* 106: 391-394, 2003.

Hammes HP, Du X, Edelstein D, Taguchi T, Matsumara T, Ju Q, Lin J, Bierhaus A, Nawroth P, Hannak D, Neumaier M, Bergfeld R, Giardino I, Brownlee M: Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. *Nat Med* 9: 294-299, 2003.

Hirai H, Ogawa M, Suzuki N, Yamamoto M, **Breier G**, Mazda O, Imanishi J, Nishikawa SI: Hemogenic and non-hemogenic endothelium can be distinguished by the activity of fetal liver kinase (*Flk*)*-1* promoter/enhancer during mouse embryogenesis. *Blood* 101: 886-893, 2003.

Issbrücker K, Marti HH, Hippenstiel S, Springmann G, Vosswinkel R, Gaumann A, **Breier G**, Drexler HCA, Suttorp N, **Clauss M**: p38 MAPK - a molecular switch between angiogenesis and vascular permeability. *FASEB J* 17: 262-264, 2003.

Koczulla R, **von Degenfeld G, Kupatt C,** Krötz F, Zahler S, Gloe T, Issbrücker K, Unterberger T, Zaiou M, Lebherz C, Karl A, Raake P, Pfosser A, **Boekstegers P,** Welsch U, Hiemstra PS, Vogelmeier C, Gallo RL, **Clauss M**, Bals R: An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* 111: 1665-1672, 2003.

Krishnan J, Kirkin V**,** Steffen A, Hegen M, Weih D, Tomarev S, **Wilting J**, **Sleeman JP**: Differential in vivo and in vitro expression of VEGF-C and VEGF-D in tumors and its relationship to lymphatic metatasis in immunocompetent rats. *Cancer Res* 63: 713-722, 2003.

Lebherz C, **von Degenfeld G**, Karl A, Raake P, Pfosser A, Pachmayr F, Scholz D, **Kupatt C**, **Boekstegers P:** Therapeutic Angiogenesis/Arteriogenesis in the Chronic Ischemic Rabbit Hindlimb: Effect of venous bFGF retroinfusion. *Endothelium* 10: 257-264, 2003.

Lucerna M, Mechtcheriakova D, Kadl A, Schabbauer G, Schafer R, Gruber F, Koshelnick Y, Muller HD, Issbrücker K, **Clauss M**, Binder BR, Hofer E: NAB2, a corepressor of EGR-1, inhibits vascular endothelial growth factor-mediated gene induction and angiogenic responses of endothelial cells. *J Biol Chem* 278: 11433-11440, 2003.

Mazur A, Aurrand-Lions M, Deylig A, Liebau M, Imhof BA, Schaper W, Meinertz T, **Ito WD**: Collateral targeting with single chain antibodies. *Endothelium* 10: 277-284, 2003.

Palmer A, **Klein R:** Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. *Genes Dev* 17: 1429-1450, 2003.

Tille JC, Wang X, Lipson KE, McMahon G, Ferrara N, Zhu Z, Hicklin DJ, **Sleeman JP**, Eriksson U, Alitalo K, Pepper MS: Vascular endothelial growth factor (VEGF) receptor-2 signaling mediates VEGF-$C_{\Delta N \Delta C}$- and VEGF-A-induced angiogenesis in vitro. *Exp Cell Res* 285: 286-298, 2003.

Vajkoczy P, Blum S, Lamparter M, Mailhammer R, Erber R, Engelhardt B, Vestweber D, **Hatzopoulos AK**: Multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis. *J Exp Med* 197: 1755-1765, 2003.

Van Trappen PO, Steele D, Lowe DG, Baithun S, Levey P, Beasley N, Thiele W, **Weich HA**, Krishnan J, Shepherd JH, Pepper MS, Jackson D, **Sleeman JP**, Jacobs IJ: Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D, and their receptor VEGFR-3, during different stages of cervical carcinogenesis. *J Pathol* 201: 544-554, 2003.

Von Degenfeld G, Raake P, **Kupatt C**, Lebherz C, Hinkel H, Gildehaus FJ, Münzing W, Kranz A, **Waltenberger J**, Simoes M, Schwaiger M, Thein E, **Boekstegers P**: Selective pressure-regulated retroinfusion of fibroblast growth factor-2 into the coronary vein enhances regional myocardial blood flow and function in pigs with chronic myocardial ischemia. *J Am Coll Cardiol* 42: 1120-1128, 2003.

Yilmaz A, Kliche S, Mayr-Beyrle U, Fellbrich G, **Waltenberger J**: The antiapoptotic role of VEGFR-2 is mediated via inhibition of p38 MAPK. *Biochem Biophys Res Commun* 306: 730-736, 2003.

Zimmer M, **Palmer A**, Köhler J, **Klein R:** Contact-mediated repulsion by ephrinB ligands and EphB receptors requires bi-directional endocytosis. *Nat Cell Biol* 5: 869-878, 2003.

2004

Bando H, Brokelmann M, Toi M, Alitalo K, **Sleeman JP**, Sipos B, Groene HJ, **Weich HA**: Immunodetection and quantification of vascular endothelial growth factor receptor-3 in human malignant tumour tissues. *Int J Cancer* 111: 184-191, 2004.

Birner P, Preusser M, Gelpi E, Berger J, Gatterbauer B, Ambros IM, **Acker T**, **Plate KH**, Harris AL, Hainfellner JA: Expression of hypoxia-related tissue factors correlates with diminished survival of adjuvantly treated patients with chromosome 1p-aberrant oligodendroglial neoplasms: therapeutic implications. *Clin Cancer Res* 10: 6567-6571, 2004.

Brühl T, Urbich C, Aicher D, **Acker-Palmer A**, Zeiher AM, Dimmeler S: Homeobox A9 transcriptionally regulates the EphB4 receptor in endothelial cell migration and tube formation. *Circ Res* 94: 743-751, 2004.

Conrad M, Jakupoglu C, Moreno SG, Lippl S, Banjac A, Schneider M, Beck H, **Hatzopoulos AK**, Just U, Schmahl W, Chien KR, Wurst W, Bornkamm GW, Brielmeier M: Essential role for mitochondrial Thioredoxin Reductase in hematopoiesis, heart development and heart function. *Mol Cell Biol* 24: 9414-9423, 2004.

Distler O, Distler J, Scheid A, **Acker T**, Hirth A, Rethage J, Michel BA, Gay RE, Müller-Ladner U, Matucci-Cerinic M, **Plate KH**, Gassmann M, Gay S: Vascular endothelial growth factor and its receptors in systemic sclerosis - Regulation by hypoxia and hypoxia inducible factor-1alpha. *Circ Res* 95: 109-116, 2004.

Eichler W, Yafai Y, Keller T, **Wiedemann P,** Reichenbach A: PEDF derived from glial Müller cells: a possible regulator of retinal angiogenesis. *Exp Cell Res* 299: 68-78, 2004.

Eichler W, Yafai Y, **Wiedemann P**, Reichenbach A: Angiogenesis-related factors derived from retinal glial (Müller) cells in hypoxia. *Neuroreport* 15: 1633-1637, 2004.

Eming SA, Lauer G, Cole M, Jurk S, Christ H, Hornig C, Krieg T, **Weich HA**: Increased levels of the soluble variant of the vascular endothelial growth factor receptor VEGFR-1 are asscociated with a poor prognosis in wound healing. *J Invest Dermatol* 123: 799-802, 2004.

Erber R, Thurnher A, Katsen AD, Groth G, Kerger H, **Hammes HP**, Menger MD, **Ullrich A**, **Vajkoczy P**: Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *Faseb J* 18: 338-340, 2004.

Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, Kriz W, Thurston G, **Augustin HG**: The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood* 103: 4150-4156, 2004.

Hammes HP, Lin J, Wagner P, Feng Y, vom Hagen F, Krzizok T, Renner O, **Breier G**, Brownlee M, **Deutsch U:** Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. *Diabetes* 53: 1104-1110, 2004.

Hegen A, Koidl S, Weindel K, **Marmé D**, **Augustin HG**, **Fiedler U**: Strong positive and negative regulation of endothelial cell specific Angiopoietin-2 expression. *Arterioscler Thromb Vasc Biol* 24: 1803-1809, 2004.

Khmelewski E, Becker A, Meinertz T, **Ito WD**: Tissue resident cells play a dominant role in arteriogenesis and concomitant macrophage accumulation. *Circ Res* 95: e56-e64, 2004.

Kirkin V, Thiele W, Matzichek R, Rohde K, Fellbrich G, Baumann P, **Weich HA**, **Waltenberger J**, Giannis A, **Sleeman JP**: MAZ51, an indolinone that inhibits endothelial cell and tumor cell growth in vitro, suppresses tumor growth in vivo. *Int J Cancer* 112: 986-993, 2004.

Licht AH, Hofmann U, Raab S, **Breier G**: Endothelium-specific Cre recombinase activity in flk-1-Cre transgenic mice. *Dev Dynam* 229: 312-318, 2004.

Martiny-Baron G, Korff T, Schaffner F, Esser N, Eggstein S, **Marmé D**, **Augustin HG**: Inhibition of tumor growth and angiogenesis by soluble EphB4. *Neoplasia* 6: 248-257, 2004.

Mueller MM, **Fusenig NE**: Friends or foes - bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer* 4: 839-849, 2004.

Raab S, Beck H, Gaumann A, Yuce A, Gerber HP, **Plate KH**, **Hammes HP**, Ferrara N, **Breier G**: Impaired brain angiogenesis and neuronal apoptosis induced by conditional homozygous inactivation of vascular endothelial growth factor. *Thromb Haemost* 91: 595-605, 2004.

Raake P**, von Degenfeld G,** Hinkel R, Vachenauer-R, Sandner-T, Beller S, Andrees M, **Kupatt C**, Schuler G, **Boekstegers P**: Myocardial gene transfer by selective pressure-regulated retroinfusion of coronary veins – comparison to surgical and percutaneous intramyocardial gene delivery. *J Am Coll Cardiol* 44: 1124-1129, 2004.

Wei J, Blum S, Unger M, Lamparter M, Jarmy G, Geishauser A, Chan G, Fischer KD, Rattat D, Debatin KM, **Hatzopoulos AK**, Beltinger C: Embryonic endothelial progenitor cells armed with a suicide gene target hypoxic lung metastases after intravenous delivery. Cancer Cell 5: 477-488, 2004.

Weich HA, Bando H, Brokelmann M, Baumann P, Alitalo K, Sipos B, **Sleeman JP:** Vascular endothelia growth factor-C is secreted from cells in culture and detectable in tumor tissue lysates – a new assay for quantification by ELISA. *J Immunol Methods* 285: 145-155, 2004.

Yafai Y, Iandiev I, **Wiedemann P**, Reichenbach A, Eichler W: Retinal endothelial angiogenic activity: effects of hypoxia and glial (Müller) cells. *Microcirculation* 11: 577-586, 2004.

Yasukawa T, Hoffmann S, Eichler W, Friedrichs U, Wang YS**, Wiedemann P:** Inhibition of experimental choroidal neovascularization in rats by an alpha(v)-integrin antagonist. *Curr Eye Res* 28: 359-366, 2004.

2005

Acker T, Diez A-J, Aragones J, Tjwa M, Brusselmans K, Moons L, Fukumura D, Moreno-Murciano MP, Herbert JM, Burger A, Riedel J, Elvert G, **Flamme I**, Maxwell PH, Collen D, Dewerchin M, Jain RK, **Plate KH**, Carmeliet P: Genetic evidence for tumor suppressor role of HIF-2α. *Cancer Cell* 8: 131-141, 2005.

Bando H, **Weich HA**, Brokelmann M, Horiguchi S, Funata N, Ogawa T, Toi M: Association between intratumoral free and total VEGF, soluble VEGFR-1, VEGF receptor-2 and prognosis in primary breast cancer. *Br J Cancer* 92: 553-561, 2005.

Dehio C: *Bartonella*-host cell interactions and vascular tumour formation. *Nat Rev Microbiol* 3: 621-631, 2005.

Diehl S, Bruno R, Wilkinson GA, Loose DA, **Wilting J**, **Schweigerer L**, **Klein R**: Expression patterns of ephrinB2 and EphB2 in congenital venous malformations and normal umbilical vessels. *Pediatr Res* 57: 537-444, 2005.

Ergün S, Tilki D, Oliveira-Ferrer L, Schuch G, **Kilic N:** Significance of vascular stabilization for tumor growth and metastasis. *Cancer Lett* 238:180-7, 2006.

Farhadi MR, Capelle HH, Erber R, **Ullrich A**, **Vajkoczy P**: Combined inhibition of vascular endothelial growth factor and platelet-derived growth factor signaling: effects on the angiogenesis, microcirculation, and growth of orthotopic malignant gliomas. *J Neurosurg* 102: 363-370, 2005.

Fiedler J, Leucht F, **Waltenberger J**, **Dehio C**, Brenner RE: VEGF-A and PIGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. *Biochem Biophys Res Commun* 334: 561-568, 2005.

Hoffmann J, Feng Y, vom Hagen F, Hillenbrand A, Lin J, Erber R, **Vajkoczy P**, Gourzoulidou E, Waldmann H, Giannis, A, Wolburg H, Shani M, Jaeger V, **Weich HA**, Preissner KT, Hoffmann S, **Deutsch U**, **Hammes HP**: Endothelial survival factors and spatial completion, but not pericyte coverage of retinal capillaries determine vessel plasticity. *FASEB J* 19: 2035-2036, 2005.

Luhmann UF, Lin J, Acar N, Lammel S, Feil S, Grimm C, Seeliger MW, **Hammes HP**, Berger W: Role of the Norrie disease pseudoglioma gene in sprouting angiogenesis during development of the retinal vasculature. *Invest Ophthalmol Vis Sci* 46: 3372-3382, 2005.

Jakupoglu C, Przemeck GKH, Schneider M, Moreno SG, Mayr N, **Hatzopoulos AK**, de Angelis MH, Wurst W, Bornkamm GW, Brielmeier M, Conrad M: Cytoplasmic Thioredoxin Reductase is essential for embryogenesis but dispensable for cardiac development and function. *Mol Cell Biol* 25: 1980-1988, 2005.

Kilic N, Oliveira-Ferrer L, Wurmbach JH, Loges S, Chalajour F, Vahid SN, Weil J, Fernando M, **Ergün S**: Pro-angiogenic signaling by the endothelial presence of CEACAM1. *J Biol Chem* 280: 2361-2369, 2005.

Kupatt C, Horstkotte J, Vlastos GA, Pfosser A, Lebherz C, Semisch M, Thalgott M, Mages J, Hoffmann R, Deten A, Lamparter M, Beck H, Büning H, **Boekstegers P**, **Hatzopoulos AK**: Embryonic endothelial progenitor cells expressing a broad range of pro-angiogenic and remodeling factors enhance vascularization and tissue recovery in acute and chronic ischemia. *FASEB J* 19: 1576-1578, 2005.

Kupatt C, Hinkel R, Lamparter M, von Brühl M-L, Pohl T, Horstkotte J, Beck H, Müller S, Delker S, Gildehaus F-J, Büning H, **Hatzopoulos AK**, **Boekstegers P**: Retroinfusion of Embryonic Endothelial Progenitor Cells Attenuates Ischemia-reperfusion Injury in Pigs: Role of PI3-Kinase/AKT. *Circulation* 112: I117-122, 2005.

Mäkinen T, Adams RH, Bailey J, Lu Q, Ziemiecki A, Alitalo K, **Klein R**, Wilkinson GA: PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. *Genes Dev* 19: 397-410, 2005.

Pfosser A, Thalgott M, Büttner K, Brouet A, Feron O, **Boekstegers P**, **Kupatt C:** Liposomal Hsp90 cDNA induces neovascularization via nitric oxide in chronic ischemia. *Cardiovasc Res* 65: 728-736, 2005.

Scharpfenecker M, **Fiedler U**, Reiss Y, **Augustin HG**: The Tie-2 ligand Angiopoietin-2 destabilizes quiescent endothelium trough an internal intractine loop mechanism. *J Cell Sci* 118: 771-780, 2005.

Vosseler S, Mirancea N, Bohlen P, **Mueller MM**, **Fusenig NE**: Angiogenesis inhibition by vascular endothelial growth factor receptor-2 blockade reduces stromal matrix metalloproteinase expression, normalizes stromal tissue, and reverts epithelial tumor phenotype in surface heterotransplants. *Cancer Res* 65: 1294-1305, 2005.

Wang YS, Eichler W, Friedrichs U, Yafai Y, Hoffmann S, Yasukawa T, Hui YN, **Wiedemann P:** Impact of endostatin on bFGF-induced proliferation, migration, and matrix metalloproteinase-2 expression/secretion of bovine choroidal endothelial cells. *Curr Eye Res* 30: 479-489, 2005.

Weigand M, Hantel P, Kreienberg R, **Waltenberger J**: Autocrine vascular endothelial growth factor signalling in breast cancer. Evidence from cell lines and primary breast cancer cultures *in vitro*. *Angiogenesis* 8:197-204, 2005.

2006

Buttler K, Kreysing A, von Kaisenberg CS, **Schweigerer L**, Gale N, Papoutsi M, **Wilting J**: Mesenchymal cells with leukocyte and lymphendothelial characteristics in murine embryos. *Dev Dyn* **235**: 1554-1562, 2006.

Erber R, Eichelsbacher U, Powajbo V, Korn T, Djonov V, Lin J, **Hammes HP**, Grobholz R, **Ullrich A**, **Vajkoczy P**: EphB4 controls blood vascular morphogenesis during postnatal angiogenesis. *EMBO J* 25: 628-641, 2006.

Fiedler U*, Reiss Y*, Scharpfenecker M*, Grunow V, Thurston G, Gale NW, Sobke A, Herrmann M, Preissner K, **Vajkoczy P**, **Augustin HG**: Angiopoietin-2 sensitizes endothelial cells to TNFα and has a crucial role in the induction of inflammation (*equal contribution). *Nat Med* 12: 235-239, 2006.

Heroult M, Schaffner F, **Augustin HG**: Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progressions. *Exp Cell Res* 312: 642-650, 2006.

Horst A, **Ito WD,** Dabelstein J, Schumacher U, Sander H, Turbide C, Brümmer J, Meinertz T, Beauchemin N, Wagener C: CEACAM1 modulates vascular remodelling in vitro and in vivo. *J Clin Invest,* 116: 1596-605, 2006.

Korff T, Dandekar G, Pfaff D, Füller T, Goettsch W, Morawietz H, Schaffner F, **Augustin HG**: Endothelial ephrinB2 is controlled by microenvironmental determinants and associates context-dependently with CD31. *Arterioscler Thromb Vasc Biol* 26: 468-474, 2006.

Langer H, May AE, Daub K, Heinzmann U, Lang P, Schumm M, Vestweber D, Massberg S, T. Schonberger T, Pfisterer I, **Hatzopoulos AK**, Gawaz M: Adherent platelets recruit and induce differentiation of murine embryonic endothelial progenitor cells to mature endothelial cells in vitro. *Circ Res* 98: 2-10, 2006.

Licht AH, Muller-Holtkamp F, **Flamme I**, **Breier G**: Inhibition of hypoxia-inducible factor activity in endothelial cells disrupts embryonic cardiovascular development. *Blood* 107: 584-590, 2006.

Mitola S, Brenchio B, Piccinini M, Tertoolen L, Zammataro L, **Breier G**, Rinaudo MT, den Hertog J, Arese M, Bussolino F: Type I Collagen limits VEGFR-2 signalling by a SHP2 protein-tyrosine phosphatase-dependent mechanism. *Circ Res* 98: 45-54, 2006.

Tilki D, Irmak S, Oliveira-Ferrer L, Hauschild J, Miethe K, Atakaya H, Hammerer P, Friedrich MG, Schuch G, Galalae R, Kilic E, Huland H, **Ergün S**: CEA-related cell adhesion molecule-1 (CEACAM1) is involved in angiogenic switch in prostate cancer. *Oncogene*, in press, 2006.

Wilting J, Aref Y, Huang R, Tomarev SI, **Schweigerer L**, Christ B, Valasek P, Papoutsi M: Dual origin of avian lymphatics. *Dev Biol* **292**: 165-173, 2006.

Witte MH, Jones K, **Wilting J**, Dictor M, Selg M, McHale N, Gershenwald JE, Jackson D: Structure-function relationships in the lymphatic system and implications for cancer biology. *Cancer Metastasis Rev* 25: 159-184, 2006.

Yao D, Taguchi T, Matsumura T, Pestell R, Edelstein D, Giardino I, Suske G, Ahmed N, Thornalley PJ, Sarthy VP, **Hammes HP**, Brownlee M: Methylglyoxal Modification of mSin3A Links Altered Glycolysis to Angiopoietin-2 Transcription: A Novel Mechanism for Regulating Gene Expression. *Cell* 124: 275-286, 2006.

Zengin E, Chalajour F, Gehling UM, **Ito WD**, Treede T, Lauke H, Weil J, Reichenspurner H, **Kilic N**, **Ergün S**: Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development* 133: 1543-1551, 2006.

List of diploma theses, doctoral theses, and habilitations pursued within the SPP

The DFG funds have been used to do science. Yet, many young scientists at an early stage in their career were involved in the research activities of the SPP1069. As such, the research network also played an important educational role to train young scientists. Training is of particular importance in a young and emerging discipline such as angiogenesis research. Likewise, even the broader field of vascular biology is only today emerging as an independent, yet interdisciplinary scientific discipline. As such, training activities play a pivotal role in contributing to the shaping of future leading scientists in the field. Following is a list of diploma theses, doctoral theses, and habilitations pursued within the SPP1069 (Table 7).

Table 7: Diploma theses, doctoral theses, and habilitations pursued within the SPP1069

Diploma theses

Gronemeyer, Thomas (Heumann lab): “Expression and molecular evolution of Y13259-scFv”. Ruhr University of Bochum, Molecular Neurobiochemistry (2001).

Khan, Friedrich (Heumann lab): „Bestimmung der Bindungsaffinitäten zwischen dem neuronal aktiven monoklonalen Antikörper Y13-259 und dem Rasp21-Protein mittels Oberflächenplasmonresonanzspektroskopie“. Ruhr University of Bochum , Molecular Neurobiochemistry (2000).

Krissl, Tanja (Fiedler/Marmé lab): "Bindung von Angiopoietin-1 und -2 an ihren Rezeptor Tie2 – Struktur- und Funktionsanalysen“. Tumor Biology Center Freiburg, Institute of Molecular Oncology (2000).

Lamparter, Mathias (Hatzopoulos lab): “Investigation of homing and activation of embryonic endothelial progenitor cells in tumor angiogenesis”. Technical University of Munich, Institute for Physiology - Wissenschaftszentrum Weihenstephan.

Mössinger, Katharina (Fiedler/Marmé lab): „Die Rolle von Tie1 bei der Angiopoietin-vermittelten Endothezell-Funktion“. Tumor Biology Center Freiburg, Department of Vascular Biology and Angiogenesis Research (2005).

Norgall, Susanne (Weich lab): “Isolation and characterization of primary human and mouse lymph endothelial cells”. University of Oldenburg.

Reiter, Sabine (Heumann lab): „Sequentiell-parallele Elektroanalytik in Mikrotiterplatten als Basis zur Optimierung der Redoxfunktionalisierung von Proteinen“ (in Kooporation with Prof. Schuhmann at RUB/Chemistry). Ruhr University of Bochum, Molecular Neurobiochemistry (2001).

Rothley, Melanie (Sleeman lab): "Regulation der VEGFR-3-induzierten Lymphangiogenese“. University of Karlsruhe, Institute of Genetics (2003).

Thiele, Wilko (Sleeman lab): "Die entscheidende Rolle der Mitglieder der Familie der vaskularen endothelialen Wachstumsfaktor-Rezeptoren (VEGFRs) bei der zellulären Differenzierung“. University of Karlsruhe, Institute of Genetics (2002).

Doctoral theses

Albrecht, Tobias (Waltenberger lab): „Aktivierung des VEGF-Rezeptors KDR in der Randzone einer regionalen Myokardischämie beim Hund als Hinweis für eine Beteiligung des VEGF-Systems an Ischämie-induzierten Reparaturvorgängen“. University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2001).

Aref, Yama (Wilting lab): „Untersuchungen des Ursprungs des Lymphgefäßsystems bei Vogel-embryonen“.

Babiak, Alexander (Waltenberger lab): “Die Etablierung eines *in vivo* Modells der Arteriogenese”. University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2004).

Bergler, Axel (Waltenberger lab): „Einfluß einer Hypercholesterinämie auf die Monozytenfunktion. Implikationen für die Ausbildung einer kollateralen Zirkulation“. University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2003).

Bergmann, Astrid (Weich lab): “Induction, modulation and blocking of the ligand/sFlt-1 interaction in a preeclampsia model with mice”. Technical University Braunschweig.

Comati, Amina (Plate lab): “Putative endothelial dysregulation of HIF as a cause for SWS vascular malformations”.

Czepluch, Frauke (Waltenberger lab): „Der Effekt einer Cholesterinsenkung mithilfe von Statinen auf die Verbesserung der Monozytenfunktion. Die VERACAD-STAT-Studie”. University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2006).

Endregat, Jan (Waltenberger lab): „Hemmung der Arteriogenese durch Diabetes mellitus: Funktionelle Untersuchungen Mouse-Hindlimb-Ischemia Modell“. University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2006).

Erber, Ralf (Vajkoczy lab): “Pathophysiologische und therapeutischen Bedeutung von Rezeptor-Tyrosinkinasen für Angiogenese, Mikrozirkulation und Wachstum maligner Gliome”. University of Heidelberg, Faculty of Clinical Medicine, Mannheim, Department of Neurosurgery.

Farhadi, Mohammad (Vajkoczy): „Mechanismen der vaskulären Initiierung maligner Gliome: Untersuchungen an einem orthotopen *in vivo* Modell an der Maus (magna cum laude, 2003).

Füller, Tim (Augustin lab): Zelluläre Effektorfunktionen von EphB/EphrinB2-Interaktionen während der Angiogenese. Tumor Biology Center Freiburg, Department of Vascular Biology and Angiogenesis Research (2004).

Hegen, Anja (Fiedler lab): **“**Transkriptionelle Regulation der endothelzellspezifischen Expression von Angiopoietin-2“. Tumor Biology Center Freiburg, Department of Vascular Biology and Angiogenesis Research (2004).

Hinkel, Rabea (Boekstegers lab): “Vascular protective treatment via AKT-eNOS dependent mechanisms: Insights from a preclinical pig model”. University of Munich Medical School.

Hoffmann, Jennifer (Hammes lab): summa cum laude (in conjunction with the graduate school 534, Prof. Piper, Giessen).

Horstkotte, Jan (Boekstegers lab): “Mechanisms of cell-cell interaction in ischemic mouse myocardium: Differences of leukocyte and embryonic EPC recruitment”. University of Munich Medical School.

Issbrücker, Katja (Breier lab): „Mechanismen der Signaltransduktion in Angiogenese und *Tissue Factor*-Produktion durch den vaskulären endothelialen Wachstumsfaktor (VEGF)“. University of Marburg Medical School (2003).

Kappel, Andreas (Breier lab): „Analyse regulatorischer Elemente des Flk-1 Gens“. University of Marburg Medical School (1999).

Kirkin, Vladimir (Sleeman lab): "Towards the development of novel cancer therapies: selection and characterisation of compounds that inhibit tumor cell proliferation, angiogenesis and lymphangiogenesis“. University of Karlsruhe, Institute of Genetics (2001).

Korff, Thomas (Augustin lab): Entwicklung eines dreidimensionalen Sphäroidmodells zur Untersuchung endothelialer Differenzierung, Apoptose und Angiogenese.

Kreysing, Alice (Wilting lab): „Untersuchungen der Entwicklung des Lymphgefäßsystems bei Mausembryonen“.

Krishnan, Jaya (Sleeman lab): "The role of VEGFR-3 and its ligands VEGF-D in tumor metastasis and haemopoesis“. University of London (2002).

Kurz, Kerstin (Waltenberger lab): „Der differentielle Einfluss der beiden VEGF-Rezeptoren VEGFR-1 und VEGFR-2 auf die Genexpression von PlGF und MCP-1“. University of Ulm, Faculty of Medicine, Department of Internal Medicine II (1999 - 2004).

Landgraf, Julia (Plate lab): “The regulation of PHD function by ROS”.

Licht, Alexander (Breier lab): „Untersuchungen zur Funktion endothelialer Transkriptionsfaktoren in der Angiogenese“. University of Marburg Medical School (2004).

Müller, Patrick (Waltenberger lab): „Der Einfluss des VEGF-Rezeptor 1 und des endothelialen PDGF-B auf die Arteriogenese“. University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2005).

Niedhammer, Thomas (Waltenberger lab): „Die Rolle des VEGF-Rezeptor-1 (Flt-1) beim Kollateralen-Wachstum in der Maus. Studien unter Verwendung gentechisch modifierter Mäuse mit funktionsgestörtem VEGF-Rezeptor-1 (Flt1-TK$^{-/-}$)“. University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2006).

Obermeyer, Natalie (Ito lab): “Proteomanalyse wachsender Kollateralarterien sowie migrierender und nicht-migrierender Endothelzellen aus der Ratte im Vergleich“. University of Hamburg, Department of Biologie (2005).

Sabine Raab (Breier lab): „Untersuchung der Funktion des Angiogenesefaktors VEGF in der Entwicklung des Zentralnervensystems“. University of Giessen (2001).

Radtke, Ina (Heumann lab): „VEGF-Rezeptor-spezifische Signaltransduktion über Ras und Stickstoffmonoxid in Endothelzellen“. Ruhr University of Bochum, Molecular Neurobiochemistry (2004).

Rhomberg, Thomas (Dehio lab): “Identification of outer membrane proteins from *Bartonella henselae* ATCC 49882 H-1 by proteomic means” (Master thesis). University of Basel, Division Molecular Microbiology.

Riedel, Johanna (Plate lab): “The role of PHD in malignant brain tumor”.

Röttgen, Marlene (birth name: Meyer) (Dehio lab): “Angiogenese als Pathogenitätsmechanismus”, University of Tübingen, Department of Biology.

Rohde, Katrin (Waltenberger lab): „Biochemische und zellbiologische Charakterisierung neuer Tyrosin-Kinase-Inhibitoren zur Prädiktion ihre Anti-Tumor- und Anti-Restenose-Potentials“. University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2005).

Schaffner, Florence (Augustin lab): Role of ephrinB2/EphB4 interactions in tumor angiogenesis, tumor progression and metastasis. Tumor Biology Center Freiburg, Department of Vascular Biology and Angiogenesis Research (2004).

Schmid, Michaela (Dehio lab): "Subversion of endothelial cell function by the human pathogen *Bartonella henselae*", University of Basel, Division Molecular Microbiology.

Schumm, Anke-Mira (Waltenberger lab): „Die Rolle verschiedener VEGF-Rezeptor-Inhibitoren auf die Arteriogenese im Maus-Hindlimb-Modell". University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2005).

Thalgott, Mark (Boekstegers lab): „Induktion von Neovaskularisierung durch Heatshock protein 90 vermittelte eNOS-Phosphorylierung". University of Munich Medical School.

Thomson, Michael (Ergün lab): „Die Arteria thoracica interna: Etablierung eines humanen Gefäßmodells". Degree: cum laude, University of Hamburg (2005).

Tilki, Derya (Ergün lab): „Duale Rolle von CEACAM1 bei der Angiogenese und der Tumorinvasion am Beispiel des Harnblasen- und Prostatakarzinoms". Degree: summa cum laude, University of Hamburg (2006).

Vosseler, Silvia (Fusenig lab): „Tumorangiogenese, Vaskularisierung und Invasion: Tumor-Stroma Interaktionenen während der malignen Tumor Progression". University of Stuttgart-Hohenheim (2001).

Wagner, Patrick (Hammes lab): magna cum laude.

Wangler, Christoph (Waltenberger lab): „Die Rolle verschiedener Vertreter der VEGF-Familie und von MCP-1 auf die Arteriogenese im Mouse-Hindlimb-Modell". University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2003).

Wu, Jianbo (Waltenberger lab): "Enhanced expression of VEGF receptor-2 in growing collateral arteries in the mouse hindlimb model". University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2001).

Yilmaz, Ali (Waltenberger lab): „Die Rolle der p38 MAPK im VEGF-induzierten Anti-Apoptose-Signalweg". University of Ulm, Faculty of Medicine, Department of Internal Medicine II (2003).

Zengin, Elvin (Ergün lab): „Gefäßwand-residente endotheliale Vorläuferzellen als Quelle für postnatale Vaskulogenese. Will be finished in 2006.

Habilitations

Breier, Georg: „Untersuchungen zur Funktion endothelialer Signalmoleküle in der Angiogenese und Vaskulogenese" (Habilitation). University of Würzburg Medical School (2004).

Hawighorst, Thomas: "Die Bedeutung der endogenen Angiogeneseinhibitoren Thrombospondin-(TSP-) 1 und TSP-2 für die Karzinogenese und Metastasierung" (Habilitation). University of Göttingen, Department of Gynecology and Obstetrics.

Sleeman, Jonathan: "Zelluläre und molekulare Mechanismen der Metastasierung" (Habilitation). University of Karlsruhe, Institute of Genetics (2000).

Vaikoczy, Peter: „Mechanismen der Angiogenese, Mikrozirkulation und Perfusion hochgradiger Gliome: Experimentelle in vivo Untersuchungen mittels der Intravitalen Multi-Fluoreszenz Mikroskopie". Neurochirurgical Clinic, University Hospital Mannheim, Faculty of Clinical Medicine, Mannheim Campus of the University of Heidelberg (2001, Prof. Dr. P. Schmiedek).

Professional promotions of PIs within the SPP

Much like publications, theses, and habilitations, professional promotions of Principle Investigators can be considered as a vital sign of growth, maturation, and success of the members of a research network. This applies particularly to the Principal Investigators of the SPP1069. Compared the

largely conservative funding principles applied by funding agencies within Germany, the SPP1069 was at its beginning a group of mostly young, not well established junior investigators. Of the PIs within the first funding period, 85% were non-tenured scientists. Consequently, the SPP1069 has given many young and emerging PIs a unique opportunity for personal growth. Within the six year funding of the SPP1069, 70% of all PIs had been non-tenured scientists and have promoted to tenured senior scientist, university professor or equivalent (Max-Planck-Director, Lab Head of pharmaceutical company, etc.) (Table 2).

Summaries of individual projects

Following are the individual reports of the research groups that have participated in the SPP1069 for 1, 2, or 3 funding periods. Projects are listed according to the theme clusters established within the SPP1069 according to the following order (some groups have switched focus during the 3 funding periods – these groups are listed in line with their primary thematic affiliation at the conclusion of the project):

Developmental angiogenesis

Bernhard Bader
Ingo Flamme
Antonis Hatzopoulos
Herbert Weich

VEGF

Christoph Dehio
Rolf Heumann

Ephrin ligands and Eph receptors

Hellmut Augustin
Rüdiger Klein / Amparo Acker-Palmer
Peter Vajkoczy / Axel Ullrich

Angiopoietin / Tie signaling

Urban Deutsch
Ulrike Fiedler / Dieter Marmé
Hans-Peter Hammes

Hypoxia

Georg Breier
Karl Plate / Till Acker

Angiogenic signaling

Matthias Clauss

Süleyman Ergün / Nerbil Kilic

Lothar Schweigerer

Vascular differentiation

Birgit Kräling / Hjalmar Kurzen

Peter Wiedemann

Cardiac angiogenesis and arteriogenesis

Peter Boekstegers / Christian Kupatt

Wulf Ito

Sigrid Nikol

Johannes Waltenberger

Tumor angiogenesis

Günther Finkenzeller / Dieter Marmé

Norbert Fusenig / Margarete Müller

Georg Martiny-Baron / Dieter Marmé

Irmgard Schwarte-Waldhoff / Wolff Schmiegel

Lymphatic angiogenesis

Thomas Hawighorst / Manuel Koch

Jonathan Sleeman

Jörg Wilting / Lothar Schweigerer

Developmental angiogenesis

Conditional αv Integrin Gene Knockout in Mice: Functional Analysis of αv Integrins during Blood Vessel Formation in Physiological and Pathological Situations

Dr. Bernhard L. Bader

Max-Planck-Institute for Biochemistry, Martinsried, Germany
Present address:
Department of Nutritional Medicine
Technical University of Munich
Am Forum 5, 85350 Freising, Germany

Member of the SPP 1069 from 1999 until 2006

SUMMARY

Gene expression and functional analysis using αv integrin-specific antibodies and antagonistic RGD-based peptidomimetics have implicated αv integrins in various morphogenetic and pathological events, e.g. vasculogenesis and angiogenesis during embryonic development, as well as tumor angiogenesis and wound healing. Previously, key functions of αv integrins were assessed by a constitutive targeted null-mutation in the αv integrin gene in mice. αv integrin deficiency is 100% lethal and leads to two classes of vascular phenotypes: Class I, 80% of all αv-nulls show deficient placentation and embryonic lethality at E 10.5; class II, 20% of all αv-nulls manifest defective embryonic intracerebral vasculature and hemorrhage at E12.5 and finally perinatal lethality. Since αv integrins can be expressed in endothelial cells of blood vessels as well as in perivascular cells it was not evident which cell type(s) and underlying mechanisms contribute to the vascular defects in αv integrin-null mice. To answer these questions we proposed in the DFG-SPP1069 to study the αv-null embryonic brains at the ultrastructural level and to generate strains of mice carrying a conditional gene knockout αv integrin allele (floxed allele) and a lacZ-reporter-knockin αv integrin allele. This should allow us to genetically dissect the cell type(s) contributing to the abnormal αv-null brain vascular morphogenesis and to bypass embryonic lethality in order to study the function of αv integrins at postnatal stages in mice. Here, major accomplishments of our project during the SPP1069-funding period are summarized: Our ultrastructural studies of αv-null embryos detect defective associations between cerebral microvessels and the surrounding brain parenchyma. These data suggest a novel role for αv integrins in the association between cerebral microvessels and central nervous system parenchymal cells. Using the Cre/loxP-approach we successfully generated strains of mice carrying floxed αv integrin and lacZ-knockin αv integrin alleles. Surprisingly, tie1-Cre-dependent ablation of the αv integrin gene in endothelial cells does not cause the vascular phenotypes or lethality as described in constitutive αv-null mice. However, nestin-Cre dependent ablation of the αv integrin gene in neuroepithelial cells results in abnormal brain vascular morphogenesis as observed in constitutive αv-null embryos. These mutant mice are viable and manifest a defective retinal vasculature postnatally. We demonstrate that first steps of retinal vascularization proceed normally, however retinal blood vessel sprouts and branches in the inner nuclear layer terminate in aberrant vascular structures and the formation of the second deep inner nuclear vascular plexus is impaired. Our data show that endothelial αv integrins are not essential for blood vessel development in mice. Surprisingly, neuroepithelial cells of the brain and retina are

important in regulating cerebral and retinal blood vessel morphogenesis via αv integrins. These results are important and consistent with the data from ß3, ß5 or ß3/ß5 and ß6 integrin gene knockout mice. These mice are viable and do not show phenotypes described for either the αv or ß8 integrin gene knockout. Since the ß8 integrin nulls phenocopies the embryonic αv-null phenotype, we suggest that the αvß8 integrin receptor dimer plays an important role in the neurovascular interface during the vascular morphogenesis.

Future studies are necessary to identify cellular and molecular interactions between neuroepithelial αv integrins and brain, and retinal blood vessels. It will be of interest to find out whether the neuroepithelial αvß8 integrin operates in region-specific blood vessel guidance and control of endothelial cell proliferation in the central nervous system.

INTRODUCTION

In recent years it has become clear that the cells which comprise blood vessels are regulated in their behavior by a large number of growth factors and their receptors. Central among these, are various growth factors, VEGF, FGF-2, TGFß, angiopoietins, neuregulin and PDGF and their corresponding receptors. FGF-2, VEGF and angiopoietins act on endothelial cells by binding to tyrosine kinase receptors, whereas PDGF and neuregulin are produced by endothelial cells and act to recruit and organize accessory cells, again by acting on tyrosine kinase receptors on those cells (Risau, 1997). Recently it could be demonstrated that ephrin-B2 and its counter receptor, Eph-B4, are involved in determining the distinction between venous and arterial development (Yancopoulos et al, 1998). Various analyses, most notably those using gene ablation methods to generate mice lacking specific factors or their receptors, have provided initial insights into the roles played by these different signalling systems and a rough sequence of inductive interactions can be formulated. Thus, VEGF, acting through two different receptors, first controls the initial determination of angioblasts and subsequently their ability to assemble into tubes. However, prior action of FGF-2 appears necessary to induce the expression of VEGF receptors in the endothelial precursors. Subsequently, angiopoietins acting on tie-receptors affect further development of the vasculature, probably including interactions between endothelial and accessory cells. PDGF, TGFß and neuregulin signalling and ephrin/Eph interactions further contribute to the differentiation of different vessel types (Hanahan, 1997).

Although the understanding of the hierarchy of controls affecting vascular development rapidly develops we still need to understand how the factors and their receptor-mediated signals actually produce vessels, induce branching and endothelial-accessory cell interactions and yield the array of different vessel types found in a mature animal. At the cell biological level, these events clearly require control of cell proliferation and survival, various cell migrations and cell adhesive events, basement membrane assembly and remodelling and stable interactions between cells and with the extracellular matrices around them. Cell-cell adhesion molecules such as cadherins are believed to play important roles and, indeed, gene ablation studies clearly implicate both N-cadherin and VE-cadherin in early steps of vessel formation. Similiar important is another family of cell adhesion receptors, the integrins, and their involvement in vascular development and remodelling (Hynes and Bader, 1997; Hynes et al, 1999).

Integrins are a family of heterodimeric cell surface receptors, which mediate adhesion of cells to extracellular matrix proteins and sometimes to other cells. In mammals, around two dozen integrins are known and endothelial cells can express at least five or six different ones (Hynes, 1992). Cell surface expression of integrins can be controlled by various growth factors, including, notably, VEGF. In addition to mediating cell adhesion to, and cell migration on, a variety of extracellular matrix

molecules relevant to vascular development (e.g. fibronectin, collagens, laminins, vitronectin, von Willebrand factor, thrombospondin), integrins also mediate intracellular signalling events involving various protein kinases, small GTPases etc. These, in turn, control aspects of cytoskeletal organization and cell motility, as well as the regulation of cell cycle progression, apoptosis and gene expression. Therefore integrins occupy a central position in any consideration of vascular development; they are regulated by growth factors known to regulate the process of vacularization, they mediate exactly those cell biological processes (adhesion, migration, proliferation, survival and differentiation) needed to organize a vasculature and they are expressed by the cells involved (endothelial cells, pericytes, smooth muscle cells). There is, in fact, a large and growing body of evidence implicating various integrins and integrin ligands in vascular development (Varner et al, 1995; Hynes and Bader, 1997; Bader et al, 1998). However, it is not clear exactly which integrins are the most important nor exactly what each of them does. One major body of work bearing on the possible roles of integrins in vasculogenesis and angiogenesis involves the use of blocking reagents (antibodies, peptides, peptidomimetics) to inhibit the functions of various integrins. This approach has been used most intensively to investigate the functions of αv integrins. These represent a subset of the integrin family sharing a common αv subunit in combination with one of five different ß subunits (ß1, ß3, ß5, ß6, ß8). Endothelial cells can express at least αvß3 and αvß5 and perhaps αvß1 (since they do express ß1, although resting endothelial cells express little or no αvß3. However, this integrin is markedly upregulated on vessels undergoing angiogenesis (Brooks, et al, 1994a; 1995; Friedlander et al, 1996). Cheresh and his colleagues have shown that monoclonal antibodies or peptides that selectively bind αvß3 or αvß5 can inhibit vasculogenesis during early quail embryo development (Drake et al, 1995) and angiogenesis in the chicken chorioallantoic membrane both during normal development (Brooks et al, 1994a) and in response to FGF-2 or VEGF (Brooks et al, 1994a; Friedlander et al, 1995) or tumor implants (Brooks et al, 1994a,b). They have also shown inhibition of angiogenesis in response to tumor implants on human skin transplants to mice (Brooks et al, 1995) and during neovascularization in the murine retina (Friedlander et al, 1996) and another group has provided corroborative data in the latter system (Hammes et al, 1996). Cheresh and colleagues could further demonstrate that different angiogenic stimuli apparently rely on either αvß3 (FGF-2, TNFa) or αvß5 (VEGF, phorbol esters) (Friedlander et al, 1995) and have shown that αvß3 can bind the matrix metalloprotease, MMP-2, in a fashion that contributes to an invasive response and to angiogenesis (Brooks et al, 1996, 1998). These results have stimulated a lot of interest, not least because of the potential use of blocking reagents for therapy of a variety of disorders including tumor angiogenesis and blindness caused by retinal neovascularization.

Results on mouse strains lacking specific integrins also implicate several different integrins in vasculogenesis and angiogenesis. Ablation of α5ß1 integrin or its ligand, fibronectin, both cause major disruptions in development of extraembryonic (yolk sac) and embryonic (heart, aorta) vasculature. In both cases, endothelial cells do differentiate; that is, the VEGF/VEGFR2-mediated induction of angioblasts is intact. However, absence of either α5ß1 or fibronectin disrupts vessel formation in a fashion somewhat reminiscent of the defects seen in embryos lacking VEGFR-1. Clearly, interactions of endothelial cells with fibronectin play an important role in these early steps and there exists a distinct possibility that there is regulation of α5ß1expression or function by VEGF/VEGFR-1 or that this signalling system cooperates with the α5ß1-FN regulated responses. This result conforms with the inhibition of early vascular development by anti-ß1 antibodies (Hynes and Bader, 1997; Hynes et al, 1999). In contrast with this concordance between antibody blocking and genetic ablation results, some other studies show less convergence. Although antibody blockade of α1ß1and α2ß1 integrins blocks angiogenesis in the CAM, ablation of the α1 gene yields viable, fertile animals with no evidence of vascular defects. Since α1ß1 and α2ß1 both act as collagen and

laminin receptors, it is possible that they serve overlapping and to some extent redundant roles (Hynes and Bader, 1997; Hynes et al, 1999). However, it is already clear that ablation of the αv integrin gene yields results that are difficult to reconcile with the results of αv-inhibitors (Bader et al, 1998). αv-null mouse embryos develop an apparently normal yolk sac and early embryonic vasculature (Bader et al, 1998) in marked contrast with the blockade of quail dorsal aorta formation (Drake et al, 1995) or chicken chorioallantoic angiogenesis (Brooks et al, 1994a) by antibodies directed against αvß3. Granted that the systems employed in these studies are different, the two sets of data differ greatly in their implications for the importance of αv integrins in early vascular development. Indeed, 20% of αv-null embryos develop to term and are born alive, although they die promptly (Bader et al, 1998). There is extensive vasculogenesis and angiogenesis in most organs and tissues in the absence of all five αv integrins. Although αv-null embryos consistently develop defects in their brain vasculature, the basic endothelial processes of proliferation, migration, tube formation and branching, and basement membrane assembly all occur.

We know of a variety of growth factors and receptors which are clearly implicated in controlling vasculogenesis and angiogenesis, although exactly what they all do is not yet clear. Most particularly we do not know how they do what they do; that is, which are the intermediate molecules that they control? That is where integrins and their ligands are functional. Some of these molecules clearly are regulated by VEGF and other factors which are not known yet. Integrins and their ligands clearly do play important roles in the cell biological processes necessary for vessel development (adhesion, migration, proliferation, survival, differentiation, matrix formation) but it is unclear exactly which ones are most important in the different processes. Indeed, the answers to those questions may differ depending on the vascular bed or the angiogenic stimulus. It may well be, that there is more than one form of angiogenesis. It could be that yolk sac vasculature relies primarily on α5ß1-FN interactions and less, or not at all, on αv integrins, whereas retinal or tumor vasculatures may be more dependent on αv integrins and their ligands. More detailed studies of the expression patterns, regulation and functions of different integrins and their ligands in response to different angiogenic growth factors are clearly necessary. Vessel development and remodelling involve multiple cell biological processes that need to be well coordinated to yield a functional vasculature. One can suppose that such a complex process, involving as it does, several different cell types acting in concert, would require regulation by multiple adhesive proteins. It will be a fascinating challenge to unravel the regulatory networks and coordinated functions of all these players. The potential results from a detailed understanding of these processes is significant both in terms of the underlying biology and in opportunities for intervention in diseases involving dysregulation of vessel growth.

Altogether, our genetic analyses are of particular interest, both scientifically and clinically, since there is considerable interest in the possibility that blockade of αv integrins, employing antibodies, peptides or peptidomimetics targeted against αv integrins, could be useful clinically in treatments of angiogenesis of tumors (Brooks et al, 1994b, 1995) or in the retina (Friedlander et al, 1996; Hammes et al, 1996). It would be valuable to have an independent genetic validation of the importance of αv integrins in these processes and to elucidate the molecular mechanisms of the regulatory networks and coordinated functions of the essential players.

METHODS

Main technical and molecular expertise of the laboratory in relation to the project

1. Screening of genomic libraries and molecular cloning of genes and cDNAs, vectors and various gene targeting constructs

2. Gene targeting in ES cells and mice: the application of the homologous recombination technique in mouse embryonic stem cells using the Cre/loxP technique as well as the flp/frt technique. Injection of targeted mouse ES cells into blastocyst and blastocyst transfer into uterus.
3. Application of transgenic mouse technology and mouse embryology: whole mount and histological analysis, analysis of organ development by light- and immunofluorescence microscopy (incl. confocal microscopy), with a special focus on blood vessel development in the brain and retina, histopathology.
4. Gene expression analyses by Northern blot and RT-PCR.
5. Protein analysis by Western blot and ELISA.
6. Culturing of primary mouse embryonic cells and mouse embryonic stem cells (ES cells), generation and analysis of embryoid bodies from ES cells under specific differentiation condition (e.g. endothelial cell differentiation).

Ultrastructural studies by transmission electron microscopy

These studies have been performed in collaboration with Dr. Hartwig Wolburg (Institute of Pathology, University of Tübingen, Germany).

Transgenic mice used for Cre-mediated gene ablation experiments

For Cre-mediated recombination the following mouse strains were used in interbreeding experiments and kindly provided by different members of the SPP1069 and scientific community.

tie-1-*Cre* (Gustafsson et al, 2000; kindly provided by Dr. R. Fässler; Max-Planck-Insitute for Biochemistry, Martinsried, Germany), tie-2-*Cre* (Theis et al, 2001; kindly provided by Dr. Urban Deutsch, Max-Planck-Institute, Bad Nauheim, Germany), PGK-*Cre* (Lallemand et al, 1998; kindly provided by Dr. Peter Lonai,), Nestin-*Cre* (Tronche et al, 1999; kindly provided by Dr. R. Klein, Max-Planck-Institute for Neurobiology, Martinsried, Germany), ROSA26R lacZ-responder line (Soriano et al, 1999). The constitutive αv gene knockout mice have been generated and analysed by myself during my postdoctorate in the laboratory of Dr. R.O. Hynes (Bader et al, 1998; kindly provided by R.O. Hynes, Massachusetts Institute of Technology, Cambridge, MA, USA).

RESULTS

The overall aim of our project was the generation of conditional αv integrin gene knockout mice to study the function of the αv integrin gene in physiological and pathological angiogenesis in mice and to reveal the underlying molecular mechanisms. Our project has been funded in all three consecutive funding periods of the SPP1069. During the first funding period we finished our ultrastructural studies on the αv-null embryos and generated the conditional αv integrin allele and lacZ-knockin αv integrin allele in ES cells and mice. This formed the base for the subsequent two funding periods, where we performed our phenotype analysis of mutant mice with selective ablations of the αv integrin gene.

Analysis of vascular morphogenesis deficits in αv integrin null embryos

Constitutive αv integrin gene knockout in mice results in two classes of αv integrin mutant mice, approx. 80% of all αv null embryo dying at around E10.5 with placental defects. The remaining approx. 20% αv null embryos do not show placental deficits and develop intracerebral hemorrhages at mid-gestation and die shortly after birth (Bader et al, 1998). At this time a key question was whether the hemorrhage arises from primary defects in vascular endothelial cells, pericytes, or from

other causes, such as defective brain parenchyma. We have previously reported normal initiation of cerebral vessels comprising branched tubes of endothelial cells in brains of αv mutant embryos (Bader et al, 1998). During the first SPP1069-funding period, we further analysed the nature of the mutant brain vasculature in my group (Dr. Markus Keller, postdoc; Matthias Pöckl, technical assistant, both funded by the SPP1069) in collaboration with Dr. H. Wolburg (Institute of Pathology, University of Tübingen) by ultrastructural studies. We could show that the onset of hemorrhage is not due to defects in pericyte recruitment. Additionally, most αv-null vessels display ultrastructurally normal-looking endothelial-pericyte associations and normal interendothelial cell junctions. Thus, endothelial cells and pericytes appear to establish their normal relationships in cerebral microvessels. However, by both light and electron microscopy we detected defective associations between cerebral microvessels and the surrounding brain parenchyma, composed of neuroepithelial cells, glia, and neuronal precursors (McCarty et al, 2002). These data suggest a novel role for αv integrins in the association between cerebral microvessels and central nervous system parenchymal cells. We published our findings together with data from the laboratory of Dr. R.O. Hynes (MIT, Cambridge, MA, USA) in the report McCarty et al (McCarty et al, 2002).

Conditional αv integrin gene knockout in mice

In the course of the first funding period we successfully applied Cre/loxP- and FLP/frt-based conditional gene knockout technology to generate the floxed αv allele and the lacZ-knockin αv integrin allele in ES cells. Subsequently, we used these targeted ES cell clones to successfully generate the corresponding mice in my laboratory. We analysed and confirmed the presence of wild type allele, the loxP-sites of the floxed αv integrin allele or transgene (lacZ-knockin) in mice by Southern blotting, PCR-based technology and DNA-sequence analysis. PCR-genotyping of mice from various lines and progeny of crosses were performed with allele-specific probes and primer combinations to analyse the distribution and survival rate of mutant embryos and adult mice. In addition, we spent considerable time and effort in our analyses to show that (i) Cre/loxP-dependent recombination of the floxed αv allele results in a recombined αv-null allele in mice and (ii) the lacZ-reporter gene is functional in αv-lacZ mice.

Generation of a conditional αv integrin gene knockout allele and a lacZ-knockin αv integrin allele in mice

We constructed two different gene targeting vectors, GT1 (floxed αv/lacZ/neo-TK, Fig. 1 and data not shown) and GT2 (floxed αv/neo-TK; Fig. 2) to generate conditional αv integrin alleles. Both gene targeting vectors have similar modules: a) exon1 of the mouse αv integrin gene is flanked by loxP-sites to inactivate the gene upon Cre-expression and b) a selection cassette (neo-Tk) for positive and negative-selection is flanked by directly repeated frt-sites which allow its removal by transient expression of FLP-recombinase in ES cells. GT1 differs from GT2 since GT1 includes a lacZ-reporter gene which is located 3' of the loxP2-site in intron 1. After Cre-mediated recombination the expression of the lacZ-reporter gene is mediated by the αv integrin promoter (Fig. 1). The lacZ-expression should be useful to detect two events (i) Cre-mediated recombination and (ii) αv integrin gene expression.

We obtained targeted ES cells carrying either the αv^{flox} or the αv^{lacZ} allele in murine ES cells in a two-step procedure. In brief, the procedure for the αv^{flox} allele is described (Fig. 2). In step 1, we targeted the αv gene by homologous recombination-approach. The gene targeting vector used to obtain the αv^{flox} allele carries two loxP-sites placed upstream of exon 1 (loxP1) and in intron 1 (loxP2)

of the murine αv integrin gene. Cre-recombination excises part of the promoter region, the entire exon 1 and a short part of intron 1 of the αv integrin gene. A pgk-neo;pgk-tk tandem selection-cassette required for ES cell targeting and flanked by Flp-recombinase recognition-sites (frt) was positioned downstream of the loxP2-site in intron 1. In step 2, we removed the selection-cassette present in the targeted alleles by FLP-frt-based recombination technology. We identified the wild type and targeted αv allele harboring loxP1- and loxP2-site and the selection-cassette in homologous ES cell recombinants of step1. After step2, heterozygous αv^{flox} ES cell clones devoid of the entire selection-cassette due to transient expression of the FLPe recombinase in targeted ES cells were characterized (Fig. 2).

We performed injections of targeted ES cells into blastocyst and obtained mouse lines derived from two separate ES cell clones for each mutation. Using Southern blotting, PCR-based technology and DNA-sequence analysis we identified the expected wild type and genetically modified αv alleles in the corresponding mice (data not shown).

The mouse lines carrying the following modified αv integrin alleles were breed to establish enough mice for further analysis: (i) a Cre/loxP-based conditional αv integrin allele (**floxed** αv **allele; Fl**) to achieve a conditional αv integrin gene knockout, such as in endothelial or perivascular cells, in mice and cells derived from mutant mice, and (ii) a lacZ-reporter gene 'knock in' αv integrin allele (**αvlacZ allele,** αv^{lacZ}) to analyze the αv integrin-specific gene expression *in situ,* and in particular, to specify its expression in vascular cell type(s) dependent on the vascular beds or organs at certain stages of embryonic development and in adult life. Using these genetic approaches, we started to evaluate the potential roles and functional properties of αv integrins *in vivo*.

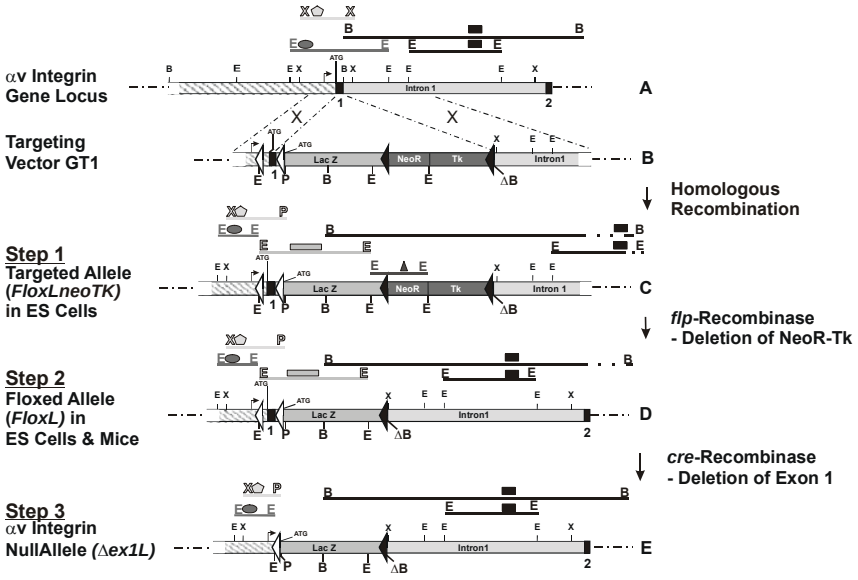


Fig. 1: Targeting of the αv integrin locus by a conditional lacZ-knockin allele. Strategy to replace exon 1 of the αv integrin gene by a lacZ reporter gene after Cre-mediated recombination. After homologous recombination of the targeting vector into the αv integrin locus (A-C) the neo-tk cassette is removed by the *flp*-recombinase (D). Exon 1 can subsequently be removed by tissue-specific expression of active *Cre*-recombinase (E). Solid triangles represent *frt* sites (recognition sequence for *flp*-recombinase, open triangles represent the recognition sequences for the *Cre*-recombinase, the *loxP* sites. Restriction fragments used for Southern blot analysis are indicated (circle: 5' probe; filled rectangle: 3' probe, half-open rectangle: lacZ probe; triangle: neo-probe). Arrow: transcription start; B, BamHI, E, EcoRI; P, PstI; X, XbaI..

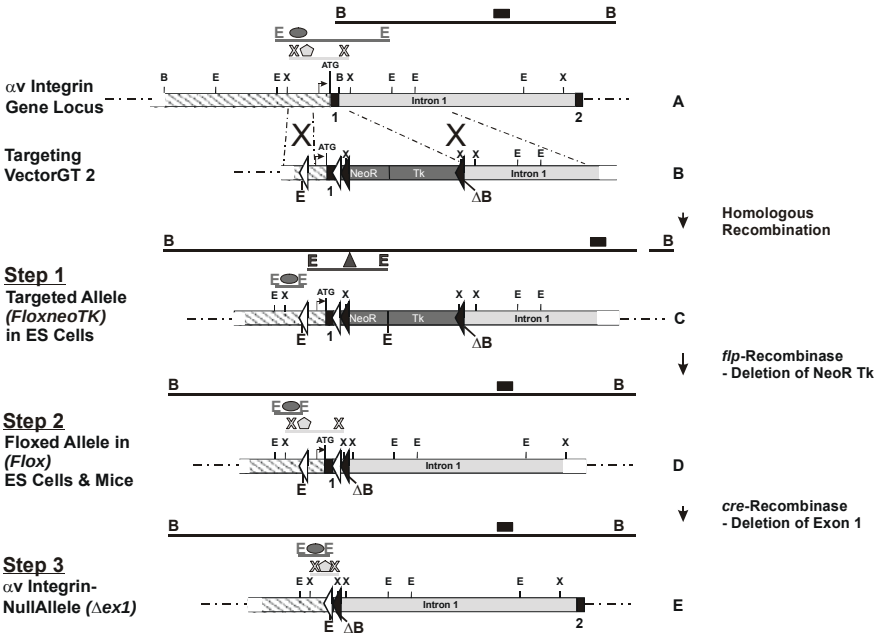


Fig. 2: Targeting of the αv integrin locus by a conditional αv integrin gene. Strategy to ablate exon 1 the αv integrin gene by active *Cre*-recombinase. After homologous recombination of the targeting vector into the αv integrin locus (A-C) the neo-tk cassette is removed by the *flp*-recombinase (D). Exon 1 can subsequently be removed by tissue-specific expression of active *Cre*-recombinase (E). Solid triangles represent *frt* sites (recognition sequence for *flp*-recombinase, open triangles represent the recognition sequences for the *Cre*-recombinase, the *loxP* sites. Restriction fragments used for Southern blot analysis are indicated (circle: 5‘ probe; filled rectangle: 3‘ probe; triangle: neo-probe). Arrow: transcription start; B, BamHI; E, EcoRI; P, PstI; X, XbaI.

LacZ-expression analysis of αv^{lacZ} mice at embryonic stages

We generated a stable lacZαv integrin allele in mice from the floxed αvlacZ mice by interbreeding floxed αvlacZ mice with the PGK-Cre deleter mouse (deleter mouse, kindly provided by Dr. Peter Lonai, Lallemand et al, 1998). This genetic approach led to the Cre-mediated recombination replacing the complete coding region of exon 1 and a short region of intron 1 in the αv gene by a lacZ-reporter gene. Therefore, the targeted allele should be a αv-null allele and the lacZ-knockin gene should be under the control of the endogeneous αv integrin gene regulatory sequences (Fig. 1). Since this recombination also occurred in the germ cells we could establish a stable αv^{lacZ} mouse line. Heterozygous αv^{lacZ} mice are viable and homozygous mice are embryonic/perinatal lethal showing the same phenotypes (data not shown) as the constitutive αv-null allele (Bader et al., 1998). Our lacZ-expression studies of embryos at different developmental stages by ß-Gal whole mount-stainings or histological sections show that the αv^{lacZ} allele is expressed in different cell types and organs, such as placenta, yolk sac, heart, AER-region of developing limbs, blood vessels, cartilage and others (Fig. 3; data not shown). We focussed our lacZ-expression analysis on the brain of heterozygous αv^{lacZ} mice at embryonic stages when αv-null mice develop the brain vasculature phenotype (dilated brain capillaries and intracerebral hemorrhages) in order to identify the cell-type(s) where the αv integrin gene is functional. LacZ-expression was detected in blood vessels of the brain, choroids plexus and in the hyaloid vascular plexus of the eye by conventional ß-Gal-stain (Fig. 3). We applied antibodies recognizing ß-Gal, endothelial-specific PECAM or the chondrosulfate proteoglycan NG2 (recognizing pericytes) in immunohistological analyses to assess whether lacZ-

expression in blood vessels occurs in endothelial cells and/or pericytes. A significant lacZ-localization was observed in blood vessel pericytes, whereas a obvious lacZ-expression in endothelial cells or other cell types of the brain parenchyma was not observed. This finding was surprising and we interpreted our results in at least two ways. First, αv integrins of pericytes may be important mediators of brain blood vessel development. Second, it is known that the ß-Gal-reporter system is not very sensitive. Therefore we may not detect very low levels of αv integrin gene activity in situ, such as. αv integrin gene expression in endothelial cells or neuroepithelial cells as we have expected from report by Hirsch et al (Hirsch et al, 1994).

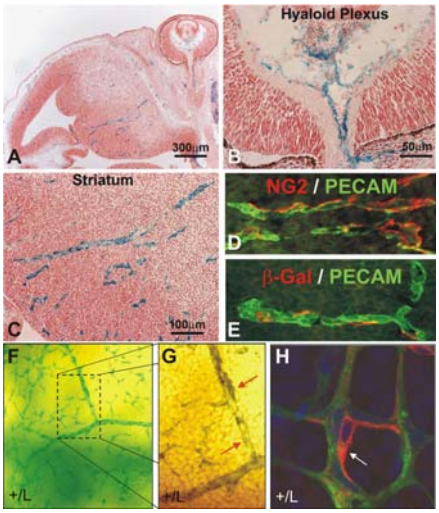


Fig. 3: LacZ-expression analysis of lacZ-αv integrin mice at embryonic stage at E12.5/13.5 (A-E) and in postnatal retina (F-H). (A-C) Brain blood vessels and the hyaloid plexus of the developing eyes are ß-Gal-positive. (D, E) Double-immunofluorescence microscopic analysis of brain blood vessels in the ganglionic eminence using anti-PECAM antibodies (endothelium; green, D, E) combined either with anti-NG2 antibodies (pericytes; red; D) or with anti-ß-Gal (lacZ, red; E). lacZ-expression in blood vessels is not localized in the endothelial lining, however anti-ßGal antibodies recognize perivascular cells (pericytes). (F-H) Whole mount ß-Gal-staining (F, G) and double-immunofluorescence microscopic analysis (H) of postnatal retina localize lacZ-expression in pericytes. (H) Endothelial cells are labelled with FITC-isolectin B4 (green) and ß-Gal is recognized with anti-ßGal antibodies (red). Periendothelial cell (pericyte) are ß-Gal-positive.

The αv^{flox} allele is functional and Cre-mediated recombination renders the αv^{flox} allele to an $\alpha v^{\Delta ex1}$ allele, which is a null allele in mice

Genotype distribution and offspring survival rate of embryos and adult mice derived from heterozygous αv^{flox} breeding pairs, PCR-genotyping of genomic DNA were performed with allele-specific primer combinations. Mice heterozygous or homozygous for the αv^{flox} allele were observed at Mendelian frequencies, were phenotypically indistinguishable from wild type littermates, and reproduced normally (data not shown). Next we tested whether the αv^{flox} allele is functional for Cre-recombination and whether the recombined $\alpha v^{\Delta ex1}$ allele represents a null allele in mice. Therefore a mouse line carrying an $\alpha v^{\Delta ex1}$ allele was generated by crossing αv^{flox} mice with PGK-Cre transgenic mice expressing Cre ubiquitously, including in the germline (deleter mouse, kindly provided by Dr. Peter Lonai, Lallemand et al, 1998). The $\alpha v^{\Delta ex1}$ allele lacked the promoter, exon 1 and intron 1 sequences of the αv integrin gene (Fig. 2). The deletion of similiar sequences in the constitutive αv integrin gene knockout (Bader et al, 1998) resulted in a null allele. Our analysis of progeny from breeding pairs heterozygous for $\alpha v^{\Delta ex1}$ showed that wild type and heterozygous embryos, neonatal and adult mice are phenotypically normal, whereas embryos or neonates homozygous for the $\alpha v^{\Delta ex1}$ allele manifested phenotypes at a frequency (data not shown) identical to those observed in αv-null mice of the constitutive αv integrin gene knockout mice as described earlier (Bader et al, 1998). This demonstrates that the floxed allele is functional and renders to an $\alpha v^{\Delta ex1}$ allele after recombination, which is a null allele.

Mating schemes devised for Cre-dependent conditional ablation of the αv integrin gene

For the nomenclature of the different mutant αv allele occurring in this study the mutant gene knockout allele of the constitutive αv integrin gene knockout is indicated as (-) αv allele (αv^{-}) and the conditional αv integrin gene knockout allele as floxed αv allele (αv^{flox}). The constitutive homozygous mutant mouse ($\alpha v^{-/-}$) is also called αv-null.

Mouse mating schemes were devised for conditional gene inactivation and aimed at discovering embryonic and postnatal functions of αv integrins. We bred mice to obtain two different groups of mice according to their gender and compound genotypes. Cre-transgenic mice were mated with heterozygous $\alpha v^{+/flox}$ or $\alpha v^{+/-}$ mice and offspring males with the compound genotype $\alpha v^{+/flox}$Cre+ or $\alpha v^{+/-}$Cre+ (heterozygous for the αv^{flox} allele or αv^{-} and transgenic for Cre (Cre+) were used as group-A mice. From our established $\alpha v^{flox/flox}$ mouse line we used females with the genotype $\alpha v^{flox/flox}$;Cre- (homozygous for the αv^{flox} allele, but without Cre-transgene; Cre-) as group-B mice.

Cre-dependent ablation of αv integrins were expected in progeny from group-A/B-breeding pairs. According to Mendel, the alleles of group-A/B breeding pairs are expected to segregate as four different compound genotypes in their progeny at the following frequency: 25% (flox/flox; Cre+) or (-/flox; Cre+); 25 % (flox/flox; Cre-) or (-/flox; Cre-); 25 % (+/flox; Cre+); 25 % (+/flox; Cre-). For simplification of the genotype terminology in the following text, mice carrying the genotype (flox/flox; Cre+) or (-/flox; Cre+) will be called mutant αv mice, since only in these mice the genetic inactivation of both αv alleles can occur. Mice carrying the genotypes (flox/flox; Cre-); (-/flox; Cre-); (+/flox; Cre+) or (+/flox; Cre-) are called control αv mice. The occurrence of a mutant phenotype in mutant αv mice depends on the efficiency of Cre-recombination and the functional importance of the floxed gene in the corresponding cellular or organotypic context. In mice carrying the genotype $\alpha v^{-/flox}$Cre+ only one floxed αv allele needs to undergo recombination to create tissue null for the gene. No phenotypes were associated with mice carrying $\alpha v^{+/flox}$, $\alpha v^{flox/flox}$, $\alpha v^{+/-}$, $\alpha v^{-/flox}$ or compound $\alpha v^{+/flox}$;Cre+ genotypes. $\alpha v^{+/flox}$, $\alpha v^{+/-}$ were used as controls in most experiments, but occasionally also $\alpha v^{+/flox}$;Cre+ were added as controls.

Tie1-Cre-dependent ablation of endothelial αv integrins does not cause embryonic or perinatal vascular phenotypes

To achieve enothelial selective gene ablation in mice we used two independent transgenic mouse lines, tie1-Cre (Gustafsson et al, 2001) and tie2-Cre (Theis et al, 2001), expressing Cre under endothelial cell-active promoters already at embryonic stage E8-8.5. First, we tested whether endothelial Cre-activity of group-A males $\alpha v^{+/flox}$Cre+ or $\alpha v^{+/-}$Cre+ occurs in their embryonic progeny by breeding the males with lacZ-responder Rosa26R female (Soriano, 1999). Whole-mount ß-Gal staining of embryos and tissue sections confirmed that in the endothelial lining of the embryonic vasculture, including brain blood vessels (data not shown). LacZ-expression was induced by Cre-mediated recombination of the lacZ-responder gene. Next, we set up specific group-A/B-breedings (see above) to generate tie1-Cre dependent ablation of the αv integrin gene in their progeny. The analysis of their genotypes, survival rate and phenotypes during embryonic development (E11.5-E18.5; n=44) and postnatal stages (> 3weeks to 6weeks; n=45) showed that mutant and control αv mice developed normally and were born at a Mendelian frequencies (data not shown). No obvious Cre-dependent lethality or vascular deficits in conditional mutant αv mice were observed during embryonic development or until approximately six weeks of age as analyzed. Older animals develop deficits in body homeostasis (data not shown). Similar results have been obtained from tie2-Cre

expressing mutant αv mice (αv flox/flox;Cre+; data not shown). The efficiencies of tie1-Cre dependent recombination of the αv^{flox} allele in various tissues was assessed by semi-quantitative PCR-analysis assuming the equal amplification efficiency of wild type, αv^{flox} and $\alpha v^{\Delta ex1}$ alleles in each PCR-reaction. The PCR-analyses showed combinations of αv allele-specific PCR DNA-fragments dependent on the tissues and genotype of mice. The abundance of the αv^{flox} derived PCR-fragment compared to the $\alpha v^{\Delta ex1}$ allele-specific PCR-fragment was higher in all examined tissues from $\alpha v^{flox/fox}$Cre+ mice. This was not surprising, since, in general, the majority of cells in tissues are nonendothelial cells, where no Cre-activity was expected, and the relative contribution of endothelial cells (Cre-active) from blood vessels is naturally minor. However, when DNA from microdissected mesenteric blood vessels was used to reduce the 'contamination' with nonendothelial tissue as template in PCR assays, we preferentially detected the recombined $\alpha v^{\Delta ex1}$ allele (data not shown). From our lacZ-expression analysis of lacZ-responder mice and the PCR-analysis we concluded that the Cre-recombination of the αv^{flox} alleles occurs efficiently in endothelial cells resulting in a grossly αv-deficient endothelial vasculature early during development and consequently, also in adult mice.

Nestin-Cre-dependent ablation of αv integrins causes intracerebral hemorrhage

To assess whether the ablation of αv integrins in the neural parenchyma causes the aberrant embryonic vascular brain morphogenesis observed in constitutive αv-null embryos (Bader et al, 1998; McCarty et al, 2002), we used conditional floxed αv mice in combination with a Cre-transgene under the control of the nestin promoter/neuron-specific enhancer (Tronche et al, 1999). We characterized the nestin-Cre-activity in embryos derived from breeding pairs of lacZ-responder ROSA26R females with group-A αv integrin males (see above). ß-Gal staining was detected throughout the embryonic neural tube as early as embryonic day E10 (data not shown) and within the developing cortical wall and ventricular neuroepithelium of transverse brain sections of E12.5 animals and in postnatal retina (data not shown). This was consistent with the known expression pattern of nestin-Cre mouse line (Tronche et al., 1999; Graus Porta et al, 2001). The nestin-Cre dependent ablation of the αv^{flox} gene was carried out following the breeding scheme and types of analysis as mentioned above. The genotypes of progeny during embryonic development (E11.5-E18.5; n=94) and postnatal stages (> P0; n=133) were observed at almost Mendelian ratios (data not shown). Most importantly, our whole-mount light microscopy and histological analysis revealed that all $\alpha v^{flox/flox}$Cre+ embryos analyzed at E12.5 or later stages showed intracerebral hemorrhages (data not shown) resembling the αv null E12.5 embryos of both constitutive gene knockouts either for αv integrin (Bader et al, 1998) or ß8 integrin (Zhu et al, 2002). Noteworthy, in these conditional mutant αv mice, we have not seen any additional αv-null or ß8-null phenotypes of the constitutive gene knockouts, such as placental deficits, cleft palate or intestinal bleeding. At neo- and postnatal stages, conditional αv mutant mice can be easily distinguished from control mice by their (i) deformed head (hydrocephalic) due to intracerebral hemorrhages, and (ii) smaller body size first obvious at around P10 (data not shown). A small fraction, approx. 20% of all αv-mutants were lost due to postnatal lethality at varying ages, most likely caused by reoccurring intracerebral bleedings and damage of ischemic brain tissue leading to neurological deficits and lethality. Adult mutant mice also manifest abnormal gait in the hind limbs several weeks after birth, which may be secondary effects due to the significant neural damage in the ischemic brain. To confirm the αv integrin genotypes and to analyse whether the αv integrin gene was inactivated in the brain parenchyma of mutant αv mice, we monitored Cre-recombination of the αv^{flox} allele in vivo at the DNA level by semi-quantitative PCR using genomic DNA. Recombination in neural tissue was readily detectable and seemed to occur efficiently. The PCR-fragment specific for the intact αv^{flox} allele was amplified at a much lower signal

level which was expected, since brain tissue contains meningeal cells, and endothelial cells, where Cre should be not active, and therefore 'contaminating' the dissected neural tissue. The relative equal amplification efficiency of wild type, αv^{flox} and $\alpha v^{\Delta ex1}$alleles in each PCR-reaction was assumed and the percentage of amplified $\alpha v^{\Delta ex1}$-specific PCR-fragments compared to the total sum of all αv allele-derived PCR-products was calculated. We detected a recombination frequency of 80% to 95% in the brain tissue, whereas in tail or liver it was only approximately 10% or less. Considering both set of data, the ß-Gal staining of Cre-expressing lacZ responder mice and the PCR-analysis, we concluded that the αv integrins were efficiently inactivated in embryonic neuroepithelial tissue resulting in a grossly αv-deficient neural tissues.

Nestin–Cre dependent ablation of αv integrins in mice causes vascular deficits in the retina leading to retinal hemorrhage

Our previous studies of αv-null embryos and this report show that the αv integrin gene is important for brain blood vessel morphogenesis (Bader et al, 1998). This rises the question whether in other places of the αv integrin deficient central nervous system vascular phenotypes can occur, e.g. during retinal vascularisation, which proceeds postnatally in mice. During the development of the inner retinal vasculature first one primary vascular plexus layer (PVPL) located in the retina nerve fiber layer (NFL)/ganglion cell layer (GCL) interface and two deeper vascular plexi are formed between postnatal days P4 and P7 and P7 and P26, respectively. The so-called 'deep plexus' develops from the primary layer by sprouting and vacularizes the inner nuclear plexus (INP) and establishes two new layers of vessels at the inner and out border of the INP referred as superficial inner nuclear layer vascular plexus (INPL-1) or deep vaascular plexus 1 (DVPL-1) and the deep inner nuclear vascular plexus layer (INPL-2) or second deep vascular plexus (DVPL-2). The outer plexiform layer (OPL) and outer nuclear layers (ONL) are normally avascular. The ONL/ photoreceptor cells are supplied with oxygen by diffusion from the choriocapillaries (Stone et al., 1995).

Since in αv-null mice of the constitutive αv gene knockout die during embryonic development or perinatally (Bader et al, 1998), we could not assess the role of the αv integrin gene during the physiological vascularisation of the retina which occurs postnatally (P4-P26). However, the generation of viable nestin-Cre and tie1-Cre dependent mutant and control αv integrin mice described in this project allowed us to examine their retinal vasculatures.

Our whole-mount analysis of eyes from tie1-Cre and nestin-Cre mutant αv integrin mice and controls observed spotlike retinal hemorrhages in nestin-Cre mutant αv integrin eyes by light and fluorescence microscopy between P4 to P12, but no hemorrhages were obvious in controls. In order to find out which step(s) of the complex vascular morphogenesis, vascular sprouting and branching are disturbed in mutant retinas, we assessed whole-mount-stained (Isolectin B4 or PECAM) and flat-mounted retinas by confocal microscopy. Our analysis of the retina and its vasculature shows that the overall thickness of the retina and the widths of the three individual retinal nuclear layers were comparable in mutant and control eyes as analyzed by counting the number of cell nuclei layers and by confocal microscopic measurements (data not shown). In addition, our histological analysis did not reveal any gross abnormalities in mutant retinae, except that the cell nuclei in the ganglion layer in the mutant retina appeared occasionally to be organized in a slighty kinky line whereas the corresponding nuclei in control retina form a more straight line. The cellular layers of the outer compartment of mutant retina, the photoreceptor cell layer, pigment cells and the choroid vasculature appeared normal. The PVPL of control and mutant retina are indistinguishable. They developed between P0 and P7, and reached the periphery of the *ora serrata* at P7. While the two deep layer vascular plexi started to form between P7-9 in control retinae, the formation of the DVPL-1 was either

delayed or less pronounced developed, and a distinct DVPL-2 never formed in mutant retinae. Sprouting blood vessels originating from the PVPL and penetrating the IPL in mutant retinae at P9 showed first signs of pathological alterations. They began to form vascular tufts or glomeruloid-like structures at their invading vascular sprouts which subsequently appear to terminate their directed invasion process. By contrast, the examination of the retina from *tie1-Cre* dependent mutant αv integrin mice, the retinal vasculature in both mutant and control mice appears normal and shows the typical three vascular layers (data not shown).

To get more information about the cellular nature of these aberrant retinal vascular sprouts or branches which appear like the result of an uncontrolled accumulation of proliferating cells, we performed BrdU-labelling and immunostaining with endothelial and pericyte-specific markers. This revealed that preferentially cell nuclei of vascular/endothelial cells are BrdU-positive including nuclei of the mutant vascular structures (data not shown). In collaboration with Dr. Hartwig Wolburg (Institute of Pathology, University of Tübingen, Germany) ultrastructural analysis of retinal vasculature and aberrant vascular structures from control and mutant mice were performed by transmission electron microscopy. At the ultrastructural level the aberrant vascular structures could be analysed as compact clusters of cells, most likely endothelial cells (Fig. 12). In addition, we were asking if the aberrant vascular structures are associated with changes in the expression or localisation of basement membrane proteins. By immunostaining with antibodies recognizing fibronectin or collagen IV, we observed strong BM stainings of blood vessels in the primary vascular network, whereas the intensity of staining was less pronounced in the blood vessels of the deep vascular layers in both control and mutant retinae. So far, we have not detected a significant accumulation of deposited basement membrane proteins at mutant retinal blood vessels (data not shown).

Moreover, those vessels which initially appeared to develop normal in the IPL formed similiar aberrant vascular structures either in the INL or shortly after the vessels reached the OPL. In some instances, they were found even in close vicinity of the first ONL. In addition to our finding that a horizontal vascular plexus in the DVPL-1 did not form at the appropriate times, we also recognized that a deep horizontal vascular plexus DVPL-2 was totally absent in mutant retina at later stages, such as P22 and P113. Therefore, one can assume that hypoxic conditions may develop in the mutant retina, which induce neovascularization either of the outer retina by the chorioid vessels or of the PVPL invading the vitreous body. However, we did not observe these pathological signs of neovascularization in the analysed mutant retina. However, immunofluorescence microscopic studies of retinae showed in Creased GFAP-staining in cellular structures of mutant retinae which resemble Müller glia cells whereas GFAP-staining of the corresponding structures in retinae of control mice was very faint or not detectable (data not shown). It will be of future interest to assess whether this is due to pathologically activation caused by hypoxic condition or a delay of differentiation. Müller glial cells express GFAP in early postnatal stages but downregulate GFAP afterwards.

DISCUSSION

In this study we addressed the function of the αv integrin gene during blood vessel morphogenesis. We performed the genetic ablation of the αv integrin gene in endothelial and neuroepithelial cells in mice and show the importance of neuroepithelial αv integrins during blood vessel morphogenesis in the embryonic brain and postnatal retina. The main findings from our project and the published results from other groups will be discussed.

Blood vessel development in the CNS

The embryonic brain and postnatal retina are initially both avascular neuroectodermal derivatives which become vascularized by the process of sprouting angiogenesis. The embryonic brain gets vascularized by invading blood vessels originating from the perineural complexes of the brain at around E10, whereas the retinal vasculature develops shortly after birth. The retinal vasculature forms first a horizontal vascular network located in the retina nerve fiber layer ganglion cell layer interface and subsequently blood vessels from this layer invade the retina to form two deeper inner retinal vascular layers (Stone etal, 1995). Blood vessels of the central nervous system are composed of endothelial cells and pericytes surrounded by a vascular basement membrane. The close neighborhood between cerebral and retinal blood vessels, surrounding neuroepithelial cells during early development and later by neurons and particularly glia, and integrin gene expression and functional studies suggest an interesting model, whereby integrins can exert various functions during vascular morphogenesis on different cell types involved. Proposed functions for integrins have been mediator of morphogenetic processes and coreceptor signalling events, as well as neural cell adhesion providing physical support that maintains proper blood vessel morphology. Further, αv integrins could also operate in a dynamic fashion as communicator between neural cells and blood vessels (Bader et al., 1998). This led us to address the following questions in our funded project: a) in which cell type is the αv integrin functional, in endothelial cells, pericytes and/or neuroepithelial cells? b) which is the functional integrin dimer αvß1, αvß3, αvß5, αvß6 and/or αvß8 operating during blood vessel development in the brain? c) does the αv integrin gene also mediates blood vessel development in the retina, which is also a neuroectoderm-derivative? d) what is the primary cellular deficit and (e) what are the underlying molecular mechanisms?

Blood vessel morphogenesis in the embryonic brain and postnatal retina depends on neuroepithelial αv integrins

We have analysed the constitutive (null), conditional (floxed) and lacZ-knockin αv integrin alleles to reveal the function of this integrin subunit in mice. Our ultrastructural studies of brains from the constitutive αv-null embryos at E12.5/13.5, the time-point when the brain phenotype manifests, show defective associations between cerebral microvessels and the surrounding brain parenchyma (McCarty et al, 2002). These data suggest a novel role for αv integrins in the association between cerebral microvessels and central nervous system parenchymal cells.

Our lacZ-knockin expression analysis demonstrates embryonic αv integrin gene activity in several cell types and organs, e.g. endothelial cells of the dorsal aorta and intersomitic vessels. This is consitent with previous αv intgrin expression data (Hirsch et al, 1994). However, to our surprise the brain from heterozygous αv^{lacZ} mice at embryonic stages when αv-null mice develop the brain vasculature phenotype, show a significant ß-Gal-localization in pericytes of brain blood vessels, whereas a obvious lacZ-expression in endothelial cells or other cell types of the brain parenchyma is lacking. Similiarly, we detect an obvious ß-Gal-staining primarily in pericytes of the retinal vasculature. We interprete our results in at least two ways. First, αv integrins are expressed in pericytes, a potential proper place to mediate brain blood vessel morphogenesis. This fact would be consistent with our ultrastructural data showing defective associations between cerebral microvessels and the surrounding brain parenchyma without an obvious basement membrane defect (McCarty et al, 2002). It could be that pericytic integrins are necessary for proper assembly and/or maintenance of distinct basement membrane components which are crucial for the initiation of neural cell-blood vessel adhesion and communication. Second, it is known that the ßGal-reporter system is not very sensitive *in vivo* and therefore it can be the limiting factor in such analysis. Thus, we may not

detect very low levels of αv integrin gene activity present in endothelial cells or neuroepithelial cells. By contrast, Hirsch et al. (Hirsch et al, 1994) show endothelial and glial αv integrin gene expression during mouse embryonic development. In case αv integrins in pericytes were be important, we could not test this at this time, because no pericyte-specific Cre-mouse line has been avaiable.

Nevertheless, in the meantime, we started our selective αv integrin gene ablation with tie1-Cre and nestin-Cre expressing mice. To our surprise, selective ablation of αv expression in endothelial cells does not manifest any obvious embryonic or neonatal developmental defects and these mice are viable. This result we confirmed using tie2-Cre mice, a second endothelial active Cre-mouse line (Theis et al, 2001). By contrast, our αv integrin ablation in neuroepithelial cells of mice causes exactly the embryonic brain blood vessel phenotype at E12.5 as described in αv-null mice (Bader et al, 1998). We show that defective vascular morphogenesis develops in the forebrain in nestin-Cre dependent αv integrin mutant embryos at E12.5 with distinct features, such as aberrant vascular sprouting vessels in deeper layers of the brain, clusters of endothelial cells at abruptly terminating sprouts and distended blood vessel lumen resulting in bilateral hemorrhages in these brain regions. However, these vessels maintain an intact basement membrane and normal endothelial cell-associated pericytes. Interestingly, this mice are viable and can survive until adulthood. Our study presents evidence that neuroepithelial integrins are functional important during blood vesssel morphogenesis, whereas endothelial αv integrins are not essential in this process. Although, we obeserved a strong αv integrin gene promoter activity in brain and retinal blood vessel pericytes of αvlacZ mice, it appears that pericytic αv integrins do not play an essential functional role in blood vessel development.

Finally, we suggest that the perinatal αv-null phenotype is most likely not lethal due to the defect intracerebral blood vessel morphogenesis, but is caused by the occurrence of a cleft palate as described in class II αv-nulls (Bader et al, 1998).

Genetic analysis of ß integrin subunits in mice

In the course of the SPP-funding period, the constitutive ß8 integrin gene knockout performed in the laboratory of Louis F. Reichardt (HHMI at University of California, CA, USA) was published (Zhu et al, 2002). The report by Zhu et al clearly shows that ß8 integrin null embryos resembling in many details the phenotype of the αv-nulls (Bader et al, 1998; McCarty et al, 2002). They have two stages of lethality, with a majority of mutant dying during embryogenesis and a minority surviving until birth with severe cerebral hemorrhage. This evidence strongly suggests that these two subunits function together as a heterodimer during CNS vascular development. This is further supported by other gene knockout studies of the relevant ß subunits of the five αv integrin dimers showing that no vascular defects were observed in ß3 (Hodivala-Dilke et al, 1999), ß5 integrin (Huang et al, 1999), ß3/ß5 double knock-out mice (McCarty et al, 2002; Reynolds et al, 2002), ß6 integrin knock-out mice (Huang et al, 1996), or nestin-Cre-derived ß1-integrin deficient mice (Graus-Porta et al, 2001).

Taken together, our neuroepithelial αv integrin gene knockout and the genetic data by others show that αvβ8 integrin expressed in embryonic neuroepithelial cells is essential for the proper development of embryonic intracerebral blood vessels and implicates αvβ8 as a neuroepithelial regulator of embryonic brain blood vessel function.

Neuroepithelial αv integrins mediate intraretinal angiogenesis

The biological process forming the retinal vasculature in the whole retina is sprouting angiogenesis guided by glial cells. In the superficial retinal vascular network endothelial cells are guided by preceeding astrocytic network, while the guidance for the formation of the deep retinal vascular networks is performed by Mueller cells (Stone et al, 1995; Fruttiger, 2002). So far, less is known which integrins are functional during this process. Studies using RGD-peptides or αvß3 or αvß5 blocking antibodies have shown to be potent antagonists for neovascularization of the retina under hypoxic conditions (Friedlander et al, 1996; Hammes et al, 1996).

Since αv and ß8 integrin null mice, both are constitutive gene knockout alleles (Bader et al, 1998; Zhu et al, 2002), die embryonically or perinatally, the role of the αvß8 integrin during the vascularisation of the retina under physiological conditions could not be tested. Our analysis of viable conditional tie1-Cre and nestin-Cre dependent αv integrin gene knockout mice demonstrates that retinal angiogenesis proceeds normal in mice where endothelial αv integrins have been ablated. However, retinal angiogenesis in nestin-Cre conditional mutant αv integrin retina is defective. The histological, immunofluorescence/confocal microscopic and ultrastructural studies present evidence that in the absence of neuroepithelial αv integrins the superficial retinal vascular network and the initial first deeper retinal vascular network can form, but the formation of the final second deeper retinal vascular layer is impaired. We show that distinct phenotypic features which develop in mutant retinal blood vessels are similiar to the defective brain capillaries in nestin-Cre dependent mutant αv integrin embryos. These are aberrant vascular sprouting vessels in deeper layers with abruptly terminating sprouts, where clusters of endothelial cells accumulate, resulting occasionally in retinal hemorrhages. Important to note, retinal vessels maintain an intact basement membrane and normal endothelial cell-associated pericytes, whereas the glia appears disorganized. Our study of the αv integrin deficient retinal vasculature is novel and has not been reported in any other genetic studies by other groups in the field. These data support the working model of our project that the αv integrin subunit is an important mediator of blood vessel morphogenesis in the central nervous system. Furthermore, we propose that the αvß8 integrin dimer is the likely functional integrin receptor during physiological retinal angiogenesis and exclude the ß3 and ß5 integrin subunits, since Reynolds et al. (Reynolds et al, 2002) reported that in ß3 or ß5 or ß3/ß5-deficient mice physiological vascularization proceeds normal, but the mice respond with a hypervascularization of the retina with invading blood vessels into the vitreous body under hypoxic conditions.

Taken together, we propose that endothelial αv integrins are not essential for blood vessel development in mice, but the αv integrin subunit on neuroepitheila cells, most likely the αvß8 dimer, is crucial for the proper brain and retinal blood vessel morphogensis in mice.

Open questions

At this point of our analysis we can not differentiate whether glial cells or neurons are the important cell types in the central nervous system which operate in an αv integrin-specific manner during vascular morphogenesis. Using nestin-Cre promoter/enhancer mice in our studies, Cre-mediated ablation of the αv integrin gene occurs already in the neuroectoderm in an early embryonic stage (<E10). Therefore, the αv integrin gene is already inactivated in neuroepithelial cells before they can fully differentiate into glial and neuronal cells. Regarding the underlying mechanisms leading to the observed vascular phenotypes we started to study the expression levels of VEGF and TGFß in extracts of brain and retina from control and mutant mice. The involvement of αvß8 in regulating TGFß bioavailability of TGFß in the brain appears an attractive possibility since it was shown that this

integrin can act in the process of activating the latent form of TGF (Mu et al, 2002). Another putative mechanism by which αvß8 expressed on neuroepithelial cells could regulate vascular morphogenesis is by indirectly controlling the release of angiogenic factors, such as VEGF, through cross talk with receptors such as neuropilin-1. Neuropilin-1 has been shown to be important for proper brain capillary development in the mouse embryro (Gu et al, 2003) and neuropilin-1mutants display aberrant endothelial cell clusters (Gerhardt et al, 2004) resembling to some extent those observed in our studies, although regional differences exists. It is tempting to speculate if the absence of the αvß8 integrin can cause an imbalance of VEGF expression which disturbs the function of the tip cell at the vascular sprouts (Gerhardt et, 2003). Certainly, there are several attractive molecules and cellular mechanisms which in cooperation with αvß8 may potentially be functional in those vascular/neural processes which are impaired in αv integrin mutant mice. For instance, recent reports show cooperative signaling between integrins and Ephs/ephrins (Zou et al, 1999), netrin (Yebra et al, 2003), slits (Stevens and Jacobs, 2002) and semaphorins (Pasterkamp et al, 2003). Since these molecules are guidance and survival factors, it will be interesting to find out (i) whether αvβ8 integrin crosstalks with these or other pathways, (ii) which cell type is functional important for the action of αvß8 – glial and/or neuron – and finally (iii) how these interactions may mediate blood vessel morphogenesis in the brain and retina.

Studies of conditional αv and ß8 integrin alleles in mice

Our data and the genetic studies described by others show that endothelial αv integrins are not important molecules during vascular morphogenesis in the central nervous system, whereas neuroepithelial αv integrins are essential. In our study, we can not differentiate whether the gial or neuronal αvß8 integrin is the functional important integrin, but with the reports by McCarty et al (McCarty et al, 2005) and Proctor et al (Proctor et al, 2005) it is now evident, that the glial αvß8 integrin is the functional integrin during brain blood vessel morphogenesis. This was possible, since in these studies neuroepithelial, glial and neuronal specific ablation of the αv integrin gene were performed, which makes the qualitative difference to our study. It is important to note that we observed the vascular defects in our nestin-Cre mutant embryos exactly at the same time-point where we detect the αv-null phenotype in the constitutive gene knockout at E12.5/E13.5. However, in the reports by the other groups the phenotypes manifest at later stages even sometimes as late as perinatally. This supports our model that the absence of neuroepithelial αv integrins already at the neuroectodermal lineage is not compatible with proper brain and retinal blood vessel morphogenesis. Whether this early ablation of the αv integrin gene is important for the development of the retinal vascular defect as reported in our study and not by others, is not clear yet.

Perspectives

It will be interesting to find out whether αvβ8 integrin crosstalks with guidance and survival factors or other pathways as discussed already in order to get a closer insight into underlying molecular and cellular mechanisms of the vascular/glial αvß8-deficiencies. This needs isolation of primary cells of the brain and retina as well as organ culture experiments. It will be of equal importance to find out which other gene-knockout in mice will phenocopy the αvß8 integrin-deficiency.

REFERENCES

Arroyo AG, Taverna D, Whittaker C, Strauch UG, Bader BL, Rayburn H, Crowley D, Parker CM, Hynes RO: *In vivo* roles of integrins during leukocyte development and traffic: Insights from the analysis of mice chimeric for α5, αv, and α4 integrins. *J Immunol* 165: 4667-4675, 2000.

Bader BL, Rayburn H, Crowley D, Hynes RO: Extensive vasculogenesis, angiogenesis and organogenesis precede lethality in mice lacking all αv integrins. *Cell* 95: 507–519, 1998.

Beck LJr, D'Amore PA: Vascular development: Cellular and molecular regulation. *Faseb J* 11: 365-373, 1997.

Brooks, PC, Clark, RA, Cheresh DA: Requirement of vascular integrin alpha v beta 3 for angiogenesis. *Science* 264: 569-571, 1994a.

Brooks PC, Montgomery AM, Rosenfeld M, Reisfeld RA, Hu T, Klier G, Cheresh DA: Integrin alpha v beta 3 antagonists promote tumor regression by inducing apoptosis of angiogenic blood vessels. *Cell* 79: 1157-1164, 1994b.

Brooks, PC, Stromblad, S, Klemke, R, Visscher D, Sarkar FH, Cheresh DA: Anti integrin alpha v beta 3 blocks human breast cancer growth and angiogenesis in human skin. *J Clin Invest* 96: 1815-1822, 1995.

Brooks PC, Stromblad S, Sanders LC, von Schalscha TL, Aimes RT, Stetler-Stevenson WG, Quigley JP, Cheresh DA: Localization of matrix metalloproteinase MMP-2 to the surface of invasive cells by interaction with integrin alpha v beta 3. *Cell* 85: 683-693, 1996.

Brooks PC, Silletti S, von Schalscha TL, Friedlander M, Cheresh DA: Disruption of angiogenesis by PEX, a noncatalytic metalloproteinase fragment with integrin binding activity. *Cell* 92: 391-400, 1998.

Drake CJ, Davis LA, Little CD: Antibodies to ß1 integrins cause alterations of aortic vasculogenesis, in vivo. *Dev Dyn* 193: 83-91, 1992.

Drake CJ, Cheresh DA, Little CD: An antagonist of integrin alpha v beta 3 prevents maturation of blood vessels during embryonic neovascularization. *J Cell Sci* 108: 2655-2661, 1995.

Francis SE, Goh KL, Hodivala-Dilke K, Bader BL, Stark M, Davidson D, Hynes RO: Central roles of α5ß1 integrin and fibronectin in vascular development in mouse embryos and embryoid bodies. *Arterioscler Thromb Vasc Biol* 22: 927-933, 2002.

Friedlander M, Brooks PC, Shaffer RW, Kincaid CM, Varner JA, Cheresh DA: Definition of two angiogenic pathways by distinct alpha v integrins. *Science* 270: 1500-1502, 1995.

Friedlander M, Theesfeld CL, Sugita M, Fruttiger M, Thomas MA, Chang S, Cheresh DA: Involvement of integrins alpha v beta 3 and alpha v beta 5 in ocular neovascular diseases. *Proc Natl Acad Sci USA* 93: 9764-9769, 1996.

Fruttiger M. Development of the mouse retinal vasculature: Angiogenesis versus vasculogenesis. *Invest Ophthalmol Vis Sci* 43: 522-527, 2002.

Gerhardt H, Golding M, Fruttiger M, Ruhrberg C, Lundkvist A, Abramsson A, Jeltsch M, Mitchell C, Alitalo K, Shima D, Betsholtz C: VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 161: 1163–1177, 2003.

Gerhardt H, Ruhrberg C, Abramsson A, Fujisawa H, Shima D, Betsholtz C: Neuropilin-1 is required for endothelial tip cell guidance in the developing central nervous system. *Dev Dyn* 231: 503–509, 2004.

Graus-Porta D, Blaess S, Senften M, Littlewood-Evans A, Damsky C, Huang Z, Orban P, Klein R, Schittny JC, Muller U: Beta1-class integrins regulate the development of laminae and folia in the cerebral and cerebellar cortex. *Neuron* 31: 367–379, 2001.

Gu C, Rodriguez ER, Reimert DV, Shu T, Fritzsch B, Richards LJ, Kolodkin AL, Ginty DD: Neuropilin-1 conveys semaphorin and VEGF signaling during neural and cardiovascular development. *Dev Cell* 5: 45–57, 2003.

Gustafsson E, Brakebusch C, Hietanen K, Fassler R: Tie-1-directed expression of *Cre* recombinase in endothelial cells of embryoid bodies and transgenic mice. *J Cell Sci* 114: 671-676, 2001.

Hammes HP, Brownlee M, Jonczyk A, Sutter A, Preissner, KT: Subcutaneous injection of a cyclic peptide antagonist of vitronectin receptor-type integrins inhibits retinal neovascularization. *Nat Med*. 2: 529-533, 1996.

Hanahan D: Signaling vascular morphogenesis and maintenance. *Science* 277: 48-50, 1997.

Hirsch E, Gullberg D, Balzac F, Altruda F, Silengo L, Tarone G: Alpha v integrin subunit is predominantly located in nervous tissue and skeletal muscle during mouse development. *Dev Dyn* 201: 108 –120, 1994.

Hodivala-Dilke KM, McHugh KP, Tsakiris DA, Rayburn H, Crowley D, Ullman-Cullere M, Ross FP, Coller BS, Teitelbaum S, Hynes RO: ß3-Integrin-deficient mice are a model for Glanzmann thrombasthenia showing placental defects and reduced survival. *J Clin Invest* 103: 229-238, 1999.

Huang XZ, Wu JF, Cass D, Erle DJ, Corry D, Young SG, Farese RVJr, Sheppard D: Inactivation of the integrin beta 6 subunit gene reveals a role of epithelial integrins in regulating inflammation in the lung and skin. *J Cell Biol* 133: 921-928, 1996.

Huang XZ, Griffiths M, Wu JF, Farese Jr RF, Sheppard D: Impaired cell migration and adhesion but normal devel-opment in b5 deficient mice. *Mol Cell Biol*. 20: 755-759, 2000.

Huynh-Do U, Stein E, Lane AA, Liu H, Cerretti DP, Daniel TO: Surface densities of ephrin-B1 determine EphB1-coupled activation of cell attachment through αvß3 and αvß5. *EMBO J* 18: 2165–2173, 1999.

Hynes RO: Integrins: versatility, modulation, and signaling in cell adhesion. *Cell* 69: 11-25, 1992.

Hynes RO: Targeted mutations in cell adhesion genes: what have we learned from them? *Dev Biol* 180: 402-412, 1996.

Hynes RO, Bader BL: Targeted mutations in integrins and their ligands: their implications for vascular biology. *Thromb Haemost* 78: 83-87, 1997.

Hynes RO, Bader BL, Hodivalka-Dilke KM: Integrins in vascular development. *Braz J Med Biol Res* 32: 501-510, 1999.

Lallemand Y, Luria V, Haffner-Krausz R,Lonai P: Maternally expressed PGK-Cre transgene as a tool for early and uniform activation of the Cre-site-specific recombinase. *Transgenic Res* 7: 105-112, 1998.

Lyden D, Young AZ, Zagzag D, Yan W, Gerald W, O'Reilly R, Bader BL, Hynes, RO, Zhuang Y, Manova K, Benezra R: Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumor xenografts. *Nature* 140: 670-677, 1999.

McCarty JH, Monahan-Earley RA, Brown LF, Keller M, Gerhardt H, Rubin K, Shani M, Dvorak HF, Wolburg H, Bader BL, Dvorak AM, Hynes RO: Defective associations between blood vessels and brain parenchyma lead to cerebral hemorrhage in mice lacking alpha v integrins. *Mol Cell Biol* 22: 7667–7677, 2002.

McCarty JH, Lacy-Hulbert A, Charest A, Bronson RT, Crowley D, Housman D, Savill J, Roes J, Hynes RO: Selective ablation of αv integrins in the central nervous system leads to cerebral hemorrhage, seizures, axonal degeneration and premature death. *Development* 132: 165–176, 2005.

Mu D, Cambier S, Fjellbirkeland L, Baron JL, Munger JS, Kawakatsu H, Sheppard D, Broaddus VC, Nishimura, SL: The integrin αvß8 mediates epithelial homeostasis through MT1-MMP-dependent activation of TGF-ß1. *J Cell Biol* 157: 493-507, 2002.

Pasterkamp RJ, Peschon JJ, Spriggs MK, Kolodkin AL: Semaphorin 7A promotes axon outgrowth through integrins and MAPKs. *Nature* 424: 398–405, 2003.

Proctor PM, Zang K, Wang D, Wang R, Reichardt LF: Vascular development of the brain requires ß8 integrin expression in the neuroepithelium. *J Neurosci* 26: 9940-9948, 2005.

Reynolds LE, Wyder L, Lievely JC, Tarverna D, Robinson S, Huang X, Sheppard D, Hynes RO, Hodivala-Dilke KM: Enhanced pathological angiogenesis in mice lacking ß3 integrin or ß3 andß5 integrins. *Nat Med* 8: 27-34, 2002.

Risau W: Mechanisms of angiogenesis. *Nature* 386: 671-674, 1997.

Soriano P: Generalized lacZ expression with the ROSA26 Cre reporter strain. *Nat Genet* 21: 70-71, 1999.

Stone J, Itin A, AlonT, Pe'er J, Gnessin H, Chan-Ling T, Keshet E: Development of retinal vasculature is mediated by hypoxia-induced vascular enothelial growth factor (VEGF) expression by neuroglia. *J Neurosci* 15: 4738-4747, 1995.

Theis M, de Wit C, Schlaeger TM, Eckardt D, Kruger O, Doring B, Risau W, Deutsch U, Pohl U, Willecke K: Endothelium-specific replacement of the connexin43 coding region by a lacZ reporter gene. *Genesis* 29: 1-13, 2001.

Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G: Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* 23: 99-103, 1999.

Varner JA, Brooks PC, Cheresh DA: The integrin alpha V beta 3: angiogenesis and apoptosis. *Cell Adhes Commun* 3: 367-374, 1999.

Yancopoulos GD, Klagsbrun M, Folkman J: Vasculogenesis, angiogenesis, and growth factors: ephrins enter the fray at the border. *Cell* 93: 661-664, 1998.

Zhu J, Motejlek K, Wang D, Zang K, Schmidt A, Reichardt LF: beta8 integrins are required for vascular morphogenesis in mouse embryos. *Development* 129: 2891–2903, 2002.

Zou JX, Wang B, Kalo MS, Zisch AH, Pasquale EB, Ruoslahti E: An Eph receptor regulates integrin activity through R-Ras. *Proc Natl Acad Sci USA* 96: 13813–13818, 1999.

Effect of endothelial cell specific transcription factors on the regulation of vasculogenesis and embryonal angiogenesis in vivo

Dr. Ingo Flamme
Bayer Inc.
51368 Leverkusen, Germany

Member of the SPP 1069 from 1999 until 2001

This project has been transferred in the course of its funding period. See project of Prof. Dr. Georg Breier (page 205).

Recruitment and Activation Mechanisms of Embryonic Endothelial Progenitor Cells in Neovascularization

Dr. Antonis K. Hatzopoulos

present address:
Cell and Developmental Biology
Vanderbilt University
Div. of Cardiovascular Medicine, PRB 383
2220 Pierce Ave, Nashville, TN 37232-6300, USA

formerly:
GSF-Research Center for Environment and Health, Munich

Member of SPP 1069 from 1999 until 2005

SUMMARY

Blood vessels growth is a prerequisite of normal organogenesis and plays a critical role in progression of pathological processes and healing of damaged tissues. We focused on the contribution of endothelial progenitor (EPCs) in these events and in ways to utilize their therapeutic potential. We have demonstrated that embryonic EPCs show a remarkable tropism for poorly vascularized and presumably hypoxic sites of tumor-induced angiogenesis, contribute to tumor vascularization and in certain instances accelerate tumor growth. Based on these findings, we used EPCs as “Trojan horses” to deliver toxic agents directly to the tumor killing malignant cells and inhibiting further tumor growth. Expression analysis and *in vivo* blocking experiments demonstrated that selectins mediate the initial EPC homing step. Furthermore, we discovered that transplantation of embryonic EPCs increases collateral vessel growth and capillary density of ischemic limbs and improves heart function after myocardial infarction in mice and pigs similarly to adult endothelial progenitor cells. Our data suggest novel ways to modulate neovascularization and enhance tissue regeneration after ischemic injury.

INTRODUCTION

The growth of blood vessels is a prerequisite of normal organogenesis during embryonic development and later in adulthood plays a critical role in healing of damaged tissues and progression of pathological processes such as cancer (solid tumor growth), diseases of the cardiovascular system (infarction, peripheral vascular disease), skin (psoriasis), and metabolic diseases (diabetes) (Carmeliet and Jain, 2000).

Cardiovascular diseases alone (i.e., stroke, myocardial infarction, atherosclerosis) are the number one cause of death and disability in the western world. Great strides have been made towards reducing mortality rates thanks to new drugs to treat blood clots, high blood pressure and cholesterol levels, improved surgical techniques to open blocked vessels, and prevention campaigns for proper nutrition and against smoking. This significant progress has conversely brought new challenges to medical care. As a result of severely damaged tissue, heart attack survivors often develop congenital heart failure that

restricts their motility, and stroke victims suffer from dementia and increasing disability. These afflictions gravely affect the patients' quality of life and require constant care placing a heavy financial burden on the health care system.

For these reasons, it is of primary interest to understand the cellular and molecular mechanisms of tissue regeneration following ischemic injury. Compelling new evidence established that adult stem cells for endothelium – as well as for heart muscle, neurons and other tissues - exist in adults, and that these cells play a major role in organ homeostasis and regeneration (Asahara et al., 1997). Circulating endothelial progenitor cells that originate in the bone marrow, home to sites of neovascularization and promote angiogenesis (Kawamoto et al., 2001). Systemic administration of EPCs has considerable beneficial effects as transplanted EPCs enhance capillary density, increase blood flow and dramatically improve tissue rescue (Takahashi et al., 1999). This has raised great hopes for the use of adult endothelial stem cells to stimulate blood vessel growth and thus enhance the repair of malfunctioning tissue.

In contrast to the benefits in tissue recovery, neovascularization plays a negative role in cancer because the growth and metastasis of solid tumors correlate with their ability to induce formation of new blood vessels (Risau, 1997). Therefore, effective control of aberrant vascular growth can have a significant contribution in the fight to stop progression of the disease. In addition to sprouting angiogenesis from neighboring resident endothelial cells, tumors also build vessels by recruitment and *in situ* differentiation of circulating endothelial progenitor cells (Lyden et al., 2001). Bone marrow-derived endothelial precursors are necessary and sufficient for tumor angiogenesis and EPCs differentiated *ex vivo* from multi-potent adult progenitor cells home to tumors (Reyes et al., 2002). Unfractionated bone marrow cells can be also recruited by tumors and, when modified to express an angiogenesis inhibitor, limit growth of subcutaneous tumor challenges (Davidoff et al., 2001). These findings offer the possibility to exploit EPCs for cancer therapy to interfere with blood vessel growth and attack the tumor stroma. However, the utility of *bona fide* EPCs for such treatment is yet unknown.

Endothelial Progenitor Cells

It was shown that EPCs reside in special niches within the bone marrow stroma. Several conditions such as vascular trauma, tumor growth or ischemia, can lead to elevated plasma levels of VEGF, PlGF and Ang1 or cytokines such as G-CSF and GM-CSF, that can mobilize endothelial progenitor cells (Asahara et al., 1999; Hattori et al., 2002). Such agents liberate EPCs from their bone-marrow microenvironment through activation of metalloproteases (MMP-9) and release of kit-ligand (or SCF) from the extracellular matrix (Heissig et al., 2002). This EPC mobilization is impaired in mutant mice lacking *Id* transcription factors (Lyden et al., 2001).

What happens next is less clear. How EPCs find their target tissue, migrate to the intended site and differentiate is unknown. It is postulated that concurrently mobilized Hematopoietic Stem Cells (HSCs) assist in this manner (Lyden et al., 2001). Studies have implicated the SDF-1 chemokine, VEGF and PlGF in the recruiting process, providing first hints in what most likely is a complex and well-orchestrated pathway (Hattori et al., 2002; Yamaguchi et al., 2003).

We have isolated endothelial progenitor cells or angioblasts from mouse E7.5 embryos at the onset of vasculogenesis, when the first blood vessels begin to form in the new organism, and established *in vitro* culture conditions (Hatzopoulos et al., 1998). The embryonic cells have unlimited stem-cell-like growth potential, express a number of endothelial specific genes, and upon stimulation with cAMP, or inside

collagen matrices (MATRIGEL), undergo differentiation to mature endothelium. Importantly, they can be easily genetically manipulated and grown in large quantities for molecular analysis. To show the biological potential of the eEPCs, we injected cells into chicken embryos. In these experiments, we were able to demonstrate that the mouse eEPCs efficiently incorporate into chick heart and brain vasculature.

During the duration of the SPP project, our laboratory used embryonic EPCs to understand how EPCs are recruited to sites of pathological angiogenesis during tumor growth or in ischemic areas in the heart after myocardial infarction. Furthermore, we investigated the effects of engrafted EPCs on tissue recorery after ischemic injury in the heart or in the hindlimbs. Finally, we showed that platelets may plate a role in EPC recruitment and differentiation (Langer et al., 2006). We have also participated in the characterization of the cardiovascular defects in two mouse knockout lines with inactivation of the thioredoxin reductase genes.

METHODS

As experimental models of pathological angiogenesis, we have used tumor-bearing mice. Three different tumor types were generated. The first was the C6 rat glioma that was implanted in the back of nude mice inside a transparent chamber allowing us to visualize tumor-induced angiogenesis in live animals using intravital videomicroscopy. Using this technique, we were able to observe the behavior of fluorescently labeled EPCs in vivo after intra-arterial injection (with the help of a catheter).

The second approach was based on the LM8 osteosarcoma cells that form cancer growths in the lung, liver and kidney simulating a model of metastatic tumors. In this case, EGFP- or DiI-labeled EPCs were injected in the tail vein. As a third model, we used Lewis Lung carcinoma cells that form tumors only in the lung. We have also used a chronic ischemia model in the rabbit hindlimb by removing a femoral artery. In this case, labeled EPCs were administered by retroinfusion though a neighboring vein to flood the ischemic bed. We used angiography to assess collateral vessel formation and immunohistochemistry and immunofluorescence to count capillary density in the thigh.

For an acute ischemic model, we used occlusion of a coronary artery in mice and pigs and administered EPCs systemically or by retroinfusion respectively. A Millar tip catheter was advanced in the heart ventricle to measure systolic and diastolic parameters of cardiac function in mice. In the pig model, sternotomy was performed and ultrasonic crystals were placed in the non-infarcted and infarcted area at risk as well as in the Cx perfusion area in a standardized manner. Subendocardial segment shortening (SES) was performed under resting heart rate as well as at 120 and 150/min atrial pacing (for 1 min each). After assessing infarct size (TTC-viability and Methylene-blue exclusion), tissue samples were analyzed for the presence of EGFP-labeled EPCs by immunocytochemistry.

We have used tissue culture techniques as well as molecular biology tools to investigate the transcriptome of EPCs by Affymetrix gene chips and Bioinformatics analysis. The angiogenic potential of EPCs was assessed in vitro using aortic rings inside MATRIGEL.

The surface markers on EPCs were studied by FACS analysis using specific antibodies. The resistance of EPCs to Natural Killer cells was investigated by a classic radioactive chromium release assay. To target metastases, we genetically engineered EPCs with the yeast cytosine deaminase cDNA fused to uracil phosphoribosyl transferase (UPRT, which shortcuts rate limiting enzymatic steps) as a suicide gene. After EPCs homed to tumors, the mice were treated with 5-FC that was converted to the toxic 5-FU product by the transgene. We used survival curves to assess the effect of this treatment.

RESULTS

The main objective of our project within the Priority Program 1069 “Angiogenesis” was to investigate if the embryonic endothelial progenitor cells could contribute to neovascularization in the adult mouse. Our long-term goals were to study the efficiency and specificity of eEPCs recruitment, their activation and differentiation once they reach their target sites, and finally to examine the way eEPCs influence tissue vascularization. This way we hoped to establish an experimental model system, parallel to adult EPCs, to examine the mechanisms of endothelial progenitor cell recruitment and to assess the potential of EPCs for future cell-based therapeutic approaches to block or enhance the growth of new blood vessels.

EPCs home specifically to tumors, but not to normal tissues

To address these issues, we have investigated homing and behavior of transplanted eEPCs in tumor-bearing mice using three different tumor types, namely C6 glioma, LM8 osteosarcoma and Lewis lung carcinoma (LLC) (Vajkoczy et al., 2003; Wei et al., 2004). In all three models, we observed that the embryonic cells home specifically to tumor vasculature, survive and contribute to the formation of new blood vessels (Fig. 1).

Homing was specific within the tumor and very few eEPCs were found in the surrounding normal tissues. Extensive analysis after tail vein injection of radiolabeled eEPCs into normal mice without tumors or eEPCs marked either by the fluorescent dye DiI or stable EGFP expression, showed that the cells sequestered at low numbers in the lung, spleen and liver but were absent from heart, kidneys, brain, gut and bone marrow (Wei et al., 2004). It is also noteworthy that eEPCs injected into the blood circulation, or eEPCs transplanted subcutaneously, did not form tumors (Wei et al., 2004).

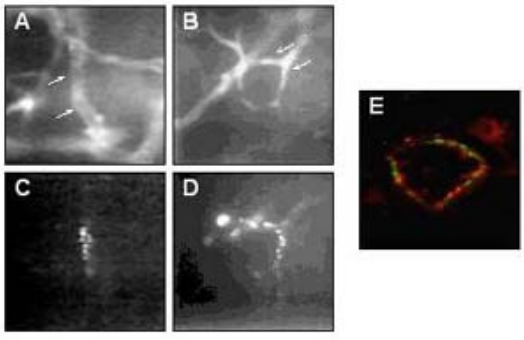


Fig. 1. Incorporation of eEPCs into the tumor microvasculature. (A and B) Tumor microvasculature after contrast enhancement by FITC-Dextran. (C and D) Same regions of interest as in (A and B) demonstrating DiI-labeled eEPCs that line perfused tumor blood vessels indicating successful incorporation into the new tumor microvasculature (arrows). (E) Cryosections of tumor specimens stained with fluorescent antibodies against PECAM-1 (red) confirming the successful integration of EGFP-labeled embryonic EPCs (green) into the endothelial lining of the tumor microvasculature.

Tumor metastases differentially recruit EPCs

The LM8 cells form many tumor nodules in the lung, liver and kidneys while LLC forms metastases only in the lung. This gave us the opportunity to analyze eEPC homing to metastases of different sizes growing in various organs. Histological analysis revealed that intravenously (i.v.) injected eEPCs homed only to a selected subgroup of tumor nodules in the lung. We observed that some nodules recruit EPCs in large numbers while others, often in close vicinity, are totally devoid of transplanted cells. This specificity correlated with the degree of vascularization of independent tumor nodules. In particular, metastases with few blood vessels recruited the majority of eEPCs while well-vascularized metastases did not attract EPCs (Fig. 2).

We reasoned that less perfused and therefore potentially less oxygenated metastases would recruit more eEPCs. Tumor cells, especially under hypoxic conditions, are known to up-regulate and secrete VEGF (Plate et al., 1993). We determined the presence of VEGF in the metastases as a surrogate assessment of tumor hypoxia by immunofluorescence using an anti-VEGF antibody. We found that some large, poorly vascularized metastases recruiting eEPCs in their periphery stained heavily for VEGF in their central areas whereas non-recruiting metastases did not. This suggests that, in these metastases, eEPCs homed preferentially to the margins of the VEGF over-expressing, probably hypoxic, central areas.

We extended the analysis to smaller metastases using immunohistochemistry. Analyzing 14 LM8 metastases and 15 LLC metastases we confirmed that eEPCs, detected by the anti-EGFP antibody, were preferentially found in metastases with decreased vascularity in both tumor models as visualized by

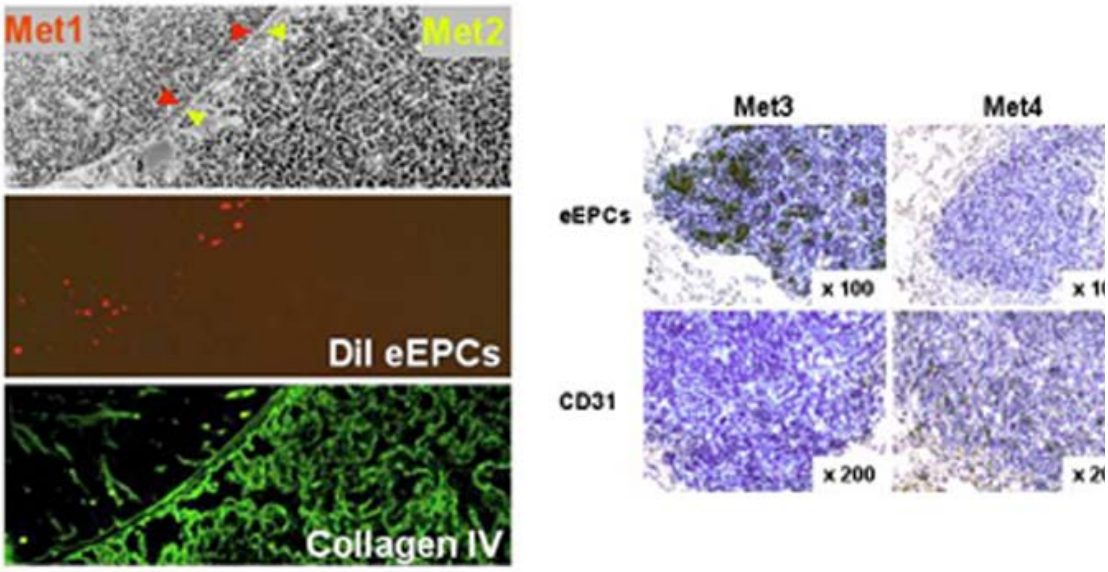


Fig. 2: eEPCs home preferentially to less vascularized tumors. Left Panels: Phase contrast microscopy (top) delineates the two adjacent metastases Met1 and Met2 (the pleural demarcation is outlined by arrowheads). Immunofluorescence microscopy detects DiI-labeled eEPCs in Met1 only (middle) which is less vascularized as shown by anti-collagen IV antibody labeling (bottom). Right panels: An LM8 metastasis (Met3) heavily recruits eEPCs as detected by anti-EGFP antibody staining, while another (Met4) does not (top). Met3 is poorly vascularized while Met4 is well-vascularized (bottom). Endothelial cells were stained using an antibody against CD31.

anti-CD31 antibody staining. Surprisingly, we found that in these smaller metastases VEGF expression co-localized primarily with the eEPCs and not with the tumor cells. Since eEPCs express very low levels of VEGF when grown *in vitro*, local cues within the metastases must have induced strong VEGF expression.

Selectins mediate EPC homing

To gain further insights in the homing mechanisms of eEPCs we grew C6 glioma tumorgrafts within skinfold chamber preparations in mice and observed the behavior of the cells *in vivo* using intravital microscopy (in collaboration with Dr. Vajkoczy). Video image analysis showed that eEPCs actively interact with the vascular wall, extravasate into the tumor stroma, form multi-cellular clusters, and finally incorporate into functional vascular networks (Vajkoczy et al., 2003).

The original steps in eEPC recruitment in tumors displayed striking similarities to the way blood borne cells such as monocytes and lymphocytes home to inflammatory sites (50, 51). This prompt us to hypothesize that the recruitment of EPCs shares molecular mechanisms with the homing of these cells. This notion was further supported by the fact that eEPCs express ligands for selectins such as PSGL-1 and ESL-1 (Vajkoczy et al., 2003). In collaboration with Dr. Engelhardt, we demonstrated that eEPCs bind to both E- and P-selectins *in vitro* and that endothelial cells within the glioma express selectins (Fig. 3).

Based on these results, we addressed the significance of PSGL-1/P-selectin binding in mediating EPC homing to tumor vessels by using blocking antibodies directed against PSGL-1 or E- and P-selectin that were generated in the laboratory of Dr. Vestweber. Pretreatment of mice with an antibody cocktail directed simultaneously against E- and P-selectin, as well as treatment of eEPCs with an antibody directed against PSGL-1 prior to injection, reduced initial eEPC arrest on the tumor endothelium and

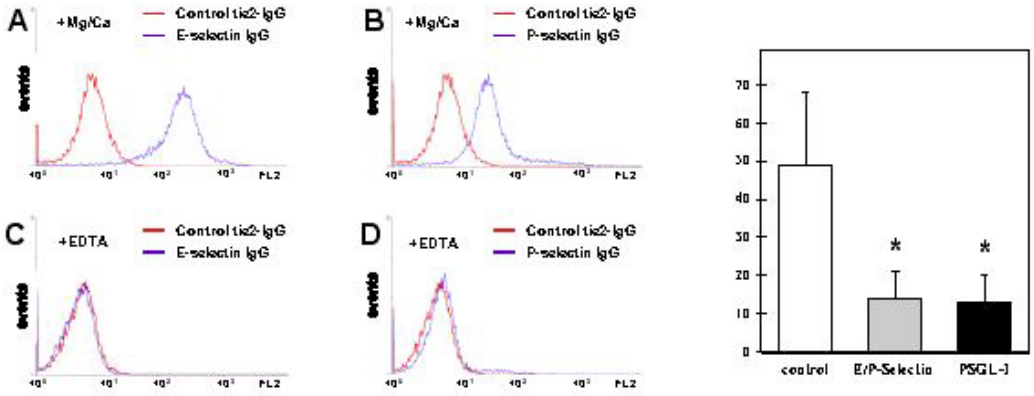


Fig. 3: Interaction of eEPCs with tumor endothelium is mediated by selectins and PSGL-1. (A-D) E- and P-selectins bind to eEPCs. Flow cytometry using an E-selectin IgG chimera (A) or a P-selectin IgG chimera (B) shows strong binding to eEPCs in the presence of Mg^{2+} and Ca^{2+} as compared to a tie2-IgG chimera control. The binding of both proteins is abolished in the presence of EDTA (C, D). Right panel: Quantitative analysis of eEPC homing to tumor endothelium using intravital fluorescence videomicroscopy 10 minutes after cell injection and following blocking of PSGL-1, or P-selectin and E-selectin. N=3 animals per experimental group. * $p<0.05$.

subsequent eEPC incorporation into the tumor microvasculature by 76% and 78%, respectively (Fig. 3). Those cells, however, that were arrested despite blocking PSGL-1 or E-/P-selectin, followed the subsequent multi-step process of eEPC incorporation suggesting that PSGL-1/selectin binding exclusively mediates the initial eEPC arrest**.**

Endothelial progenitor cells for systemic gene therapy of experimental tumors

The remarkable tropism of eEPCs for tumors suggested that they could be used as cellular vehicles to deliver therapeutic load to metastases through the blood stream. To treat metastases, we armed the eEPCs with the suicide gene cytosine deaminase from yeast fused to uracil phosphoribosyltransferase that enhances the formation of the cytotoxic compound 5-fluorouracil from the harmless pro-drug 5-fluorocytosine. In collaboration with Dr. Beltinger at the U. of Ulm, we chose a suicide gene system because it allows for inducible cell death giving time for the eEPCs to home to, integrate into and proliferate within the metastases before triggering their death. eEPCs expressing CD/UPRT (eEPC-CD) exhibited a significant bystander cytotoxic effect *in vitro* on both endothelial and tumor cells (Wei et al., 2004).

Given systemically followed by the pro-drug 5-FC, eEPCs significantly prolonged the lifetime of mice with multiple established lung metastases. Interestingly, metastases-bearing mice that received eEPC-CD without 5-FC lived shorter. Because eEPCs injected into mice without metastases did not shorten survival, the decreased survival in tumor-bearing mice cannot be attributed to a toxic effect of the eEPC-CD, suggesting instead that eEPC-CD without 5-FC treatment increased tumor growth.

In the mice that had received eEPC-CD and 5-FC, the metastases at the time of death were strikingly fewer and the remaining metastases were highly vascularized (Fig. 4). This is in line with the finding that eEPC-CD home preferentially to less vascularized metastases and is consistent with the notion that these metastases are eradicated following 5-FC, leaving only the more vascularized metastases to grow further. We believe that the most likely explanation that no cures were achieved is that eEPCs did not

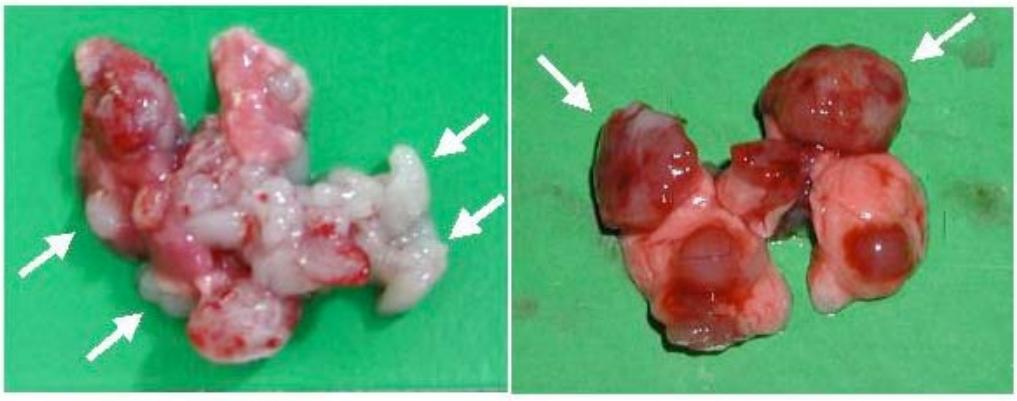


Fig. 4: Not all metastases in the lung are affected by eEPC-CD with 5-FC. The left panel shows a representative lung from the control group that received LLC cells and PBS only. The right panel depicts a typical lung from the treatment group (eEPC-CD and 5-FC). Lungs were procured at the time when the mice appeared moribund. In mice without treatment numerous poorly vascularized metastases are present (left panel, arrows). In contrast, metastases in the treatment group (right panel, arrows) are fewer but larger and well vascularized.

home to all tumors in this hard-to-treat, multiple metastases model. They homed poorly to well-vascularized metastases that continued to grow and killed the mice. On the other hand, the eEPCs' ability to home to poorly vascularized, hypoxic metastases may be one of their major advantages because hypoxic tumors are notoriously resistant to chemotherapy, radiation and anti-angiogenic drugs given as single modalities.

EPCs enhance tissue vascularization after ischemic injury

It is noteworthy that the lung metastastes-bearing mice that received eEPCs survived a shorter time, suggesting an unwanted paracrine effect of eEPCs on tumor or tumor endothelial cells possibly due to production of VEGF and/or other growth factors (Wei et al., 2004). In collaboration with Dr. Kupatt, we investigated if eEPCs can enhance vascularization in a rabbit hind limb ischemia model (Fig. 8). Retrofusion of eEPCs had a positive effect both on collateral vessel growth and capillary density (Kupatt et al., 2005a). Using a myocardial infarction/reperfusion model in mice we observed a marked improvement on heart function after eEPC administration as compared to control untreated mice (Kupatt et al., 2005a). Taken together these results indicate that embryonic EPCs have a positive effect on promoting neovasculogenesis.

EPCs are immunoprivileged

An advantage for cell-based therapeutic approaches would be access to donor material that can be applied to a large number of recipients avoiding rejection from the host's immune system. Cells isolated from early embryonic stages such as the embryonic EPCs might possess such features since early embryonic tissue evades the maternal immune system until the immunological barrier between mother and fetus is established. Therefore we examined the level of MHC I expression on eEPCs using a pan anti-MHC class I antibody. FACS analysis indicated that MHC I antigens were not expressed on three independently isolated eEPC clones while control fibroblasts, T-cells and mast cells from this strain showed strong antibody binding (Fig. 9). This result suggests that eEPCs are protected against T-cell-mediated immune responses (Wei et al., 2004).

However, rejection of transplants is determined not only by MHC I expression, but also by sensitivity towards natural killer (NK) cells. Cells that do not express MHC I can elicit a strong NK cell response. In collaboration with Dr. Fischer at the U. of Ulm**,** we tested whether NK cells can target eEPCs in cell lysis assays using T cell-depleted splenocytes. We found that eEPCs are resistant to NK cell cytotoxic activity suggesting that eEPCs do not engage activating NK receptors (Wei et al., 2004). The escape of early surveillance by NK cells, coupled to lack of MHC I surface molecules may allow recruitment and persistence of eEPCs in non-syngeneic hosts.

To test this notion, LM8 osteosarcoma cells (which are derived from C3H mice, H-2^k haplotype) were mixed with DiI-labeled eEPCs from 129Sv (H-2^b), C57/BL6 (H-2^b) or FVB (H-2^q) mice and transplanted subcutaneously into C3H mice. Tumor growth was followed for up to 24 days before FITC-labeled HPA lectin was injected i.v. to decorate tumor vessels five minutes prior to tumor isolation. Tumors examined by whole mount immunofluorescence microscopy showed abundant eEPCs incorporated into the tumor vessels. Thus, eEPCs survive, proliferate and build tumor vessels in non-syngeneic hosts.

Bone marrow-derived cells in tissue repair following stroke

In the long term, we are interested in transferring knowledge gained in the eEPC homing studies to enhance recruitment mechanisms of endogenous bone-marrow derived EPCs. As a first step to this direction, we investigated the participation of bone marrow-derived cells in remodeling processes after experimental stroke in mice. We transplanted bone marrow from C57BL/6-TgN(ACTbEGFP)1Osb mice, that express enhanced green fluorescent protein (EGFP) in all cells, into lethally irradiated C57BL/6J mice. The recipient mice underwent permanent occlusion of the middle cerebral artery, and bone marrow-derived cells were tracked by fluorescence. We analyzed the involvement of bone marrow-derived cells in repair processes six weeks and six months following infarction. Six weeks after occlusion of the artery, more than 90% of the GFP-positive cells in the infarct border zone were microglial cells. Only few GFP-positive cells expressed endothelial markers in the infarct/infarct border zone, and no bone marrow-derived cells transdifferentiated into astrocytes, neurons or oligodendroglial cells at all time points investigated (Beck et al., 2003). Our results indicate that the therapeutic efficacy of non-selected bone marrow may be limited. It is likely that the number of stem cells is very small under physiological conditions, and that clinical applications would be more effective using isolated enriched progenitor cell populations.

Transplantation of EPCs enhances neovascularization, decreases the area of ischemic injury and improves global heart performance after myocardial infarction

To assess the effect of EPCs on tissue vascularization in an acute ischemia model, we delivered EGFP-labeled EPCs in the pig heart after myocardial infarction. Briefly, a balloon was placed in the left anterior descending (LAD) artery distal to the bifurcation of the first diagonal branch, and inflated with 4 atm (0.41Mpa). Correct localization of the coronary occlusion and patency of the first diagonal branch was ensured by injection of contrast agent. In all groups, the PTCA balloon was deflated at 60min of ischemia, and the onset of reperfusion was documented angiographically. The infusion of EPCs ($5x10^6$ cells per animal) was done either systemically or into the anterior interventricular vein (AIV) draining the parenchyma perfused by the LAD. Each regimen started at 55min of ischemia and lasted until 5min of reperfusion. Systemic infusion was performed via the external jugular vein. In retrogradely treated groups (including saline treated control experiments), we used continuous pressure regulated retroinfusion of isothermic Tyrode solution (25ml/min) with or without $5x10^6$ EPC or mature endothelial cells for 10 min (Kupatt et al., 2005b).

We then examined the fate and localization of EPCs using immunohistochemical techniques. After 24hours, numerous EPCs were found in the ischemic area in smaller and larger microcirculatory vessels, whereas the cell recruitment in the non-ischemic area was limited (Fig. 5). At day 7, the number of cells decreased by 42% with EPCs found either adjacent to or integrated in the vessel wall (Fig. 5). The presence of EPCs in the vicinity of ischemic sites in the pig heart led to an increase of capillary density in the peri-infarct area and a decrease in infarct size (Fig. 6). This benefit could be seen after local retro-infusion of EPCs through the AIV, allowing “flooding” of the vascular beds with EPCs, but not after systemic injection. This finding indicates that local delivery and ample contact time between transplanted cells and host vasculature increases the homing efficiency. Of note, decrease in infarct size and improvement of heart performance under pacing conditions was obtained by infusing endothelial progenitor cells, but not mature endothelial cells, suggesting that the progenitor phenotype is important for the positive effects (Kupatt et al., 2005b).

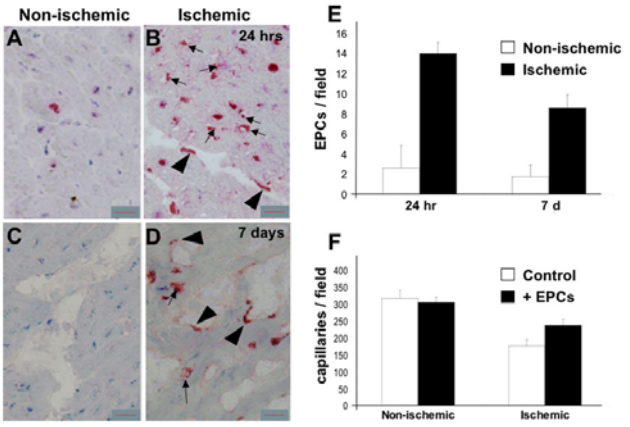


Fig. 5: EPCs home to ischemic myocardium and increase capillary density. Detection of EGFP-positive EPCs with an anti-EGFP antibody in the non-ischemic (A, C) and ischemic areas (B, D) after 24 hours (A, B) and 7 days (C, D) of treatment. After 24 hours, EPCs were present in the microcirculatory vessels (B, arrows), or adherent to the venular vessel wall (arrowheads). At d7, EPCs integrated in the wall of large vessels (arrowheads), or in the vicinity of small vessels (D, arrows). (E) Quantification of EPCs in the ischemic and non-ischemic areas 24 hrs and 7 days after retroinfusion. (F) EPC engraftment increases capillary density in the infarct area.

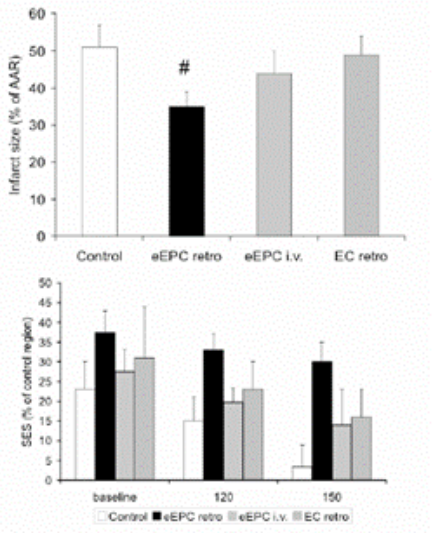


Fig 6: EPC treatment decreases infarct size and improves cardiac function. Top. Infarct size (% of Area at Risk -AAR) 7 days after ischemia and transfusion of $5x10^6$ EPCs via retroinfusion (EPC retro), systemic infusion (EPC i.v.) and endothelial cell retroinfusion (EC retro). **Bottom.** Subendocardial segment shortening (SES) in the apical LAD perfused region (% of the non-ischemic Cx-region) at rest, 120/min and 150/min atrial pacing (n=5 per group, #=p<0.05 vs. Control).

We obtained similar results in an acute myocardial infarction model in mice after injection of EPCs in the tail vein. Using a Millar pressure tip catheter, we documented an improvement in LVDP, as well as systolic and diastolic parameters (dP/dt_{max}, dP/dt_{min}) in mice treated with EPCs as compared to control animals (Kupatt et al., 2005a).

EPC-transplantation confers acute cardioprotective effects

Surprisingly, we found that the beneficial effects of the EPC retroinfusion in the pig heart after myocardial infarction were evident 24 hours following cell therapy, e.g., treated heart tissue showed less inflammation and cell death, as well as smaller infarct size (Fig. 7). This time frame is too short for angiogenesis or transdifferentiation to take place, suggesting, instead, that the major EPC-contribution to heart recovery appears to be mediated through paracrine effects on surrounding myocardium. We postulated that proteins secreted by the EPCs have a protective effect on cardiomyocytes injured by ischemia. Further analysis revealed that EPCs activate PI3 Kinase/AKT in cardiomyocytes during co-culture, a critical survival pathway for cardiac cells (Kupatt et al., 2005b).

The acute cardioprotective properties of EPCs suggest that paracrine effects may also account for the benefits of cell therapy following cardiac tissue injury. We reached a similar conclusion using EPC-therapy in rabbit hindlimbs after excision of the femoral artery (Kupatt et al., 2005a). EPC-treatment increased both collateral vessel formation and capillary density. However, the association of EPCs with host vasculature lasted less than 10-14 days, illustrating that the increased perfusion was primarily due to induction of angiogenesis and arteriogenesis from host endothelial cells.

Mechanisms of EPC-mediated cardioprotection

Since post-ischemic inflammation plays a detrimental role after myocardial infarction, we analyzed the activity of myeloperoxidase, a characteristic enzyme of PMN, in post-ischemic heart tissues with or without EPC-transplantation. Interestingly, EPC treatment limited the increase in myeloperoxidase activity within the infarct zone (Kupatt et al., 2005b).

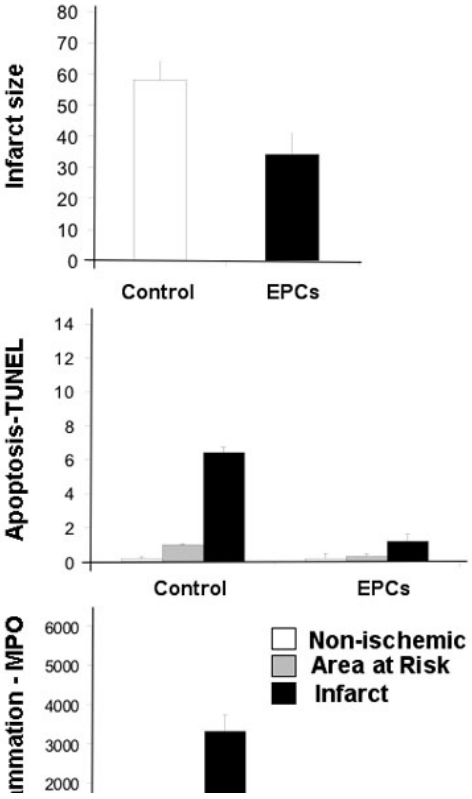


Fig. 7: EPC engraftment confers acute cardioprotection. Infarct size (top panel), apoptosis (middle) and inflammation (bottom) are reduced in EPC-injected pig hearts 24 hours after cell treatment.

We and others have proposed that besides contributing to the newly built blood vessels, one reason of improved vascularization is the ability of EPCs to induce endogenous angiogenesis through secretion of pro-angiogenic factors (Kupatt et al., 2005a). Consistent with this notion, we find that EPCs enhance sprouting in explanted rabbit aortic rings similar to that seen with angiogenesis-inducing agents such as FGF-2 (Fig. 8). This ability, coupled with the close association of EPCs with host blood vessels, probably induces a strong, localized, angiogenic response after transplantation.

Fig. 8: EPCs induce angiogenesis *ex vivo*. Rabbit aortic rings were placed in Matrigel. After polymerization, control medium, medium containing 10µg/ml FGF-2, or $5x10^5$ EPCs was placed on top. EPCs or FGF-2 induced extensive capillary networks (arrows).

DISCUSSION

Taken together, our results indicate that endothelial progenitor cells home to ischemic tissues through mechanisms resembling recruitment of immune cells to sites of inflammation. Furthermore, our data indicate that the propensity of EPCs to home to ischemic areas, but not in normal organs could be exploited to target hypovascular tumors, which are otherwise difficult to kill with conventional chemotherapy or ant-angiogenesis strategies. The unexpected immunopriviledged status of EPCs, which seems to be shared by other stem cells as well, may facilitate the use of stem cells for therapeutic purposes.

We also discovered that stem cells probably have two beneficial roles after transplantation: one linked to regeneration and the ability of stem cells to form new tissue; the other to the positive influence of stem cells on the healing process enhancing cardiomyocyte survival, attenuating inflammation, increasing vascular density and preventing the spread of the necrotic core (Fig. 9). Examining the literature and probing further into the effects of cell therapy, we believe that our conclusions are not unique to EPCs. Instead, our pre-clinical results are representative of similar findings by other investigators using bone marrow or mesenchymal stem cells to treat MI and even the data regarding neural stem cells to treat brain or spinal cord injury (Dimmeler et al., 2005).

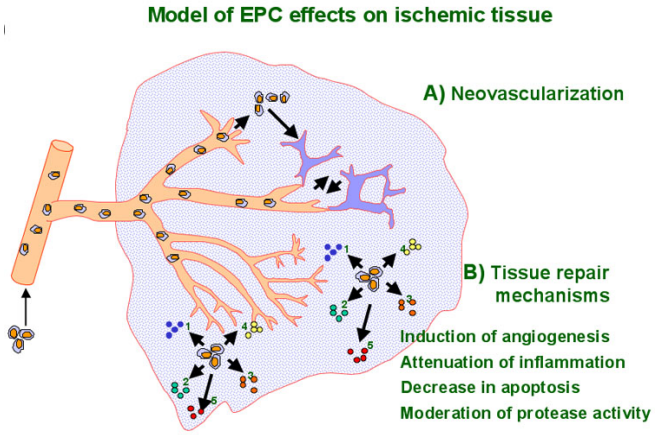


Fig. 9: Direct (A) and indirect (B) effects of EPCs on tissue recovery.

In this light, it is reasonable to assume that successful cell therapies may depend on the following parameters:

1) standardized preparation procedures to achieve a cellular product with reproducible consistency;
2) enriched progenitor cell populations at the expense of mature derivatives;
3) potent cellular mixtures -vs single cell populations- with enhanced diversity of secreted growth factors;
4) sufficiently high yields of cells to restore balance in the diseased area;
5) strong pro-angiogenic potential;
6) enhanced delivery of cells to damaged tissues.

From SPP 1069 to the clinic

There is strong evidence that cell therapy can improve cardiac function, but the exact mechanisms through which cell therapy exerts beneficial effects remain unclear. It is evident that some engraftment of cells into the tissue is necessary, but it is also apparent that these cells do not act solely by regeneration of new myocytes or by angiogenesis. We and others have demonstrated that the cardioprotective effects of endothelial progenitor cells (EPCs) following myocardial infarction are mediated, in part, by the cells secreting multiple cytokines which can favorably enhance myocardial survival and function in a paracrine fashion (Kupatt et al., 2005a,b). For these reasons, we postulate that an enriched, mixed, stem cell population may offer an advantage over a single cell type. It is also clear from our initial studies that active interaction between stem cells and the vascular wall during systemic delivery is necessary for efficient grafting to occur.

Stem cells for the cardiovascular system are scarce in adults. Endogenous stem cells may be able to take care of the natural “wear and tear”, but are not capable of restoring extensive loss of heart tissue in cardiomyopathies. In these conditions, stem cells also face two additional obstacles. The first is massive infiltration of immune cells creating a hostile environment for cell survival; the second is scar deposition

that builds a formidable structural and functional barrier to regeneration and restoration of cardiac function.

We postulate that future therapeutic strategies may need to target simultaneously two fronts: a) generating enough stem cells by *in vitro* enrichment to provide adequate therapy for cardiac repair; and, b) creating a favorable microenvironment in the host to secure a beachhead for transplanted, or endogenous stem cells. Interestingly, cell therapy itself may be an effective way to achieve this second goal. The pro-angiogenic and anti-inflammatory properties of stem cells as well as secretion of cytokines may help to create a fertile milieu for ailing cardiomyocytes, endogenous and possibly exogenously delivered stem cells. This notion supports the notion to combine bone-marrow endothelial and mesenchymal progenitor cells for therapy, next to their potential to regenerate vascular and cardiac tissues.

We have applied for FDA approval to deliver bone marrow-derived cells to the heart by direct intramyocardial injection to treat patients with severe ischemic cardiomyopathy. Building on our experience gained during the “SPP Angiogenesis” studies, we propose to deliver a mixture of EPCs and Mesenchymal Stem cells (MSCs). We postulate that this enriched mixture of bone marrow-derived progenitor cells will be superior for cell therapy than unfractionated bone marrow containing relatively minor populations of stem cells, or highly purified populations of a single cell type (e.g., $AC133^{+}$ or *ex vivo* expanded EPCs). Furthermore, we hypothesize that co-engraftment of these two cell populations may be advantageous in itself, since neovascular growth induced by EPCs will nourish not only cardiomyocytes, but also co-transplanted MSCs. This may extend the survival of MSCs inside the host and thus augment their differentiation to cardiac cells.

REFERENCES

Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM: Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 275: 964-967, 1997.

Asahara T, Masuda H, Takahashi T, Kalka C, Pastore C, Silver M, Kearne M, Magner M, Isner JM: M. Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization. *Circ Res* 85: 221-228, 1999.

Beck H, Voswinckel R, Wagner S, Ziegelhoeffer T, Heil M, Helisch A, Schaper W, Acker T, Hatzopoulos AK, Plate KH: Participation of bone marrow-derived cells in long-term repair processes following experimental stroke. *J Cerebral Blood Flow Metab* 23: 709-717, 2003.

Carmeliet P and Jain RK: Angiogenesis in cancer and other diseases. *Nature* 407: 249-257, 2000.

Conrad M, Jakupoglu C, Moreno SG, Lippl S, Banjac A, Schneider M, Beck H, Hatzopoulos AK, Just U, Schmahl W, Chien KR, Wurst W, Bornkamm GW, Brielmeier M: Essential role for mitochondrial Thioredoxin Reductase in hematopoiesis, heart development and heart function. *Mol Cel Biol* 24: 9414-9423, 2004.

Davidoff AM, Ng CY, Brown P, Leary MA, Spurbeck WW, Zhou J, Horwitz E, Vanin EF, Nienhuis AW: Bone marrow-derived cells contribute to tumor neovasculature and, when modified to express an angiogenesis inhibitor, can restrict tumor growth in mice. *Clin Cancer Res* 7: 2870-2879, 2001.

Dimmeler S, Zeiher AM, Schneider MD: Unchain my heart: the scientific foundations of cardiac repair. *J Clin Invest* 115: 572-583, 2005.

Hattori K, Heissig B, Wu Y, Dias S, Tejada R, Ferris B, Hicklin DJ, Zhu Z, Bohlen P, Witte L, Hendrikx J, Hackett NR, Crystal RG, Moore MA, Werb Z, Lyden D, Rafii S: Placental growth factor reconstitutes hematopoiesis by recruiting VEGFR1(+) stem cells from bone-marrow microenvironment. *Nat Med* 8: 841-849, 2002.

Hatzopoulos AK, Folkman J, Vasile E, Eiselen GK, Rosenberg RD: Isolation and characterization of endothelial progenitor cells from mouse embryos. *Development* 125: 1457-1468, 1998.

Heissig B, Hattori K, Dias S, Friedrich M, Ferris B, Hackett NR, Crystal RG, Besmer P, Lyden D, Moore MA, Werb Z, Rafii S: Recruitment of stem and progenitor cells from the bone marrow niche requires MMP-9 mediated release of kit-ligand. *Cell* 109: 625-637, 2002.

Jakupoglu C, Przemeck GKH, Schneider M, Moreno SG, Mayr N, Hatzopoulos AK, de Angelis MH, Wurst W, Bornkamm GW, Brielmeier M, Conrad M: Cytoplasmic Thioredoxin Reductase is essential for embryogenesis but dispensable for cardiac development and function. *Mol Cel Biol* 25: 1980-1988, 2005.

Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi JI, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM, Asahara T: Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemia. *Circulation* 103: 634-637, 2001.

Kupatt C, Horstkotte J, Vlastos GA, Pfosser A, Lebherz C, Semisch M, Thalgott M, Mages J, Hoffmann R, Deten A, Lamparter M, Beck H, Büning H, Boekstegers P, Hatzopoulos AK: Embryonic endothelial progenitor cells expressing a broad range of pro-angiogenic and remodeling factors enhance vascularization and tissue recovery in acute and chronic ischemia. *FASEB J* 19: 1576-1578, 2005a.

Kupatt C, Hinkel R, Lamparter M, von Brühl M-L, Pohl T, Horstkotte J, Beck H, Müller S, Delker S, Gildehaus F-J, Büning H, Hatzopoulos AK, Boekstegers P: Retroinfusion of Embryonic Endothelial Progenitor Cells Attenuates Ischemia-reperfusion Injury in Pigs: Role of PI3-Kinase/AKT. *Circulation* 112: I117-122, 2005b.

Langer H, May AE, Daub K, Heinzmann U, Lang P, Schumm M, Vestweber D, Massberg S, T. Schonberger T, Pfisterer I, Hatzopoulos AK, Gawaz M: Adherent platelets recruit and induce differentiation of murine embryonic endothelial progenitor cells to mature endothelial cells in vitro. *Circ Res* 98: 2-10, 2006.

Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L, Wu Y, Hicklin D, Zhu Z, Hackett NR, Crystal RG, Moore MA. Hajjar KA, Manova K, Benezra R, Rafii S: Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nat Med* 7: 1194-1201, 2001.

Reyes M, Dudek A, Jahagirdar B, Koodie L, Marker PH, Verfaillie CM: Origin of endothelial progenitors in human postnatal bone marrow. *J Clin Invest* 109: 337-346, 2002.

Risau W: Mechanisms of angiogenesis. *Nature* 386: 671-674, 1997.

Takahashi T, Kalka C, Masuda H, Chen D, Silver M, Kearney M, Magner M, Isner JM, Asahara T: Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization. *Nat Med* 5: 434-438, 1999.

Vajkoczy P, Blum S, Lamparter M, Mailhammer R, Erber R, Engelhardt B , Vestweber D, Hatzopoulos AK: Multistep nature of microvascular recruitment of *ex vivo* expanded embryonic endothelial progenitor cells during tumor angiogenesis. *J Exper Med* 197: 1755-1765, 2003.

Wei J, Blum S, Unger M, Lamparter M, Jarmy G, Geishauser A, Chan G, Fischer KD, Rattat D, Debatin KM, Hatzopoulos AK, Beltinger C: Embryonic endothelial progenitor cells armed with a suicide gene target hypoxic lung metastases after intravenous delivery. *Cancer Cell* 5: 477-488, 2004. *Editorial: Arap W, Pasqualini R: Engineered embryonic endothelial progenitor cells as therapeutic Trojan horses. Cancer Cell* 5: 406-408, 2004.

Yamaguchi J, Kusano KF, Masuo O, Kawamoto A, Silver M, Murasawa S, Bosch-Marce M, Masuda H, Losordo DW, Isner JM, Asahara T: Stromal cell-derived factor-1 effects on ex vivo expanded endothelial progenitor cell recruitment for ischemic neovascularization. *Circulation* 107: 1322-1328, 2003.

Soluble VEGFR-1: Overexpression and Function in Different Animal Models

Herbert A. Weich, Ph.D.

Dept. Gene Regulation and Differentiation
Div. Molecular Biotechnology
German Research Centre for Biotechnology
Mascheroder Weg 1, D-38124 Braunschweig.

Member of the SPP 1069 from 2000 until 2006

SUMMARY

One of the hallmarks of physiological and pathophysiological angiogenesis is the strong up-regulation of the VEGF receptors, whereas expression is almost not detectable in normal, mature blood vessels in most of the organs. In opposite to VEGFR-2 and VEGFR-3, an natural occurring soluble form of VEGFR-1 (sVEGFR-1) generated by differential splicing can be detected in the conditioned media of cultured human endothelial cells and other cell types positive for VEGFR-1 gene expression (e.g. trophoblast cells). The following work report reflects our project data from the first four years followed by a different subproject worked the last two years. In this time we constructed several SP-C sflt-1 vectors for the generation of transgenic animals overexpressing sflt-1 in their lungs. These new vectors have been carefully tested *in vitro*. We generated several chimeric transgenic embryos from different developmental stages to study their lung development. For the pronucleus injections DNA from both – the original SP-C flt-1 D1-6 and some new plasmids have been used. Further, we put much effort into the analyses of the resulting transgenic embryos. Finally, all this studies on RNA and protein level could show that the transgene expresses the soluble receptor protein at a very low level and no histological changes in the lung vasculature could be found. This was finally confirmed by studies on lung metastasis, where a decrease of tumour angiogenesis or tumour growth could not be detected.

The aim of the second project which is still running is the establishment of a mouse model for preeclampsia regarding the hypothesis that symptoms are provoked by a systemic overexpression and increase in the circulation of the soluble receptor molecule sVEGFR-1/sFlt-1. We were able to show that a titer of $5x10^8$ plaque forming units of Ad-sFLT1 injected in mice induces an effect in the glomerular endothelium. That could be demonstrated by kidney sections of sFLT1 treated mice showing a partial occlusion of capillary loops of juxtamedular glomeruli, probably as a result of the swollen endothelium and mesangiom. Electronic microscopy was performed to detect further characteristics of glomerular damage like basal membrane splitting. Finally, our results indicate that a titer of $5x10^8$pfu Ad-sFLT1 induces glomerular damage in treated Balb/c mice. Therefore we are confident that we have established the mouse model to investigate the imbalance of angiogenic molecules in the circulation. These studies will be followed including blood pressure measurements after induction of preeclampsia and reduction of preeclamptic symptons in animals by the treatment with neutralizing antibodies and corresponding ligand to the soluble receptor protein.

INTRODUCTION

The formation of the vascular system is regulated by endogenous positive and negative acting signaling molecules. These are growth and differentiation factors together with an increasing amount of specific inhibitors and modulating molecules. Positive acting regulators of the development of the vascular tree are all members of the vascular endothelial growth factor (VEGF) family together with the members of the angiopoietin (Ang) family. A natural occurring soluble form of the VEGFR-1 (sVEGFR-1) is a classical example of an endogenous produced inhibitor of VEGF action and it is used for fine tuning of VEGF bioavailability. The soluble receptor contains all structural information for the high affinity binding of VEGF-A, VEGF-B and PlGF.

The soluble receptor can be detected in the conditioned media of human primary endothelial cell, VEGFR-1 positive tumour cells and cells isolated from the placenta. This form is biological active and sequesteres VEGF as determined by Far Western studies, chemical cross-linking with iodinated VEGF and acts as a VEGF antagonist for binding studies. We have started to develop a transgenic mouse system where the overexpression of the sVEGFR-1 in the lung under control of the SP-C promoter will be studied.

The initial steps on the first part of the project was to develop different construct for the overexpression of sflt-1 in the lung of mice. In the last work report two years ago (1. founding period) we reported the first steps of the beginning transgenic SP-C sflt-1 mouse project. At that time point we had constructed our SP-C flt-1 D1-6 SV40pA plasmid, which was based on the eucaryotic expression vector pSP-C-SV40pA (a generous gift from J.A. Whitsett, Cincinnati, Ohio, USA). The pSP-C-SV40pA vector contains a 3.7kb fragment of the human SP-C (surfactant protein C) promoter and the SV40 small T intron and polyA. The SP-C promoter permits expression in the alveolar epithelial type II cells of the mouse lung from embryonic day E13 on. The human SP-C promoter has been used so far successfully to overexpress different proteins like the diphtheria toxinA (Korfhagen et al., 1990), TNF-alpha (Miyazaki et al., 1995), TGF-beta1 (Zhou et al., 1996), Sonic hedgehog (Bellusci et al., 1997), VEGF (Zeng et al., 1998), PDGF (Hoyle et al., 1999) and FGF-10 (Clark et al., 2001). Into the pSP-C-SV40pA expression cassette the extracellular IgG-like domains D1-6 of the human flt-1 (VEGFR-1, D1-6) cDNA had been cloned.

Two years ago, we had been able to generate 4 viable transgenic SP-C flt-1 D1-6 mouse founders, which bred already in the F1 generation. These transgenic SP-C flt-1 mice seemed to be phenotypically normal. After controlling the SP-C flt-1 transgene integration, the lungs from founders (F0) and F1 animals had been analysed with Northern blot, RT-PCR, Western blot and ELISA (mVEGF, total hFlt-1). We could reveal, that the lungs of the SP-C flt-1 mice were expressing the transgenic human flt-1 mRNA, but we had not been able to detect any human Flt-1 protein – neither in the Western blot nor in the total hFlt-1 ELISA. Further, analyses had been undergone with chimeric transgenic embryos from embryonic day E16.5. Again these embryos could be positively detected for the transgenic hflt-1 mRNA in the RT-PCR (RNA isolated from whole embryos), but we could not show a hFlt-1 protein expression in Western blot or total hFlt-1 ELISA .

The following work report (2. founding period) reflects our project data from the last two years. In this time we constructed new SP-C flt-1 vectors to overcome certain problems we have had with the original expression vector. These new vectors have been carefully tested *in vitro*. We generated several chimeric transgenic embryos from different developmental stages to study their lung development. For the pronucleus injections DNA from both – the original SP-C flt-1 D1-6 and the new – plasmids has been used. Further, we put much effort into the analyses of the resulting transgenic embryos. That included to establish immunohisto-chemical methods, which had not been used before in our laboratory. Thus the following work report summarizes and discusses all our recent data concerning the transgenic SP-C flt-1 mouse project.

The aim of the second project (last funding period) is the specific modulation and blockade of the ligand/ sFLT1 interaction in a mouse model for preeclampsia (Maynard et al., 2003). In a first step a preeclamptic state will be simulated by adenoviral overexpression of the soluble receptor molecule. Afterwards the angiogenic balance should be modified by adenoviral co-overexpression of regulators like VEGF-A and PlGF-2. In the next step the ligand/ sFLT1 interaction will be neutralized via an antibody against the soluble receptor. Therefore adenoviruses have to be produced used for the mouse model, a neutralizing anti-FLT1 antibody should be developed by construction of a immune V-gene phage display library and produced adenoviruses as well as the blocking antibody should be applied in the mouse model.

METHODS

Cell culture

For cell culture experiments we used human and mouse primary endothelial cells. Human endothelial cells were either purchased from PromoCell (Heidelberg) or from Cambrex (Belgien). Adenovirus preparation was done with wt low passage 293 cells using standards protocols. For adenovirus protein production we used A549 cells. Mouse tumour cell lines and primary cells from lung were cultured in standard medium according to the supplier of the cells.

Molecular cloning and PCR

Molecular cloning was done according to the standard laboratory protocols for subcloning and sequencing. PCR and RT-PCR was done using different primer sets and primer pairs for mouse and human cDNA and genomic DNA (e.g. sflt-1, flt-1, VEGF-A, SP-C, PlGF, KDR/flk-1).

Transgenic animals

All blastocyst injections in order to generate transgenic mice were done in collaboration with Dr. Urban Deutsch in the mice fascilities of the MPI in Bad Nauheim. Tail clip analysis of newborn mice were done using PCR and genomic DNA. Transgenic founder animals were kept in Bad Nauheim and in Braunschweig. Embryos from transgenic animals for structural analysis and protein detect were prepaired on day E16.5 and E18.5.

Phage display library

The construction of native phage display libraries was done with cDNAs from mice immunizied with soluble VEGFR-1. The generation of light and heavy chain fragments with specific primers was done using a kit from Amersham Pharmacia (Expression module recombinant phage antibody system). Additionally primers for heavy and light chain amplification have been used according to published reports.

Adenovirus preparation

Adenovirus production in 293 wt low passage cells and purification using CsCl gradient centrifugation was done using standard protocols established in our institution since many years. The titer of the virus preparation was estimated by serial dilution assay and indicated as pfu/ml. The production of the recombinant proteins after infection were documented by Western blot and quantified by ELISA assay. All ELISA assay for total and free sFlt-1, total and free VEGF-A, KDR and VEGF-C are established in our group in the last 6 years.

Animal model for preeclampsia

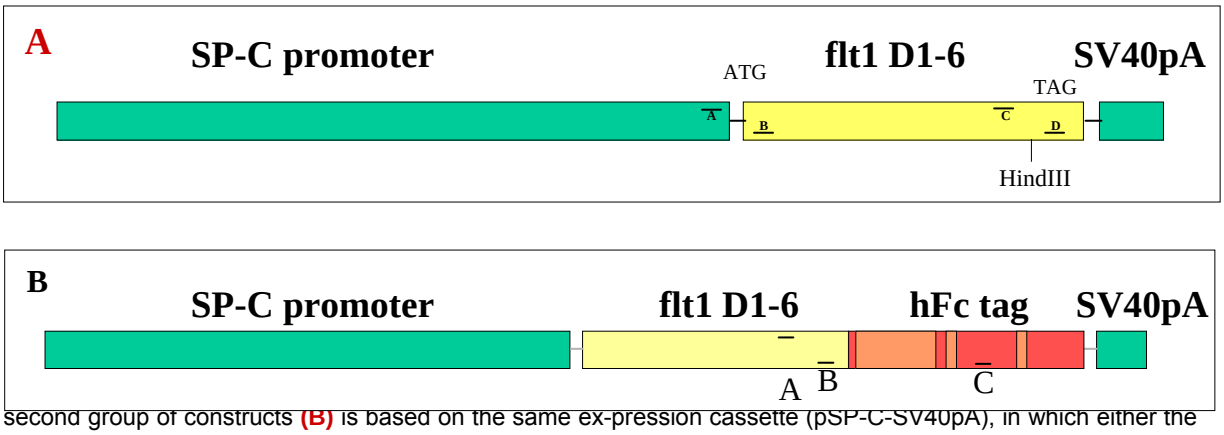
For all experiments female Balb/c mice were used 10-11 week old with full developed gomerulus structures. Virus preparations were dialysed against low salt or high salt buffer and injected (50-100 μl) into the tail vein of mice. Mice were sacrified on day ten or day twenty after adenovirus injection. Urine and blood samples were collected and frozen. Kidneys and livers were removed and one half used for paraformaldehy fixation and the other half stored at -70°C for structural and histochemical analysis.

RESULTS

1. The Transgenic mouse project

1.1 Construction of the SP-C flt-1 expression plasmids

We started the transgenic mouse project with the working hypothesis that the angiogenic factor VEGF-A must be very well regulated in the body in order to develop and maintain the blood system in a proper manner. The soluble VEGFR-1 (sFlt-1) is believed to be a crucial part in this balance. As a model we have chosen the mouse lung with its wide capillary network. During lung development endothelial cells invade the lung mesenchyme, coming into close proximity to the developing epithelial cells and growing with them simultaneously to the edges of the tissue. This process implicates, that both cell types influence each other. We hypothesize, that VEGF-A will be an important part of that process. So we asked, what would occur, if the epithelial cells overexpress and secrete the VEGF antagonist sFlt-1?To elucidate the role of sFlt-1 (VEGFR-1, D1-6) in the developing mouse lung we decided to design constructs, which drive the human sflt-1 gene (cDNA) under control of the human SP-C (surfactant protein C) promoter. The resulting protein should be expressed in the mouse lung in alveolar type II cells from embryonic day E13 on.



second group of constructs **(B)** is based on the same ex-pression cassette (pSP-C-SV40pA), in which either the human flt-1 D1-6 or the human sflt-1 (native sflt-1: splice variant) cDNA has been fused to the genomic human Fcγ tag (receptor globulin protein) resulting in a **flt-1 D1-6 Fc exon/intron** chimera or a **sflt-1 Fc exon/intron** chimera. The constructs **(B)** offer the great advantage that one can distinguish between the genomicaly integrated transgenic DNA and the resulting expressed RNA (primers A+C).

1.2 Generation of transgenic SP-C flt-1 mice

To generate transgenic SP-C flt-1 mice a purified promoter-gene-cassette from the **SP-C flt-1** plasmids was microinjected into the pronucleus of fertilized oocytes. From these injections we obtained various embryos at different stages (E16.5, E18.5, P1) and four viable founders (from construct **A, chapt.1.1**). Two of the lines from construct **(A)** breed now in the F5 generation. All animals were tested for the transgene via PCR analysis from tail clip DNA.

1.3 Analysis of transgenic mice

Relatively early in this project, we were able to generate 4 viable founders for them **SP-C flt-1 D1-6** construct**.** Three of these founders bred to the F1 generation, from two of them we could obtain transgenic lines (14380, 14377).

The lungs from founders and heterozygous transgenic F1/F2 mice were analysed for flt-1 mRNA and protein expression.

For the RT-PCR reaction mRNA from mouse lungs was isolated and transcribed into cDNAs. The expression of the transgenic human flt-1 mRNA in the F0 and F1 mice is relatively low, but all analysed transgenic mice showed a specific positive signal in the RT-PCR (Fig. 2).

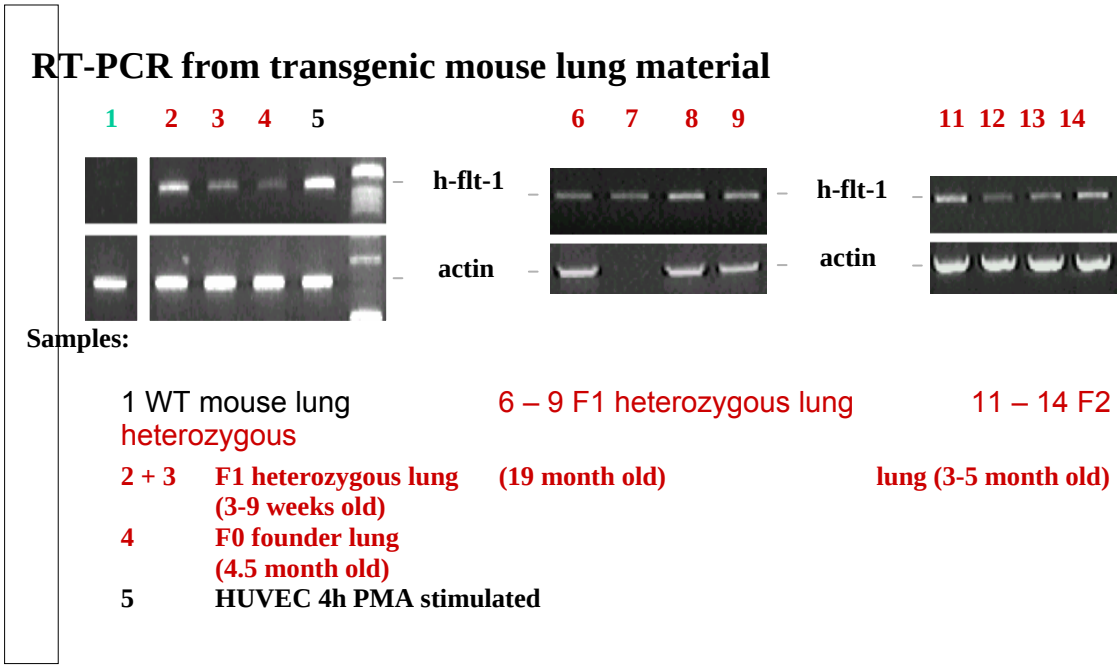
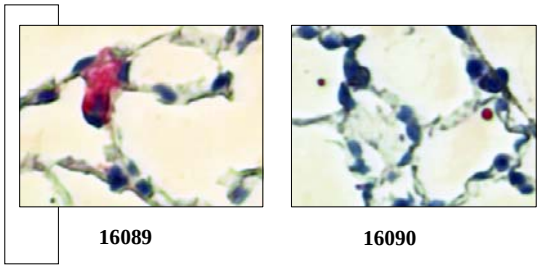


Fig. 2: RT-PCR reactions from different mRNAs from mouse lungs. The analysed F0 and F1 transgenic SP-C flt-1 mice all expressed a human flt-1 mRNA. Still we could not find an obvious phenotype in the tested animals. Therefore we focused our work on the detection of the transgenic human Flt-1 protein via immunohistochemistry.

One of our major problems during the transgenic mouse project was to find an anti-human Flt-1 antibody, which was specific against the human Flt-1 but did not detect murine Flt-1. Though we tested several anti Flt-1 antibodies in the immunohistochemistry: **1**) A suitable candidate seemed to be the mouse monoclonal **Flt-19** (e.g. SIGMA) antibody. That antibody works perfectly in the anti human Flt-1 ELISA and it recognizes Flt-1 on human placenta cryosections. This antibody has several disadvantages – it does not recognize Flt-1 after Super oxide (H2O2) treatment, after

Methanol or Paraformaldehyd fixation and it makes a relatively high background in murine tissues, especially in embryo tissue. Additionally it seems, that the Flt-19 antibody also recognizes murine Flt-1. **2**) There is another monoclonal antibody from rat – the **MF-1** antibody from Imclone, which is not commercially available. That antibody is, unfortunately specific for murine Flt-1 and does not recognize human Flt-1. **3**) Further, we tested several mouse monoclonal antibodies from our lab and from R&D. They all did not work on human placenta cryosections and thus do not recognize human Flt-1. **4**) Our last attempt was to antigen affinity purify a rabbit polyclonal anti Flt-1 antibody from our lab against human Flt-1. Also this antibody could not distinguish between human and murine Flt-1. Next to the antibody testing we also tried different immunohistochemical procedures, e.g. Biotin-Streptavidin-HRP or Biotin-Streptavidin-AP based protocols or immunoflourescences.



Immunohistochemical staining of the lung
Lung tissue from heterozygous SP-C flt-1 D1-6 F1 littermates was stained with an anti-Flt-1 antibody (**Flt-19**). Mouse no.16090 shows no expression at all, while in animal no.16089 a Flt-1 signal for a few type II cells was detected.

Fig. 3: Detection of human Flt-1 protein in a few type II cells in the lung of adult transgenic mice.
Finally, we have been able to detect human Flt-1 protein in a few type II cells in the lung of adult transgenic mice (Fig. 3). For the immunohistochemistry the lungs were infiltrated and embedded with OTC compound, frozen and cut at a cryotom into 6-10µm cryosections. These cryosections were exclusively Acetone fixed. For the detection of the human Flt-1 protein the mouse monoclonal **Flt-19** antibody in a Biotin-Streptavidin-AP based method (Immunotech) was used. Nevertheless, we had to realize, that we could not detect human Flt-1 expressing type II cells in every transgenic mouse. That means, although all examined transgenic lungs synthesized the human flt-1 D1-6 mRNA, not all transgenic lungs could be stained positively for a human sFlt-1 signal, and within the positively stained lungs only less than 1% of all type II cells could be revealed as overexpressing cells. Thus it remains, that the detection of the human sFlt-1 protein in the transgenic mouse lungs is a critical point within our project.

1.4 Summarizing embryo data

In chapt.1.3 we stated, that we have been able to generate viable transgenic SP-C hFlt-1 mice. These mice transcribed a hum. Flt-1 D1-6 mRNA and overexpressed the protein in rare cases in a few alveolar type II cells in the lung. But the lung phenotype in these transgenic mice seemed to be not affected. That was the beginning of our embryo work, which we started with several questions, listed in chapt.1.5. We wanted to know, if the lack of an obvious phenotype in the adult transgenic human Flt-1 mice could be due to a very mild phenotype in the surviving transgenic mice or due to an embryonic phenotype, which is rescued during the first days of the newborn mouse.

Several embryos at the stages E16.5, E18.5 and P1 (newborn) have been generated with different SP-C flt-1 plasmids (chapt.1.1, p2) and analysed. For the generation of these embryos the frequency of transgenic embryos after the pronucleus injection was in most cases much better than for the first chimeric founder mice (table p6, chapt.1.3). Though we concluded, that we did not loose transgenic Flt-1 embryos before birth. That would enable us, to have a broad variety of Flt-1 expressing transgenic pups with differently pronounced phenotypes.

Our first attempt was to analyse **E16.5 embryos**. Some but not all of the transgenic embryos expressed the human flt-1, as shown in RT-PCR. That is a normal phenomenon for transgenic animals, because the transgenic DNA is randomly integrated into the genome, thus being influenced by the genomic background and the transcriptional activity of the integration site. Further, even at a defined E16.5 stage do not all embryos have exactly the same develop-mential level. Since the SP-C

positive cell islands just start to appear, it might be, that some of the embryos still do not express enough flt-1 mRNA from the SP-C promoter to be detected with our RT-PCR. Anatomically and immunohistochemically there had been no differences between wildtype and transgenic human Flt-1 embryos. The detection of the human Flt-1 on the lung tissue had not been possible, because of the lack of a proper anti human Flt-1 antibody.

Between E16.5 and E18.5 the lung undergoes several changes: the terminal buds dilate and the mesenchyme thins. The developing pulmonary vasculature comes into increased proximity to the epithelial cells in the periphery. Though we proceeded the experiments with **E18.5 embryos**. Both, the wildtype (WT) and the transgenic human Flt-1 lung show a distinct pattern with well organized tubules and a well organized capillary network at that developmental stage. First time we could observe with these embryos a slight difference between WT and transgenic lungs: Some of the transgenic lungs appeared to look more fragile, the terminal buds and saccules seemed to be enlarged, the alveolar septa appeared thinner. As mentioned before, only a few embryos showed – corresponding to different genomic integration sites – that phenotype. Unfortunately, we were again not able to detect the human Flt-1 on the sections because of the lack of a proper antibody.

Finally, we turned to **newborn P1 embryos**, which were delivered via Caesarean section after E18.5–E19 and animated to breathe. For the generation of these P1 embryos also new SP-C flt-1 plasmids were cloned: They were designed as SP-C flt-1 Fc exon/intron vectors, which express a spliced Flt-1 Fc mRNA, thus distinguishing between genomically integrated plasmids and the mRNA. Further, the human Flt-1 protein is expressed as a chimeric human Flt-1 Fc protein, which should enable us to detect the human Fc tag within the murine background with specific antibodies.

The frequency of transgenic embryos after the pronucleus injection was this time relatively low with 13%. Nevertheless, we could isolate 5 transgenic Flt-1 Fc embryos, which were all healthy during the first 2 hours after birth and had no respiratory problems. All these 5 transgenic P1 pups expressed the transgenic human flt-1 Fc mRNA, as we could clearly show with RT-PCR. Anatomically and histochemically there is only little difference between wildtype and transgenic embryos: Some of the transgenic lungs appear to look more fragile, the alveoli are enlarged and the alveolar septa appear thinner. The same observations we have had already studying E18.5 embryos. Our attempt to use the Flt-1 Fc chimeric protein for better transgene detection did not succeed: None of the anti-human Fc antibodies could visualize a phenotypical difference between wildtype and human Flt-1 Fc transgenic animals. Further, we could not use the anti human Fc ELISA for the detection of the human Flt-1 Fc protein in mouse lungs, because the expression values had been under the detection limit.

Consequently we decided to undergo morphometric analyses for the P1 pups, which were analysed for its amount of alveoli, the number of alveolar type II cells, the ratio of type II cells per alveolus, the largest alveolar extension and the thickness of the alveolar septa. Most of the parameters were similar between wildtype and human Flt-1 transgenic embryos. Interestingly we could observe, that wildtype pups have only 75% of the alveoli measured in transgenic embryos. While exhibiting the same number of alveolar type II cells, that leads to a different ratio of type II cells per alveolus: 9.8 cells in wildtype and 6.8 cells (69%) in transgenic P1 pups, respectively. Further, the transgenic P1 pups happen to have thinner alveolar septa than their littermates. All these results emphasize the previous observations, that some of the human flt-1 transgenic embryos appear to be more mature than their wildtype littermates.

Summarizing the results we could obtain with the embryo experiments, we have to conclude, that SP-C promoter driven flt-1 constructs are not able to induce a clear phenotype in the mouse lung. Either the SP-C promoter used could not permit a really high expression of the human flt-1 transgene, or the secreted human Flt-1 was not stable in the lung environment or the overexpression

of human flt-1 was tolerated in the murine lung during embryonic development. A really critical point in our project had been the circumstance, that we were not able to detect the transgenic human Flt-1 protein in our transgenic animals, except for the results we could generate with adult lungs, were the Flt-19 antibody detected a very few overexpressing alveolar type II cells in some transgenic mice. That convinced us, that the SP-C constructs we used, did not allow a high expression of the human flt-1, thus being not optimal for an overexpression of human Flt-1 in the murine lung. One evidence for that fact had been the failure, to detect the transgenic human Flt-1 at least as Flt-1 Fc protein with specific anti human Fc antibodies.

Nevertheless, there seems to be a mild phenotype due to the additional expression of human Flt-1 in the murine lung: a slightly advanced maturation of the alveoli between E18.5 and the first days of birth. To verify these data, it would be necessary to analyse more transgenic and wildtype embryos and to evaluate the results statistically.

Finally, we can answer the question we started with in chapter 1.5 at least partially: Transgenic human Flt-1 embryos do not die at birth because of a respiratory dysfunction. If there is a human Flt-1 specific phenotype, it is a rather mild phenotype, which does not affect the functionality of the murine lung. The embryo experiments brought us a little bit closer to a possible Flt-1 phenotype, but without analysing larger numbers of embryos it is not possible to verify the results statistically.

1.5 Tumor growth in SP-C human flt-1 mouse lungs

Finally, we would like to report about a tumor experiment we have done with our transgenic SP-C human Flt-1 mice. That experiment was performed as a single test experiment in collaboration with the animal facility at the GBF.

It is known for a long time, that many tumors produce high amounts of VEGF. We have generated transgenic mice, which should express human Flt-1 (D1-6) under control of the SP-C promoter in the murine lung. Though we have been interested, if the expression / overexpression of the VEGF antagonist human sFlt-1 would influence the settling and the growth of tumor cells. Even if we can hardly measure a human Flt-1 expression in these transgenic mice, we wanted to test, if the SP-C controlled human Flt-1 expression could be activated / challenged with tumour cells, thus preventing the settling of tumors in the lung.

To induce tumour growth in the lungs of the SP-C flt-1 D1-6 mice we have been using the **B16 F10** cell line. The B16 F10 cell line is a black melanoma cell line originated from C57BL/6J mice, which is tumourigenic in syngeneic mice (Fidler, 1973, 1975; Fidler & Kripke, 1977). If the B16 F10 cells are injected through the tail vein into a syngeneic mouse they induce primarily tumours in the lung (Fidler, 1974; Fidler & Kripke, 1977).

Our transgenic mice obtained from pronucleus injections are generated in a mixed C57BL/6 ***/*** CBA background. To get syngeneic mice for the experiment with the B16 F10 cells it was necessary, to breed the mice out against the C57BL/6 background over several generations. Heterozygous transgenic SP-C flt-1 D1-6 mice and wildtype littermates in the F4 generation against C57BL/6 background turned out to be suitable for our B16 tumour experiments. For the injection of B16 F10 cells both transgenic founder lines (**14377** and **14380**) have been used.

For the founder line **14380** nine SP-C flt-1 D1-6 transgenic mice and 3 wildtype littermates were injected. For the **14377** founder line eight SP-C flt-1 D1-6 transgenic mice and 3 wildtype littermates were used. All animals were between 8 – 12 weeks old and healthy, when injected into the tail vein with $1x10^6$ B16 F10 cells/ml saline ($2x10^5$ cells/200µl). The mice of the 14380 founder line were kept for 4 weeks post injection, the 14377 founder line animals 3 weeks following injection. Afterwards the

mice were killed. The lungs were isolated, examined for tumor growth and photographed. Every analysed mouse was genotyped again via PCR to verify the transgenic or wildtype phenotype.

As the table summarizes, every mouse was injected with up to 200 µl of B16 F10 cells ($2x10^5$ cells). But not all mice developed tumors. There had been transgenic and also wildtype mice without tumors. One possible explanation for the failure of tumor development in these animals might be due to the mouse strain background: The transgenic mouse lines originated – like mentioned before – from a C57BL/6 ***/*** CBA background, which was in the F4 generation of backcrossing against C57BL/6. It is possible, that some of the wildtype and transgenic mice in that generation are still not completely syngeneic for the C57BL/6 B16 F10 cells. Their immunosystem recognizes the B16 F10 cells as foreign cells and develops an immunoreaction against them, thus preventing the settling of tumour cells and tumour growth. In the following evaluation we therefore focuse only on mice with tumours.

The B16 F10 injected mice were analysed for the number of tumors, their size and the formation of metastasis. **Diagr.A** shows the number of tumors for the individual mice. We could count between 1 and 18 tumors of different size, which had been either white or black. The mice of the founder line 14380 exhibited in general more tumors than animals from the founder line 14377. That could be due to a technical aspect: The 14380 mice were in average 1-2 weeks older and larger than 14377 mice, and it was easier to inject them into the tail vein with more B16 F10 cells.

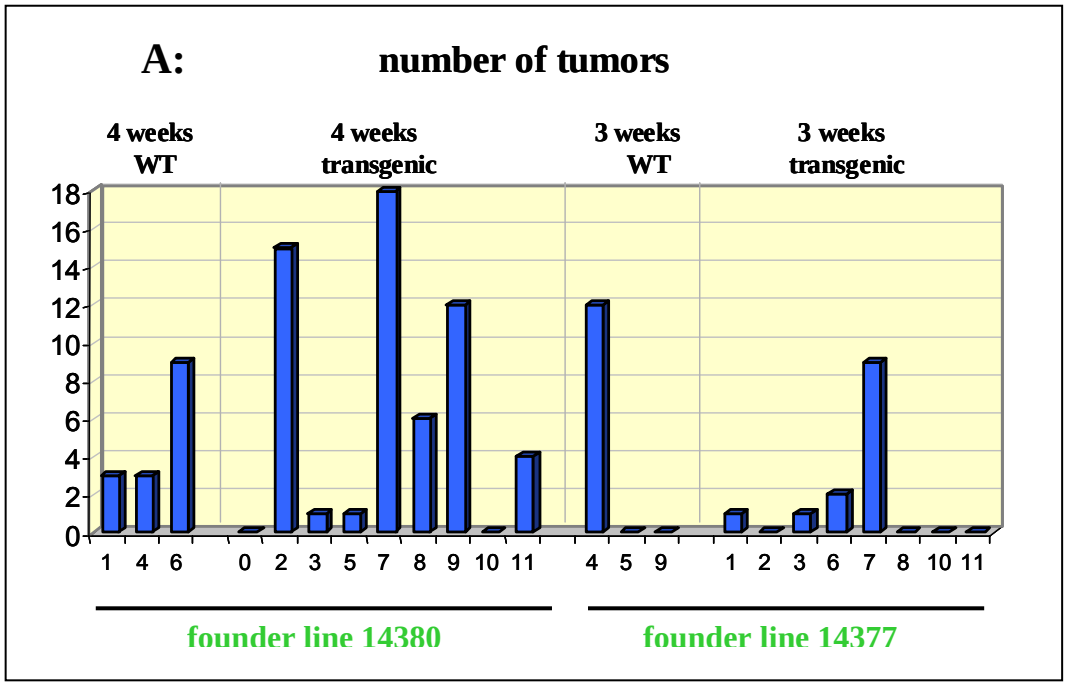


Fig. 4: Number of lung tumors in different transgenic mouse lines

Table 1

average				
of all positive	WT lungs	6.8 tumors	transgenic lungs	6.4 tumors
after 4 weeks (founder 14380)	WT lungs	5 tumors	transgenic lungs	8.1 tumors
after 3 weeks (founder 14377)	WT lungs	12 tumors	transgenic lungs	3.2 tumors

Table 1: Summary of the results from the diagram concerning tumor numbers. If we compare wildtype and transgenic lungs in respect to their tumor spots, there is not much difference between wildtype and transgenic animals: Wildtype and transgenic mice developed tumors. In average we could count in wildtype 6.8 and in transgenic animals 6.4 tumors at the affected lungs. There are some differences in tumor number between the founder lines 14380 and 14377: While in the 14380 line the wildtype mice have less tumors than the transgenic mice (5/8.1), the 14377 line exhibits more tumors in the only wildtype mouse. We are very careful with these results, because we performed that experiment only with a few animals which probable have not all been syngenic jet. The important result for us had been that transgenic mice express tumors at a similar rate as wildtype littermates.

Table 2

average				
of all positive	WT lungs	**3.8 mm**	transgenic lungs	**3.4 mm**
after 4 weeks (founder 14380)	WT lungs	**3.7 mm**	transgenic lungs	**3.3 mm**
after 3 weeks (founder 14377)	WT lungs	**4 mm**	transgenic lungs	**4.5 mm**

Table 2: Tumor size is again very similar if we compare wildtype and transgenic animals. In average the largest wildtype tumor is 3.8mm, the largest transgenic mouse tumor 3.4mm. For the just discussed parameter there is only a slight difference between both founder lines. In the line 14377 the largest tumor is in average a little bit larger than in the 14380 line (4.5 / 3.3mm), what could have been caused by the smaller amount of tumors in the lung. Overall, in both founder lines very small tumours (1mm) and relatively large tumors (6-8mm) could be found.

Finally, we want to discuss the grade of metastasis within the breast area. Tumour growth was very divergent in the animals analysed. As we could detect different numbers of tumour spots and different tumour sizes, we could also detect a very divergent degree of metastasis formation. Some of the tumour bearing mice had no metastasis, some had severe metastasis formation.

Table 3

average				
of all positive	WT lungs	2.9	transgenic lungs	3
after 4 week (founder 14380)	WT lungs	2.7	transgenic lungs	3.4
after 3 weeks (founder 14377)	WT lungs	3.5	transgenic lungs	1

Table 3: For the metastasis formation the time of tumour growth (3 or 4 weeks) is an important factor. The mice have been injected in 2 series (founder line 14380, founder line 14377). None of the injected mice showed symptoms from the tumour growth, but we found, that some of the animals had developed massive tumours and metastasis after 4 weeks. Therefore we decided to shorten the tumour growth time from 4 weeks in the first series (founder line 14380) to 3 weeks in the second series (founder line 14377). The settling of tumour cells in the lung should not be affected by this change. But we see clear differences in the grade of metastasis: While animals from the 14380 founder line show particularly severe metastasis formation with grade 4 to 5, the animals of the 14377 line exhibit metastasis only in 2 mice with a 1 to 3 degree. If we compare wildtype and transgenic lungs there are grad 2.9 metastasis in wildtype to grad 3 metastasis in transgenic lungs in average. Though we consider, that the transgenic lungs have not only a similar number of tumor spots, but also a comparable sensitivity for metastasis formation. The following pictures give an overview over the different tumour expression on the murine lungs of the SP-C flt-1 D1-6 x C57BL/6 F4 transgenic (**T**) and wildtype (**WT**) mice (Fig. 5).

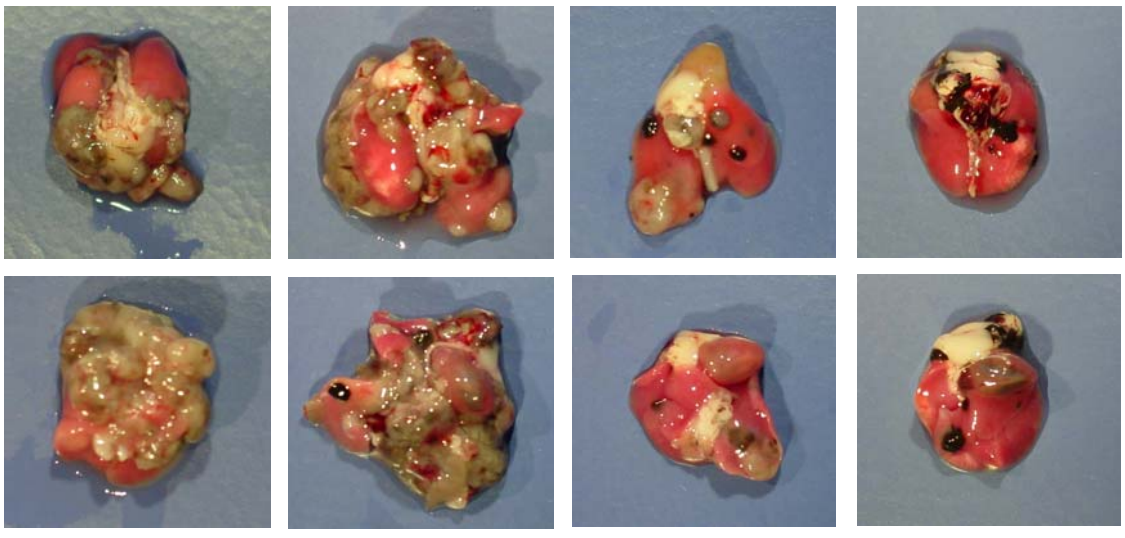


Fig. 5: B16 F10 lung tumors 4 weeks after injection (14380 founder line)

2. The preeclamptic mouse model

2.1 Construction of msFLT1 adenovirus

In the mouse model the use of a foreign protein in the mouse (human sFLT-1) may lead to artificial results attributed to fast protein degradation or not assessable immune response. To evaluate results obtained with adenoviral shuttles bearing human sFLT-1we additionally started to design a shuttle vector for infection and *in-vivo*-expression of mouse sFLT-1. The mouse sFLT-1-protein in this vector is fused to an antibody Fc-domain to ensure higher stability and lower clearance of the recombinant protein.

For the expression of the msFLT1 the adenoviral expression vector pGEM IRES eGFP, containing an E1/ E3- deleted viral genome, was choosen. The expression vector was already established and originally generated by Dr. W. Lindenmeyer as basis for the construction of recombinant adenoviruses for several other projects. The vector contains a CMV– promotor, an SV40 small T intron and polyA site from pKC4, and an IRES (internal ribosomal entry site) in front of the eGFP reporter gene (enhanced green fluorescent protein) in a pGreenLantern based backbone plasmid (GibcoBRL). The CMV-promotor permits expression in 293HEK cells, a human kidney cancer line that encloses the E1 and E3 fragment of adenoviral genome responsible for replication of the virus.

The mouse cDNA coding for the first 7 IgG-like domains of the mflt1-gene was isolated from the baculoviral vector pVL1393msFLT1 D7-hFc, which has already successfully used for synthesis (Dr. B.Barleon, RELIATECH, Braunschweig). Two strategies were used for subcloning the gene from the baculoviral vector pVL1393msFLT1 D7-hFc: i) isolation by restriction digest and ii) isolation by amplification of the gene.

The cloning strategy is shown in detail in Fig. 6. Since the sequence of the sFLT1 gene contained many restriction sites we decided to isolate the gene in two fragments: the fragment coding for sFLT1 (EcoRI-MluI, 2273bp) and the fragment coding for the antibody Fc-domain (MluI-XbaI, 693bp). To allow subcloning of these fragments the adenoviral expression vector pGEM IRES eGFP was modified: The MfeI and the PciI restriction site were deleted to permit introduction of additional restriction sites into the multiple cloning site. An AvrII-XmaI adapter, generated by annealing of two 76bp-oligos, inserted additional restriction sites (MfeI, PciI, NheI) into the expression vector. To yield the final construct both the fragment coding for the msFLT1 gene and the fragment coding for the Fc-domain were ligated in parallel into the modified adenoviral expression vector by MfeI-EcoRI and NheI-XbaI. Ligated fragments were afterwards transformed into competent cells, either chemical competent *E.coli* DH5alpha or electrocompetent *E.coli* TG1. Clones received from transformation were verified by restriction digest. Transformation was not successful until now. Reasons for that could be ineffective cleavage of restriction sites resulting in incomplete ligation. Further ligation of three fragments is a technique rarely used because of its complexity. A defined molar ratio between the fragments has to be found to get the circular construct. Therefore different ratios and formulas for evaluating ratios were tried to find the wanted ratio. Another point could be the fast degradation of ATP in the ligation reaction, important for the enzyme T4 ligase. In addition fragments used for the reaction were purified, nevertheless they could be contaminated either by deposits of column matrix after gel purification, or by residues of phenol known to inhibit ligation.

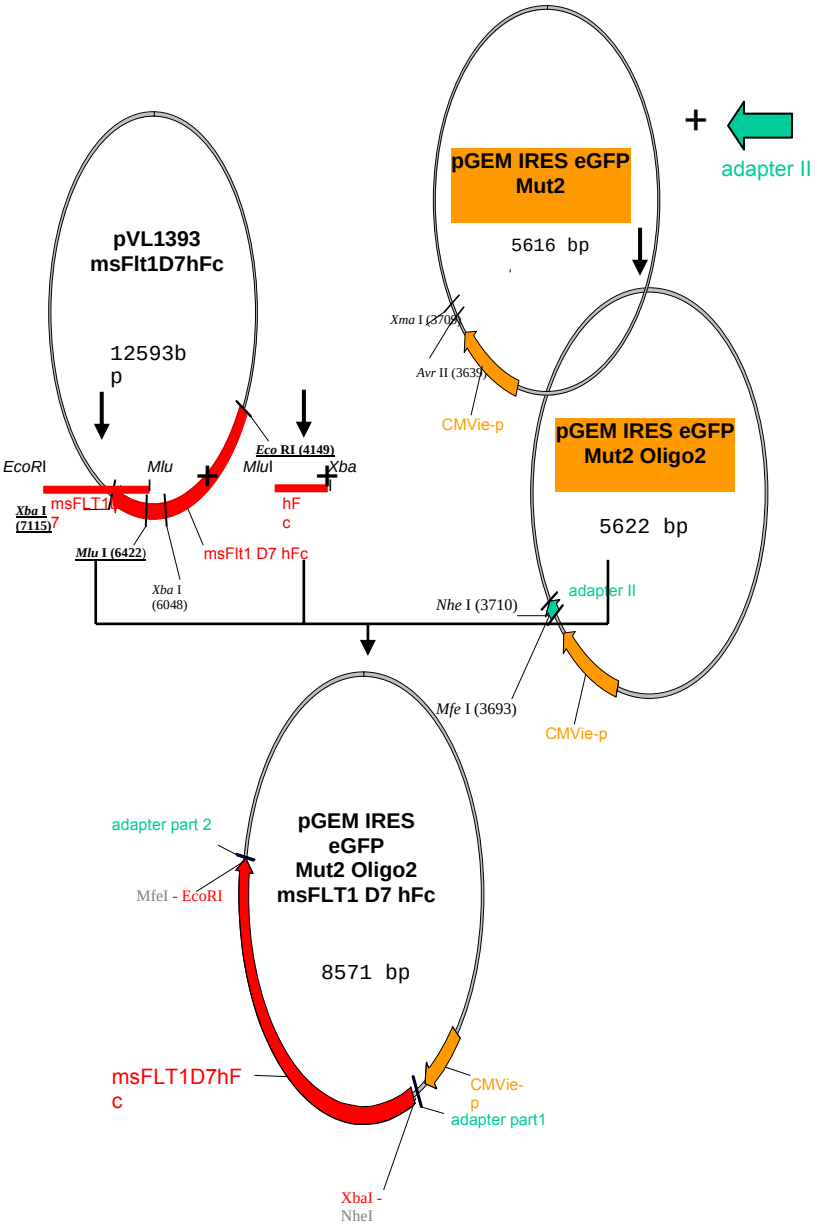


Figure 6: Cloning strategy for subcloning msFLT1 D7 hFC: Isolation of the gene by restriction digest followed by cloning two fragments of msFLT1 D7 hFc and the vector fragment in parallel.

2.2 Production of adenoviruses used into the mouse model

Adenoviruses have been generated in our department together with Dr.W.Lindenmeyer for several other projects. In our mouse model we used Ad-eGFP acting as negative control, containing the gene for enhanced green fluorescent protein (eGFP) behind an internal ribosomal entry site (IRES) to monitor infection processes in-vitro. Further Ad-VEGF-A and Ad-sFLT1were also applied to the mouse model, constructed by using the Ad-eGFP construct as basis for cloning. The construction of adenoviral constructs was already published (Mayer et al, 2004). In addition to the generated viruses as described above we will use another negative control. The Ad-msFLT4-mFc contains a homologue tyrosine kinase receptor permitting signalling via VEGF-C and VEGF-D resulting in regulation of lymphangiogenesis. Hence overexpressed protein should not crossreact with regulators of angiogenesis and the inducer of preeclampsia used in the model. The pAd-msFLT4-mFc cDNA could be obtained by cooperation (G.Christofori, University Basel)

A defined titer of viral particles is required to induce symptoms in the model described by preeclampsia. Therefore production followed by enrichment of viruses is required to yield adequate amounts of viral particles for several experiments.

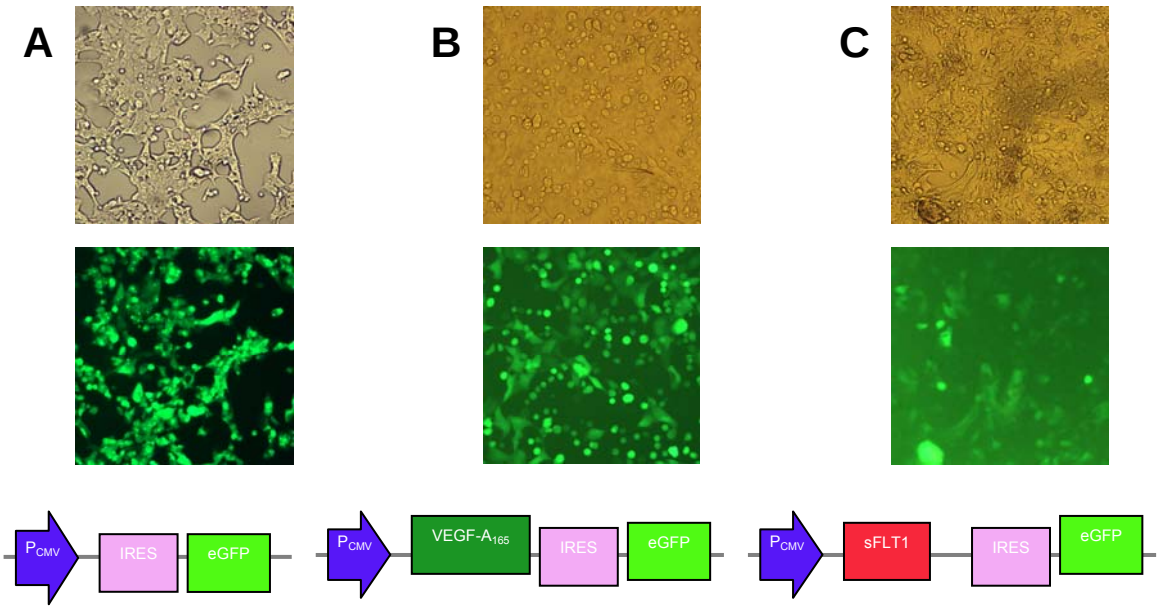


Figure 7: Recombinant adenoviruses used in the mouse model. Detection of eGFP by fluorescence microscopic analysis in adenoviral infected 293HEK cells indicates expression of adenoviral introduced proteins. **A)** Ad COS 45-eGFP as control **B)** Ad 45 VEGF-A 165 IRES-eGFP **C)** Ad 45 sFLT1 D6 IRES-eGFP

2.3. Establishment of a mouse model for preeclampsia

So far, a rat model has been reported before for preeclampsia investigating the influence of excess sFLT1 (7). We decided to use a mouse model for preeclampsia.

Within this model the preeclamptic state should be simulated by adenoviral overexpression of sFLT1. Rat models have the advantage that hypertension and renal defects can more easily measured. Compared to rats a wide range of tools is known to analyse mouse-derived materials. Preeclampsia is a pregnancy-associated disease whose symptoms are caused by excess sFLT1 in the maternal blood circulation, secreted by the placenta according to our work hypothesis. Indeed the placenta of mice is different from human but our work hypothesis focus on the overexpression of the soluble receptor molecule and the resulting symptoms, thus mice are adequate as model organism.

In addition mouse models are better established in our research centre and less adenoviruses have to be injected for sFLT-1 expression in the animals. The mouse model orientates on symptoms mainly described as preeclampsia that are caused by excess sFLT1 in the maternal blood circulation:

i) the damage of the kidney

ii) proteinuria

ii) hypertension

i) Damage of the kidney. The so-called Bowmans capsule, made from podocytes, surrounds the filtration capillaries of the glomeruli, the functional units of the kidney. During healthy pregnancy podocytes release VEGF-A, which is required for the maintenance of the fenestrated state of this sensible endothelium resulting. In the case of preeclampsia the released VEGF-A is bound by excess sFLT1, the fenestrated state cannot be maintained resulting in a decrease or loss of filtration of blood. This can be shown in histological sections by swollen endothelium combined with loss of capillary lumen and splitted basal membranes. The kidney is the organ with the highest amount of VEGF indicating a thight regulation. Therefore dysregulation of the VEGF level are first seen in the kidney. Hence we used the kidney as an indicator for the mouse model.

ii) Proteinuria. In proteinuria globular proteins are found in the urine as a result of increased permeability of the glomerular endothelium.

iii) Hypertension. Finally, hypertension is also detected due to the lack of the vasodilatory growth factor VEGF-A.

The model reflects a state of VEGF-A dysregulation by binding to a soluble receptor molecule decreasing the protein level of the ligand. Further experiments are planned as already described in the proposal to modify the model by co-expression of different players in angiogenesis like VEGF-A, sFLT1 and PlGF.

In a first step the titer of AdV-sFLT1 required for a distinct effect in the kidney had to be determined. As the amount of available virus was limited we decided to start with single mice till the essential titer is found. We chose female Balb/c mice, 4-6 weeks old, because kidney defect can easier be studied in this strain. Three adenoviral constructs were used: Ad- eGFP as negative control, Ad-VEGF-A as positve regulator and Ad-sFLT1 as negative regulator. The experiments were carried out as shown by the time course as in Fig. 8.

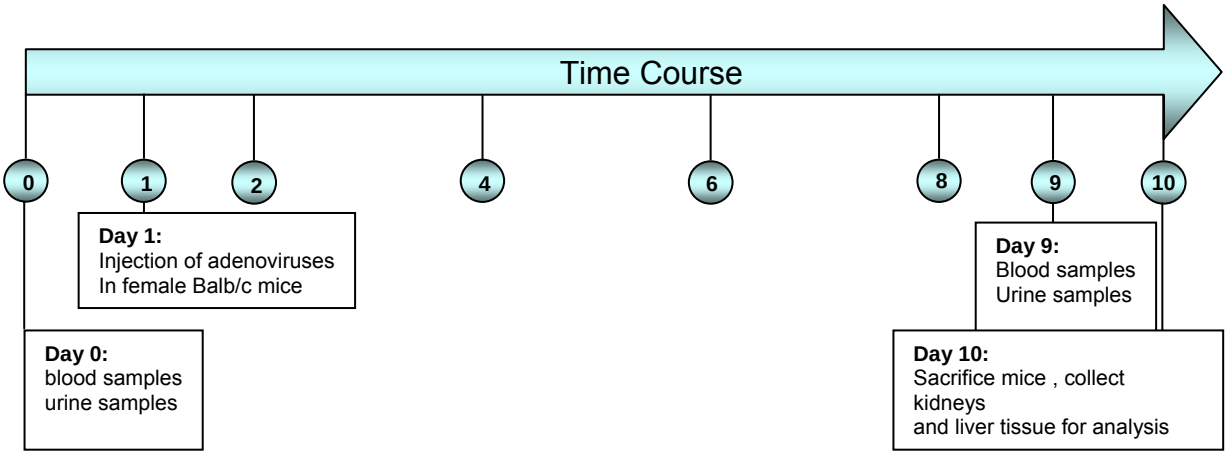


Figure 8: Time course of the preeclamptic mouse model.

Blood and urine samples were taken one day before injection of viruses, and at the end of the experiment, whereas blood were obtained by puncturing the retrobulbary vein. Viruses were applied diluted in PBS and two doses were used of each virus. For the eGFP virus we used only the high dose. One untreated mouse completed experimental set up. Viruses were generally applied to the mice by injection into the tail vein. Summarized we used six mice per experiment.

TV 1: In the first experiment 50µL PBS containing $2{,}5x10^6$ pfu, and 50µL PBS containing $7{,}5x10^6$ pfu of each virus were injected into the tail vein. All the six Mice were observed daily regarding behaviour and quality of skin and eyes to check health. After ten days blood and urine samples were taken again and mice were sacrificed. Liver and Kidney were removed and divided, one half shock frozen for RNA-preparations and analysis, the other half saved for histological sections by deposition in 10% formaldehyde pH 7.4.

The defect in the kidney especially in the filtration units of the kidney is recognized in histological sections due to the lack of lumens as a consequence of occlusion of capillaries, the swollen endothelium. Further proliferative damage can be detected in an elevation of mesangiomal cells, fixing the capillaries. Membraneous glomerular nephritis can be found only by electronic microscopy (EM), and is characterized by splitting of basal membranes surrounding glomerular endothelium. EM was not used until first signs of kidney damage are seen by light microscopy indicating a strong and clear damage. Histological sections, EM and evaluation of sections were performed due to collaboration (Prof .Gruber, Dept Veterinary Medicine, University Berlin). In the end of the first experiment no defect could be observed described above.

TV 2: A second experiment was performed using $0{,}5x10^8$ pfu and $1x10^8$pfu per mouse according to published data describing damage of glomerular endothelium in rats induced by $1x10^8$pfu of sFLT1 expressing adenovirus (7). But only slight morphologic differences in the glomeruli were detected in haematoxylin stained sections. Interestingly defects could be found selectively in juxtamedular located glomeruli. A reason could be that glomeruli located in the cortex are immature and therefore regenerate more than matured glomeruli.

TV 3: Hence for the third experiment we used adult mice (11 weeks), and increased the titer of adenoviruses again injecting $3.5x10^8$pfu and $5x10^8$pfu per mouse. In this experiment the PBS diluted volume of sFLT1 virus injected in mice were augmented from 50µL to 150µL because of the high content of caesium chloride. In the end of this experiment, kidney sections of sFLT1virus injected mice showed occlusions of lumens of glomerular endothelium (data not shown). We performed EM sections but still waiting for the outcome. This was a good result but we had to show that the defect induced by overexpressed soluble receptor protein is reproducible.

TV 4: To enforce the effect seen in experiment 3 we elevated viral concentration and tried to inject $1x10^9$pfu and $2x10^9$pfu per mouse. Due to the low titer of sFLT1 virus preparation the content of caesium chloride was very high. Both animals died during injection. We suppose that caesium chloride displayed important ion pumps in the animals.

TV 5: We decided to repeat experiment three injecting $3.5x10^8$pfu and $5x10^8$pfu per mouse, and could fortunately verify results obtained in this experiment (Figure 4: Methylenblue-stain).

TV 6: As already mentioned the concentration of sFLT1 virus preparation was found to be the limiting factor. In order to circumvent this fact we repeated the experiment again but over a period of 20 days instead of 10 days expecting amplification of the defect. Investigation of sections showed same results observed in the last experiments (Figure 9: HE-stain).

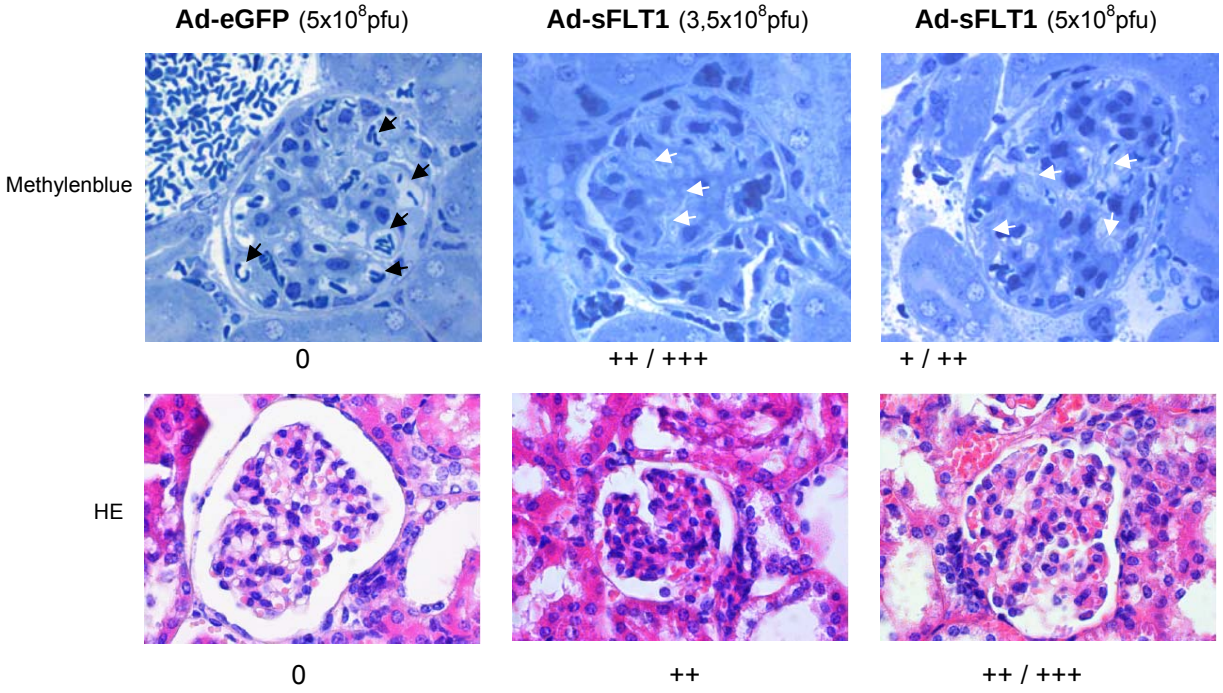


Figure 9: Overexpression of sFLT1 induces glomerular endotheliosis.
Histological analysis of renal tissue of adenoviral treated mice: Representative glomeruli were selected. ***Methylenblue-Azure-stain***: synthetic resin sections, 1µm thick, magnification 40x. Black arrowheads show open lumens of glomerular capillaries filled with erythrocytes. White arrowheads show occlusions of glomerular capillaries indicating a swollen endothelium as consequence of dysbalanced VEGF level provoked by sFLT1 overexpression. ***Haematoxylin-Eosin (HE)-stain***: marks preferentially nuclei, paraffine sections 3-5µm thick, 40x magnification, display closed capillaries compared to the Ad-eGFP treated animal, no rise of mesangiomal ncuclei could be detected. Semiquantitative analyses were carried out by the pathologist mentioned above: 0: no damage, +: low-grade damaged, ++: moderately damaged, +++: high-grade damaged.

DISCUSSION

During the first 4 years of the project we generated two transgenic SP-C flt-1 D1-6 founder lines which breed now in the F5 generation. Further, we generated several chimeric SP-C flt-1 embryos at the embryonic stages E16.5, E18.5 and P1. Different SP-C flt-1 have been used. As an example the SP-C flt-1 Fc exon/intron constructs have been tested for their *in vitro* function. They could be verified at the mRNA and protein level to be functional – though the expression was relatively low.

Via RT-PCR we could verify, that most of the generated human Flt-1 transgenic mice expressed a human flt-1 mRNA. But between all of these animals we could only detect the transgenic human Flt-1 protein in some adult F0 and F1 transgenic mice. They expressed the transgenic Flt-1 protein in a few (1%) alveolar type II pneumocytes.

From the very beginning the critical point of our project had been the problem to detect the transgenic human Flt-1 protein in transgenic mice. This was partially due to difficulties with the anti human Flt-1 antibodies, which either recognized human and murine Flt-1 at the same time or gave a high background in mouse embryos. In one of our embryo experiments we switched therefore to new SP-C flt-1 constructs with a human Fc tag. These plasmids would express a human Flt-1 Fc protein, which could be detected with a suitable anti human Fc-tag antibody. None of the antibodies could reveal any difference between wildtype and transgenic embryo, though also that approach failed.

Tumor induction experiments with mice from the 14380 and 14377 founder lines have shown, that transgenic SP-C flt-1 D1-6 mice express tumors to a similar degree like their wildtype littermates.

Starting this study our main question had been, if the expression of human Flt-1 in the transgenic mouse lungs could prevent tumor cell settling and tumor growth. Now we can answer, that the week human Flt-1 expression has no effect on tumor growth. Further it seems, that human Flt-1 expression cannot be enhanced after the injection of tumor cells.

There is a slight evidence for the exhibition of a human Flt-1 related phenotype: Some of the transgenic embryonic lungs at E18.5 and P1 looked more mature than the lungs of their wildtype littermates. The morphometric analyses revealed, that these transgenic lungs have more alveoli and thinner alveolar septa. Overall these lungs are better unfolded and aerated, leading to a better gas exchange directly after birth. But between all analysed transgenic embryos only a few embryos exhibited that special phenotype. Further, we could – even in these embryos – not detect the human Flt-1 protein.

Another attempt to detect the transgenic human Flt-1 protein in mouse lungs was the tissue culture of transgenic lungs. Transgenic lungs from E18.5 embryos were taken into cell culture. There mainly the type II cells proliferate. The supernatants were analysed for its human Flt-1 content via anti human Flt-1 ELISA. That experiment, which had been done so far only ones, revealed that primary transgenic lung cells secrete human Flt-1 into the cell culture medium 4 weeks after starting cell culture. With 90-220 pg/ml the sFlt-1 values had been relatively low, but significant compared with the wild type littermate culture supernatants (13-38 pg/ml).

Though, we have to conclude, that we are not able with the existing SP-C flt-1 plasmids to generate human sFlt-1 expressing mice or embryos, which overexpress the transgenic protein in alveolar type II cells. The SP-C flt-1 plasmids we have used permit a low human Flt-1 expression – as we can measure with the RT-PCR. We could detect a few alveolar type II cells in F0 and F1 adult mice expressing human Flt-1. We could identify a slightly chanced phenotype in some transgenic E18.5 and P1 lungs being more mature, better unfolded and aerated. And we could measure human sFlt-1 in primary cell culture supernatants from transgenic lungs. Finally, we have to ask the question, if an overexpression of human Flt-1 in the murine lung would have any effect on the development of the mouse lung and its phenotype, because it seems, that the lung tissue may be able to tolerate the additional expression of sFlt-1 to a certain degree.

In the last two years we started a different animal model concerning the adenoviral overexporession of sFlt-1 for the induction of preeclamptic symptoms in mice. The aim of the last project was the establishment of a mouse model for preeclampsia regarding the hypothesis that symptoms are provoked by an overexpression of the soluble receptor molecule FLT1. Therefore the sFLT1 protein should be adenoviral overexpressed and in the resulting model we wanted to modify the balance of aniogenic molecules regarding the FLT-1 pathway by adenoviral co-overexpression of regulators and blockade of the sFLT1 by an antibody. For this reason a blocking anti-FLT1 antibody should be developed, recombinant adenoviruses produced and a mouse model established using the adenoviruses and the antibody.

From the beginning the critical point of our project has been the virus production being the major precondition for the mouse model. As an Ad-sFLT1 was available, we additionally started to produce an adenovirus containing the mouse sFLT1 combined with the Fc-domain expecting more stability and a lower clearance during experiments with mice. First we tried to clone the msFLT1 D7-hFc gene, derived by restriction from a baculovirus vector in the adenoviral expression vector. Thus we were not successful until now, we applied an alternative strategy and started to amplify the msFLT1 D7-hFc. To perform experiments in mice adequate amounts of viral particles are required for several experiments. We therefore produced, enriched and purified different adenoviruses like Ad-eGFP, Ad-sFLT1, Ad-VEGF and Ad-msFLT4-mFc numerous times followed by determination of titer.

The adenoviral expressed proteins sFLT1 D1-6 and rhVEGF-A_{165} could be detected by *in-vitro* experiments. We verified the accumulation of protein over time and the relation of expressed protein to the applied virus concentration. Thus we could show that adenoviral infected A549 cells secreted up to 80ng/mL sFLT1 and up to 710ng/mLVEGF-A during a 3-day time period.

We were able to show that a titer of $5x10^8$ plaque forming units of Ad-sFLT1 injected in mice induces an effect in the glomerular endothelium. That could be demonstrated by kidney sections of sFLT1 treated mice showing a partial occlusion of capillary loops of juxtamedular glomeruli, probably as a result of the swollen endothelium and mesangiom. Electronic microscopy was performed to detect further characteristics of glomerular damage like basal membrane splitting, and this method could confirm a dysregulation of the endothelum insite the glomerulus.

Finally, our results indicate that a titer of $5x10^8$pfu Ad-sFLT1 induces glomerular damage in treated Balb/c mice. Therefore we are confident that we have established the mouse model to investigate the imbalance of angiogenic molecules in the circulation. Further experiments are planned with the different adenovirues for sFlt-1and sFlt-Fc to find out, if both viruses are equal potent to induce preeclamptic like lesion in the kidney. From other report we expect, that sFlt-Fc Ad will have a more dramatic effect in endotehlial dysfunction because of the much longer half life of the solube receptor if tagged with an Fc. After finishing this comparision we plan to measure the blood pressure in mice online after the induction of preeclamptic lesion. This will be done in collaboration with the MDC in Berlin-Buch and will be the first time, that a defined induction of endothelial dysfunction by reduction endogenous VEGF supply of the endothelium will allow to measure the increase of blood pressure. In a series of experiments we plan to modulate the effect of sflt-1 in the animal by coinfection of certain amount of Ad coding for VEGF and PlGF. Because these are antagonist of sFlt-1 action (agonist for endothelial function) the net effect by sflt-1 can be analyzed and may be helpful for a model, can sFlt-1 can interact in an animal model and which amount of corresponding ligand are necessary for neutralization. At the end of our planned investigations with our animal we want to analyze the effect of sFlt-1 blocking antibodies, which are available for us by a collaboration with Dr. M. Shibuya. ‘these final experiments will allow to answer our hypothesis, that fast induced lesion like preeclampsia can be diminished by treatment with blocking antibodies.

REFERENCES

Bando H, Weich, HA, Brokelmann M, Horiguchi S, Funata N, Ogawa T, Toi M: Association between intratumoral free and total VEGF, soluble VEGFR-1, VEGF receptor-2 and prognosis in primary breast cancer. British Journal of Cancer 92:553-561, 2005.

Bellusci S et al.: Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Dev* 124: 53-63, 1997.

Briles EB and Kornfeld S: Isolation and metastatic properties of detachment variants of B16 melanoma cells. *J Natl Cancer Inst* 60: 1217-1222,1978.

Clark JC et al. : FGF-10 disrupts lung morphogenesis and causes pulmonary adenomas in vivo*. Am J Physiol Lung Cell Mol Physiol* 280: L705-715, 2001.

Compagni A, Wilgenbus P, Impagnatiello MA, Cotton M, Christofori G: Fibroblast growth factors are required for efficient tumor angiogenesis. *Can Res* 60:7163-7169, 2000.

Eming SA, Lauer G, Cole M, Jurk S, Christ H., Hornig C, Krieg T, Weich HA: Increased levels of the soluble variant of the vascular endothelial growth factor receptor VEGFR-1 are asscociated with a poor prognosis in wound healing. *J. Investigative Dermatology* 123: 799-802, 2004.

Fidler IJ: Inhibition of pulmonary metastasis by intravenous injection of specifically activated macrophages. Cancer Res 34:1074-1078, 1974.

Fidler IJ: Biological Behaviour of malignant melanoma cells correlated to their survival in vitro. *Cancer Res* 35:218-224, 1975.

Fidler IJ and Kripke ML: Metastasis results from preexisting variant cells within a malignant tumor. *Science* 197: 893-895, 1977.

Gruemmer R, Motejlek K, Berghaus D, Weich HA, Neulen J: Regulation of soluble vascular endothelial growth factor receptor (sFlt-1/sVEGFR-1) expression and release in endothelial cells by human follicular fluid and granulosa cells. *Reprod. Biol. Endocrinol.* 3:57, 2005.

Helske S, Vuorela P, Carpen O, Hornig C, Weich H, Halmesmäki E: Expression of VEGF receptors 1, 2 and 3 in placentas from normal and complicated pregnancies. *Molecular Human Reproduction* 7:205-210, 2001.

Hornig C, Behn T, Bartsch W, Yahon A, Weich HA: Detection and quantitation of complexed and free soluble human vascular endothelial growth factor receptor 1. *J Immunol Meth* 226:169-177, 1999.

Hoyle GW et al. : Emphysematous lesions, inflammation, and fibrosis in the lungs of transgenic mice overexpressing platelet-derived growth factor. *Am J Pathol* 154: 1763-1775, 1999.

Kanno S, Oda N, Abe M, Terai Y, Ito M, Shitara K, Tabayashi K, Shibuya M, Sato Y: Roles of two VEGF receptors, FLT1 and KDR, in the signal transduction of VEGF effects in human vascular endothelial cells. *Oncogene* 19:2138 – 2146, 2000.

Korfhagen TR et al.: Cis-acting sequences from a human surfactant protein gene confer pulmonary-specific gene expression in transgenic mice. *Proc Natl Acad Sci U S A* 87: 6122-6126, 1990.

Lenz T, Gauer S, Weich HA, Haak T, Bergner R and Gossmann J (2005) VEGF and Flt-1 are not correlated to EPO in patients with diabetic nephropathy. Nephrology 10. 84-89.

Machens HG, Salehi J, Weich H, Munch S, Siemers S, Krapohl BD, Herter KH, Kruger S, Reichert B, Berger A, Vogt P, Mailander P: Angiogenic effects of injected $VEGF_{165}$ and sVEGFR-1 (sFLT-1) in a rat flap model. *J Surg Res.* 111:136-142, 2003.

Mayer H, Bertram H, Lindenmaier W, Korff T, Weber H, Weich HA: Vascular endothelial growth factor (VEGF-A) expression in human mesenchymal stem cells: autocrine and paracrine role on osteobalstic and endothelial differentiation. *J Cell Biochem* 95: 827-839, 2005.

Maynard SE, Min JY, Merchan J, Lin KH, Li J, Mondal S, Libermann TA, Morgan JP, Sellke FW, Stillmann IE, Epstein FH, Sukathme VP, Karumanchi SA: Excess placental soluble fms-like tyrosine kinase 1 (sFLT1) may contribute to endothelial dysfunction, hypertension and proteinuria in preeclampsia. *J Clin Invest* 111:649-658, 2003.

McCafferty J, Hoogenboom HR, Chiswell DJ: Construction and use of antibody gene repertoires. Antibody Engineering. Practical Approach Series. IRL Press/ Oxford University Press (Oxford, New York, Tokyo, 1996.

Miyazaki Y et al. :Expression of a tumor necrosis factor-alpha transgene in murine lung causes lymphocytic and fibrosing alveolitis. A mouse model of progressive pulmonary fibrosis. *J Clin Invest* 96: 250-259, 1995.

Neulen J, Wenzel D, Wünsch E, Weissenborn U, Büttner R Weich, HA: Poor responder- high responder: the importance of soluble vascular endothelial growth factor receptor 1 (sVEGFR-1) in ovarian stimulation protocols. *Human Reproduction* 16:621-626, 2001.

Spearman C, Kärber G: Titerbestimmung von Viren. Virologische Arbeitsmethoden. In: Bibrack B.W.G. (Ed.), pp37-39, 1974.

Toi M, Bando H, Ogawa T, Muta M, Hornig C, Weich HA: Significance of vascular endothelial growth factor (VEGF)/ soluble VEGF receptor-1 relationship in breast cancer. *Int. J. Cancer* 98: 14-18, 2002.

Vuorela P, Helske S, Hornig C, Alitalo K, Weich HA, Halmesmäki E: Amniotic fluid soluble vascular endothelial growth factor receptor-1d in pre-eclampsia *Obstet & Gynecol* 95: 353-357, 2000.

Wert SE, Glasser SW, Korfhagen TR and Whitsett JA: Transcriptional elements from the human SP-C genedirect expression in the primordial respiratory epithelium of transgenic mice. *Dev Biol* 156:426-443, 1993.

Wikenheiser KA et al.: Production of immortalized distal respiratory epithelial cell lines from surfactant protein C/simian virus 40 large tumour antigen transgenic mice. *Proc Natl Acad Sci USA* 90:11029-11022, 1993.

Wikenheiser KA, Clark JC, Linnoila RI, Stahlman MT, Whitsett JA: Simian virus 40 large T antigen directed by transcriptional elements of the human surfactant protein C gene produces pulmonary adenocarcinomas in transgenic mice. *Cancer Res* 52: 5342-5352, 1992.

Zeng X, Wert, SE, Federici, R, Peters, KG, Whitsett, JA: VEGF enhances pulmonary vasculogenesis and disrupts lung morphogenesis in vivo. *Dev Dyn* 211:215-227, 1998.

Zhou L, Dey CR, Wert SE, Whitsett JA: Arrested lung morphogenesis in transgenic mice bearing an SP-C-TGF-beta1 chimeric gene. *Dev Biol* 175: 227-238, 1996.

VEGF

Pathogen-encoded Angiogenesis Factors

Prof. Dr. Christoph Dehio

Focal area Infection Biology
Biozentrum, University of Basel
Klingelbergstrasse 70, 4056 Basel, Switzerland

Member of the SPP 1069 from 1999 until 2002

SUMMARY

Human infection with the Orf parapox virus or the bacterial pathogen *Bartonella henselae* can trigger pathological angiogenesis resulting in vascular tumor formation. In previous work, the Orf virus-encoded vascular growth factor VEGF-E has been identified as a homolog of human vascular endothelial cell growth factor A (VEGF-A), and a purely characterized component of the outer membrane of *B. henselae* was assigned as the bacterial angiogenesis factor. The goal of the project was a better molecular characterization of these pathogen-encoded angiogenesis factors. Several Orf virus-encoded variants of VEGF-E were identified, sequenced, and characterized regarding their receptor specificity and biological activity. All identified VEGF-E variants displayed mitogenic activity for endothelial cells to various degrees, which could be correlated with the binding activity to VEGFR-2. Interestingly, enhancement of VEGFR-2 binding has been observed due to the interaction with the co-receptor neuropilin-1. In the frame of several collaborations within or outside of the SPP 1069, VEGF-E was used as a VEGFR-2-specific (and VEGFR-1-independent) growth factor to characterize the role of VEGF-receptors in various biological processes. To identify the angiogenic activity within the outer membrane of *B. henselae*, the corresponding subproteome was characterized by 2D-gel electrophoresis and mass spectrometry. Experiments to identify the angiogenic activity in this subproteome are under way.

A final report on the project DE 539/4-2 was already submitted to the DFG on June 20th 2003. After review by an external expert, this report was officially approved by the DFG on October 1st, 2003. Here I provide an English translation of this report originally written in German. Given that I did not received further funding from the DFG in the frame of this project, I did not update the report regarding the progress of subsequent studies on this topic funded by other agencies after October 2003.

RESULTS

Orf virus-encoded VEGF-E as angiogenesis factor

Receptor-specificity of variant forms of VEGF-E

Further to the VEGF-E variant D1701 originally described by my group (Meyer et al. 1999) several VEGF-E variants were cloned and sequenced in a collaboration with Dr. T. Büttner and Dr. H.-J. Rziha from the “Bundesforschungsanstalt für Viruskrankheiten der Tiere”. The viral isolates BO74, MRI, BO29, BO23, CA11 and S1 are variant forms of VEGF-E, which were all expressed as recombinant proteins in *E. coli* and purified to homogeneity as originally described for the D1701 prototype (Meyer et al. 1999). All recombinant variants displayed VEGFR-2-dependent mitogenic activity for human umbilical vein endothelial cells (HUVEC). Biacore binding assays to VEGFR-2 and the VEGFR-2-dependent Ca^{2+}-influx measured in transfected PAE cells displayed differences in their

capacity of these VEGF-E variants to bind to VEGFR-2: VEGF-E_{D1701} and VEGF-E_{BO74} interacted most strongly with VEGFR-2, while VEGF-E_{BO23} and VEGF-EBO29 display reduced binding activity. as compared to VEGF-E_{D1701}. Strikingly, VEGF-E_{MRI} did not display any interaction with VEGFR-2 under these conditions. This variant required the co-expression of neuropilin-1 (NP-1) to cause the characteristic biphasic Ca^{2+}-signal, which was at that time the first hint for a physiological role of this VEGF coreceptor for the angiogenic stimulation. Due to my appointment as a professor of Molecular Microbiology at the Biozentrum of the University of Basel in April 2000 and the resulting reorientation of my research towards bacterial pathogenesis, this promising study was unfortunately not continued and thus did not result yet in a publication.

Biological activities of the VEGFR-2-specific growth factor VEGF-E

In the frame of different collaborations within and outside of the SPP 1069, VEGF-E was used as a VEGFR-2-specific (and VEGFR-1-independent) vascular growth factor. This was very instrumental to study the intra- and intermolecular “cross-talk” of the VEGF receptors VEGFR-1 and VEGFR-2 (Auterio et al., 2003).

In another study, recombinant VEGF-E carrying a heparin-binding domain of VEGF-A displayed the role of heparin-binding for enhancement of endothelial cell proliferation as well as for the mobilization of stem cells from bone marrow (Heil et al., 2003).

Two other studies using VEGF-E allowed to demonstrate that the chemotactic migration of either primary human osteoblasts (Mayr-Wohlfahrt et al., 2002) or human mesenchymal progenitor cells (Fiedler et al., 2005) is VEGFR-1-dependent. Regarding the role of VEGF-E for the pathogenesis of parapox viruses, we could show that angioproliferative Orf virus isolates always contained a VEGF-E homolog, while the non-proliferative parapox virus isolates from bovine did not encode a VEGF-E homolog. Thus, the presence of a VEGF-E-encoding gene in parapox virus isolates correlates with their capacity to cause angioproliferative processes (Rziha et al., 2003).

Characterization of the angiogenic activity of *B. henselae*

The angiogenic proliferation of endothelial cells by *B. henselae* can result from either a direct mitogenic activation by a bacterial angiogenesis factor, or via an angiogenic loop resulting from the infection of macrophages and the subsequent release of VEGF-A, which acts in a paracrine manner to stimulate the proliferation of endothelial cells (Dehio, 2003; Dehio, 2005). In the frame of SPP 1069 we have investigated the direct stimulation of endothelial cell proliferation by *B. henselae*.

Subcellular fractionation experiments allowed us to localize the angiogenic activity to the outer membrane of the Gram-negative bacterial cell. This activity revealed to be heat- and protease sensitive and is therefore likely of proteinacious nature. We therefore used 2D-PAGE and mass spectrometry to determine the subproteome of the *B. henselae* outer membrane. In the frame of a collaboration with the group of S. Andersson in Uppsala, Sweden, which sequenced the at that time unpublished *B. henselae* genome sequence, we were able to identify 53 distinct protein species associated with the outer membrane (Rhomberg et al., 2004). Experiments to assign the angiogenic activity to any of the identified protein are ongoing.

OUTLOOK

The results obtained in this project in the frame of SPP 1069 improved our understanding of the molecular mechanism of action of Orf virus-encoded VEGF-E. Due to the selective binding of VEGF-E to VEGFR-2, but not to VEGFR-1, this growth factor is an important receptor-specific tool for probing the role of VEGF receptors in angiogenesis and related physiological responses. The proteomic identification of angiogenesis factors in the outer membrane of *B. henselae* has not been achieved in the frame of this project. However, the characterization of the outer membrane subproteome represents an important basis for the identification of this factor in ongoing studies.

REFERENCES

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, May-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin D, Persico G, Herbert J-M, Communi D, Shibuya M, Collen D, Conway EM, Carmeliet P: Role of PlGF in the intra - and inter molecular crosstalk between the VEGF receptors Flt1 and Flk1. *Nat Med* 9: 936-943, 2003.

Dehio C: Recent progress in understanding *Bartonella*-induced vascular proliferation. *Curr Opin Microbiol* 6: 61-5, 2003.

Dehio C: *Bartonella*-host cell interactions and vascular tumour formation. *Nat Rev Microbiol* 3: 621-631, 2005.

Fiedler J, Leucht F, Waltenberger J, Dehio C, Brenner RE: VEGF-A and PIGF-1 stimulate chemotactic migration of human mesenchymal progenitor cells. *Biochem Biophys Res Commun* 334: 561-568, 2005.

Fuhrmann O, Arvand M, Krull M, Hippenstiel S, Seybold J, Schmid M, Dehio C, Suttorp N: *Bartonella henselae* induces NF-kB-dependent upregulation of adhesion molecules in cultured human endothelial cells: possible role of outer membrane proteins as pathogenic factors. *Infect Immun* 69: 5088-5097, 2001.

Heil M, Mittnacht-Krauss R, Issbrücker K, van den Heuvel J, Dehio C, Schaper W, Clauss M, Weich HA: An Engineered Heparin-Binding Form of VEGF-E (hbVEGF-E). *Angiogenesis* 6: 201-211, 2003.

Mayr-Wohlfart U, Waltenberger J, Hausser H, Kessler S, Gunther KP, Dehio C, Puhl W, Brenner RE: Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts. *Bone* 30: 472-477, 2002.

Meyer M, Lanz C, Clauss M, Lepple-Wienhus A, Waltenberger J, Augustin H, Ziche M, Büttner M, Rziha H-J, Dehio C: A novel vascular endothelial growth factor encoded by Orf virus, VEGF-E, mediates angiogenesis via signalling through VEGFR-2 (KDR) but not VEGFR-1 (Flt-1) receptor tyrosine kinases. *EMBO J* 18: 363-374, 1999.

Rhomberg TA, Karlberg O, Mini T, Zimny-Arndt U, Wickenberg U, Rottgen M, Jungblut PR, Jenö P, Andersson SGE, Dehio C: Proteomic Analysis of the Sarcosine-Insoluble Outer Membrane Fraction of the Bacterial Pathogen *Bartonella henselae*. *Proteomics* 4: 3021-3033, 2004.

Rziha HJ, Bauer B, Adam KH, Rottgen M, Cottone R, Henkel M, Dehio C, Buttner M: Relatedness and heterogeneity at the near-terminal end of the genome of a parapoxvirus bovis 1 strain (B177) compared with parapoxvirus ovis (Orf virus). *J Gen Virol* 84: 1111-1116, 2003.

Selective Functions and Intracellular Signaling Mechanisms of Vascular Endothelial Growth Factor Receptor VEGFR1 (Flt-1)

Prof. Dr. Rolf Heumann

Molecular Neurobiochemistry
Biochemistry II, NC7/174
Ruhr University
Universitätsstr. 150, 44780 Bochum, Germany

Member of the SPP1069 from 1999 until 2001

SUMMARY

Vascular endothelial growth factor (VEGF) binds to tyrosine kinase receptors VEGFR1 and VEGFR2. The relative contributions of each receptor signaling for endothelial cell key functions are important for the development of future concepts in the field of tumor prevention and therapeutic angiogenesis.

We were using a combined approach including receptor-specific endogenous ligands (PlGF, VEGF-E), heterologous chimeric receptor ligand mouseCSF-1 for selective VEGFR1 signaling and intracellular immunization of the small GTP-binding protein Ras in transformed human umbilical vein endothelial cells (T-HUVEC) to measure effects on proliferation, migration, survival, and formation of tubular-like structures. VEGFR1 and VEGFR2 both activated Ras/MAPK stimulating cell division, migration and formation of tubular-like structures *in vitro*. Conversely, these effects were each sensitive to inhibition of Ras using function-blocking, single-chain antibody fragments. However, survival of serum deprived T-HUVEC or of normal HUVEC was mainly mediated through VEGFR2. *In vivo*, conditional intracellular inhibition of Ras resulted in a strongly attenuated rate of malignant T-HUVEC-derived tumor growth, mitotic activity and tumor volume in transplanted nude mice.

In order to further characterize the selective survival-promoting effect by VEGFR2, measurements of time-resolved NO release were performed using electrochemical microsensor techniques. Inhibition of NO synthesis by L-NAME abolished the VEGF-mediated survival effect. Addition of NO-donors mimicked the effect of VEGF in survival. Intracellular inhibitors of Src kinase family, 4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo [3,4-d]pyrimidine (PP2) also abolished VEGFR2-mediated cell viability. Ras activation was necessary for cell division and formation of tubular-like structures. Conditional inhibition of Ras with a single-chain antibody prevented tumor growth of human endothelial cell xenografts. Cell survival was mediated selectively by VEGFR2 through release of NO. Migration of T-HUVEC was regulated by dual signaling pathways involving Ras/MAPK and Src/NO.

Future perspective: These results may have implications for future therapeutical concepts towards a combined inhibition of Ras and Src in normal host endothelial cells preventing tumour angiogenesis by using improved nano-particle delivery systems.

Ephrin ligands and Eph receptors

Functional Analysis of Endothelial Cells during Angiogenesis

Prof. Dr. Hellmut Augustin

(*until 2/2001*):
Cell Biology Lab., Dept. of Gynecology & Obstetrics
University of Göttingen Medical School
Robert Koch Str. 40, 37075 Göttingen, Germany

(*since 3/2001*):
Dept. of Vascular Biology & Angiogenesis Research
Institute of Molecular Oncology, Tumor Biology Center
Breisacher Str. 117, D-79106 Freiburg, Germany

Member of the SPP 1069 from 1999 until 2006

SUMMARY

The field of angiogenesis research has experienced a change of paradigm during the last few years in that the simplistic concept of an invading and migrating capillary sprout is increasingly being replaced by an appreciation of vascular morphogenesis as a complex three-dimensional process. Several classes of molecules are associated with this process. Genetic studies have shown that ephrin molecules are involved in the remodeling/maturation step of vascular morphogenesis. In order to shed further light on vascular remodeling mechanisms during angiogenesis, we have established a three dimensional model of endothelial cells (EC) and smooth muscle cells (SMC) that mimics the physiological assembly of the vessel wall in an inside out orientation (Korff and Augustin, 1998; Korff et al., 2001). Furthermore, we have established a novel three dimensional spheroidal in collagen gel assay that very much mimics the molecular and functional properties of endothelial cells during angiogenesis (Korff and Augustin, 1999). Applying these assays, we could demonstrate that endothelial EphB/ephrinB expression is primarily not an inherent property of arterial and venous EC, but rather controlled by microenvironmental cues (Korff et al., 2006). Likewise, functional cellular experiments revealed that ephrinB2-mediated reverse signaling in endothelial cells is compatible with an invasive and pro-angiogenic phenotype, whereas forward EphB4 signaling mediates cellular repulsion and endothelial cell segregation (Füller et al., 2003). Expression profiling experiments have shown that Eph receptors and ephrin ligands are not just expressed by neuronal and vascular cells, but in fact by many tissues including tumors. Manipulatory tumor experiments provided proof-of-principle evidence that soluble monomeric EphB4 receptor bodies can be employed to therapeutically interfere with tumor progression and tumor angiogenesis (Martiny-Baron et al., 2004). These findings also guided experiments aimed at deciphering the role of bi-directional EphB/ephrinB signaling in mediating interactions between endothelial cells and metastasizing tumor cells (TC). The combined anti-angiogenic and anti-tumor effect of sEphB4 suggested that EphB/ephrinB interactions do not just control EC-EC interactions or EC interactions with their microenvironment, but also TC-TC interactions and TC-EC interactions (Martiny-Baron et al., 2004). Based on these findings, we pursued experiments aimed at systematically studying the role of adhesive and angiogenic EphB/ephrinB crosstalk in controlling interactions between TC and EC that are involved in tumor progression and metastasis. We could demonstrate that TC expressing EphB4 adhere preferentially to ephrinB2 EC and that tumor cell expressed EphB4 plays a critical role in the organ distribution of metastasizing tumor cells (Schaffner et al., manuscript in preparation). Taken together, the project

pursued within the SPP1069 has led to the development of important novel experimental systems for the field of endothelial cell biology and angiogenesis research and yielded important insights into the gene-regulatory, functional, and tumor biological involvement of the EphB/ephrinB system. The identification of a role of EphB/ephrinB interactions in controlling site-specific metastasis has opened an important and exciting novel field of research that is the focus of ongoing and future studies.

INTRODUCTION

The angiogenic cascade has become increasingly complex involving distinct steps of organization and anastomotic network formation, asymmetric flow-driven orientation into arteries, capillaries and veins, mechanisms of maturation by recruitment of covering mural cells, and eventually molecularly poorly understood mechanisms of organotypic differentiation. The field is heavily driven by the application of advanced conventional and conditional genetic manipulation to study vascular morphogenesis in complex developmental *in vivo* models. In turn, the complexity of *in vivo* models makes mechanistic interpretation of observed phenotypes on the cellular level difficult. Yet, a mechanistic understanding of these molecules in the vascular system on the cellular level is largely missing due to the lack of appropriate cellular model systems. Based on the unique set of experimental tools developed in our lab, we proposed to utilize these tools to study the biology of the Eph/ephrin receptor/ligand system in endothelial cells (EC). Vascular functions of Eph receptors and ephrin ligands were uncovered in 1998 by the surprising findings of genetic loss-of-function experiments in mice which showed that EphB4 and ephrinB2 act as critical determinants of developmental arteriovenous differentiation and vessel remodeling (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). These experiments provided unambiguous evidence about the critical role of the Eph/ephrin system in the vascular system.

Eph receptors and ephrin ligands were originally identified as supposedly neuron-specific molecules that control axonal guidance and tissue-border formation. These pioneering experiments were surprising at the time and have not just led to the identification of a novel critical molecular system controlling the functions of the vasculature, but they have also opened the field of the molecular analysis of arteriovenous and organotypic vascular differentiation mechanisms (Heroult et al., 2006; Augustin and Reiss, 2003). Expression profiling experiments have shown that Eph receptors and ephrin ligands are not just expressed by neuronal and vascular cells, but in fact by many cell populations including intestinal epithelial cells, lung epithelial cells, hematopoietic cells, and many tumor cells (TC) (Hafner et al., 2004). Conceptually, the broad expression of Eph receptors and ephrin ligands in normal tissues and in tumors suggests that Eph and ephrin molecules are likely to exert much broader cell-cell communication roles as had originally been deduced from the phenotypes of the corresponding knock-out mice. As membrane molecules regulating functions of cells that are in direct juxtapositional contact, Eph/ephrin molecules are likely candidates to regulate multiple cell-cell interactions in the vascular system and to regulate the communication of vascular cells with non-vascular cells (e.g., metastatic tumor spread). This broader view of Eph/ephrin molecules as key regulators of cellular guidance and trafficking is also supported by recent experiments demonstrating that Eph/ephrin interactions transduce positional guidance cues in epithelial cells, most notably during colon epithelial cell differentiation (Batlle et al., 2002). Collectively, these data demonstrate that the Eph/ephrin system, originally thought to be a neuronal cell guidance controlling molecular system, has now evolved to become a rather universal cell guidance and trafficking-mediating molecular system. The literature also provides a strong rationale for an important role of the EphB/ephrinB system in the control of metastatic cell dissemination. Consequently, we have followed experiments aiming at systematically studying the role of adhesive and angiogenic EphB/ephrinB crosstalk in controlling interactions between TC and EC that are involved in tumor progression and metastasis.

Less than 40 angiogenesis-related original Eph/ephrin papers were published in the last 5 years before begin of the funding period. This reflects that, mechanistically, not much progress beyond the phenotype of the respective knockout mice has been previously made in the molecular and mechanistic understanding of the role of these molecules in the vascular system since the original description of their critical rate-limiting role in vascular morphogenesis and acquisition of arteriovenous vessel identity. This is largely due to 2 different reasons: First, the study of Eph and ephrin molecules is still restricted by the limited availability of relevant experimental tools (mostly suitable antibodies). Secondly, the field is hampered by the lack of appropriate experimental models in which to study Eph and ephrin molecules. These molecules are not just migration- or proliferation-regulating molecules for which functional readouts would be readily available. Instead, they are molecules that provide positional information cues to different cells. In the context of angiogenesis and vessel differentiation, these are functions for which few mechanistic functional experimental setups are available. Based on this background, we have proposed a broad and systematic structural and functional analysis of the Eph/ephrin system (initially focusing on EphB4 and ephrinB2) on EC functions and angiogenesis.

Focus of our lab's activities is the analysis of the molecular and functional properties of EC during angiogenesis. We pursued a broad program of individual projects aiming at functionally studying EC during angiogenesis in complex *in vitro* and *in vivo* experiments. In summary, the specific aims of this grant proposal were:

1. To establish a 3D co-culture system mimicking physiological vessel wall assembly and analyze the formation of complex capillary networks *in vitro* and *in vivo* originating from spheroids of different populations of EC.
2. To functionally analyze propulsive vs. repulsive functions of EphB4 and ephrinB2 that influence EC differentiation and vascular morphogenesis.
3. To assess endothelial ephrinB2 expression and microenvironmental cues that influence its expression and cellular localization.
4. To characterize the involvement of the EphB/ephrinB interaction during tumor growth and angiogenesis.
5. To establish functional *in vivo* experiments in different models of tumor cell dissemination and metastasis in order to characterize the involvement of ephrin molecules.

METHODS

A number of cellular assay systems have been developed to study the mechanisms of angiogenesis. These include *in vivo*, *in situ*, and *in vitro* assays. *In vitro* assays employing cultured EC provide the benefit of allowing the analysis of angiogenic EC functions under well defined and controlled experimental conditions. *In vitro* experimental systems of individual steps of the angiogenic cascade include migration assays (2D lateral migration, 3D vertical migration [Boyden chamber]) and proliferation assays. Correspondingly, more complex assays, such as the Matrigel alignment assay, the chemoinvasion assay, and the microcarrier bead assay have been developed to study complex morphogenic behavior of EC *in vitro*. Yet, the established assay systems have some inherent limitations. Some of them are restricted due to the fact that they only study individual steps of the angiogenic cascade (e.g. migration or proliferation). To circumvent this restriction we developed different *in vitro* assays described below.

In vitro spheroid sprouting assay

The 3D spheroid angiogenesis assay is based on the use of EC spheroids as a focal delivery device in a complex extracellular matrix. The cells are allowed to establish capillary-like sprouts over a period of 24-48h and the assay is quantitatively analyzed by determining the cumulative sprout length of all sprouts using an automated image analysis system.

Establishment of a 3D coculture system mimicking physiological vessel wall assembly

The physiological assembly of the blood vessel wall with a quiescent monolayer of endothelial cells covering a multilayered assembly of smooth muscle cells (SMC) can be mimicked in culture by a three-dimensional co-culture system of endothelial cells and smooth muscle cells. In this model, smooth muscle cells form the core of a spheroid which is covered by a monolayer of EC. This organization develops as a result of a distinct cellular organization phenomenon involving cell migration and Ca^{2+}-dependent cell-cell contacts. We have used this model to analyze SMC mediated crosstalk phenomena on cocultured EC and characterized that SMC control the quiescent phenotype of cocultured EC as described:

a) SMC contact of cocultured EC leads to an increased number of interendothelial junctional complexes
b) SMC contact of cocultured EC leads to a complete downregulation of endothelial PDGF-B expression
c) SMC contact of cocultured EC leads to reduced levels of endothelial apoptosis

Ephrin tools

Research into vascular functions of Eph receptors and ephrin ligands is hampered by the limited availability of generally available experimental tools. We have spent considerable efforts aimed at establishing our own unique experimental tools and a number of specific techniques.

- Reliable and routine RT-PCR techniques to screen for EphB receptor and ephrinB ligand expression in human and murine cells and tissues.
- EphB4-Fc and ephrinB2-Fc receptor body based staining techniques for the detection of Eph receptor and ephrin ligand expression profiling *in vitro* and *in situ*.
- Adaptation of ephrinB2 and EphB4 immunohistochemical staining protocols according to the recently published protocols for paraffin-embedded tissues.
- Endothelial (PAEC) EphB4, ephrinB2, and ∆ephrinB2 transfectants.
- A baculovirus-based expression and purification system for the production of soluble monomeric ephrinB2 and EphB4 protein.
- Tumor transfectants expressing full length and truncated soluble EphB4 and ephrinB2.
- Generation of EphB4, ∆EphB4, ephrinB2, ∆ephrinB2 expressing retroviral vectors for the transduction of HUVEC and other primary cell populations. These vectors contain the target molecules in fusion with a GFP tag allowing the tracing of expression in living cells.
- Establishment of tumor transfectants overexpressing full length and cytoplasmic truncated EphB4 in fusion with a GFP tag and a luciferase reporter gene allowing the tracing of tumor cells in living animals. Correspondingly, we have developed luciferase-based non-invasive imaging of established metastases in mice that allows the detection of small metastatic nodules containing approximately 50.000 cells using a CCD camera device (Fig. 1).

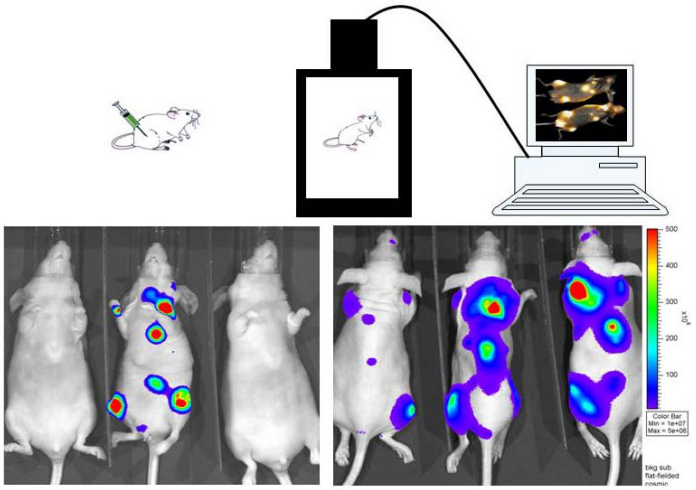


Fig. 1: Luciferase-based detection of metastatic tumors following the left-ventricular injection of tumor cells.

RESULTS

The important findings accomplished during the grant period funding are summarized below in order to answer to each specific aim.

1. **Establishment of a 3D coculture system mimicking physiological vessel wall assembly and analysis of the formation of complex capillary networks *in vitro* and *in vivo* originating from spheroids of different populations of EC.**

<u>Establishment of a 3D co-culture system mimicking physiological vessel wall assembly</u>

When pursuing confrontation experiments with EC spheroids together with tumor cell or SMC spheroids, we made some surprising observations of SMC controlling in gel sprouting activity of cocultured EC. These observations led to systematically analyzing EC/SMC crosstalk phenomena in coculture spheroids. Following these suspended coculture spheroids over time, we observed a spontaneous differentiation process. SMC organize to form the core of a coculture spheroid, whereas the EC form a thin monocellular surface layer (Fig. 2). This organization develops as a result of a distinct cellular organization phenomenon involving cell migration and Ca-dependent cell-cell contacts. In sum, these first experiments indicated that 3D coculture spheroids of EC and SMC provide a unique and highly reproducible approach to studying cellular interactions in the vessel wall.

We have used this model to analyze SMC mediated crosstalk phenomena on cocultured EC and characterized that SMC control the quiescent phenotype of cocultured EC as described:

a.) SMC contact of cocultured EC leads to an increased number of interendothelial junctional complexes.

We have performed ultrastructural analysis of EC/SMC coculture spheroids cultured for different times. The number of visible electron dense intercellular junctional complexes of the surface EC layer was quantitated as readout of the degree of EC differentiation. The number of interendothelial cell junctional complexes was found to increase over time in both, solo EC spheroids as well as in EC/SMC coculture spheroids. EC in coculture spheroids, however, establish more than twice as many junctional complexes after 4 days as solo EC spheroids.

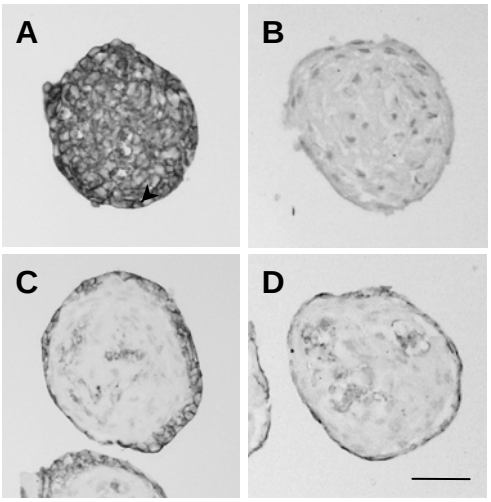


Fig. 2: Spontaneous differentiation of coculture spheroids of human umbilical vein endothelial cells (EC) and human umbilical artery smooth muscle cells (SMC). EC spheroids (**A**), SMC (**B**), and EC/SMC coculture spheroids (**C,D**) were cultured for various periods of time after which they were fixed, embedded, sectioned and stained for the expression of the endothelial cell marker CD31. (**A**) Section of a 2 d EC spheroid uniformly staining for CD31. (**B**) SMC in spheroids (2 d) do not express CD31. (**C**) EC/SMC coculture spheroid (2 d) with CD31 positive surface layer of EC and core of CD31 negative SMC. Few single CD31 positive EC are trapped in the center of the spheroid. (**D**) EC/SMC coculture spheroid (4 d) with flattened CD31 positive surface layer of EC and core of CD31 negative SMC. Scale bar in **D**: 50 µm.

b.) SMC contact of cocultured EC leads to a complete downregulation of endothelial PDGF-B expression.

Monolayer EC expresses abundant levels of PDGF under various culture conditions. This has repeatedly been interpreted to reflect the inability to properly mimic the *in vivo* quiescent EC phenotype in tissue culture. We consequently performed an analysis of PDGF-B expression in monolayer culture in comparison to the expression in solo and coculture spheroids. SMC in spheroids do not express PDGF. Co-culture spheroids of EC and SMC have detectable levels of PDGF in their center as well as in the surface monolayer after 1 day in culture. No PDGF expression is detectable in EC/SMC co-culture spheroids after 4 days. The surface monolayer of EC, which is in contact with the underlying SMC, becomes completely PDGF negative in these fully differentiated coculture spheroids.

c.) SMC contact of cocultured EC leads to reduced levels of endothelial apoptosis.

Solo EC and SMC spheroids as well as EC/SMC coculture spheroids were cultured for 2 days under low serum conditions. Under these conditions, EC in solo EC spheroids undergo massive apoptosis as evidenced by ELISA measuring the amount of fragmented DNA. In contrast, SMC cultured in spheroids have very low baseline levels of apoptosis.

SMC mediated endothelial nonresponsiveness to VEGF in coculture spheroids

a.) Effect of SMC on endothelial CD34 expression upon VEGF stimulation.

We had shown in our previous work that VEGF selectively stimulates the surface endothelium in 3D solo spheroids to reexpress CD34 (Korff and Augustin, 1998). Based on this finding, we analyzed CD34 expression in EC and EC/SMC spheroids as a functional readout for VEGF dependent activation of EC. Untreated EC cultured in spheroids do not express CD34, whereas treatment with VEGF induces the surface EC to express CD34. This effect can be quantitated by counting the percentage of positive cells of the surface monolayer (Fig. 3). EC cocultured with SMC in spheroids do not express CD34. Surprisingly, EC in coculture spheroids can not be induced by stimulation with VEGF to express CD34 (Fig. 3) suggesting that the surface layer of EC has become refractory to the stimulation with VEGF upon contact with SMC.

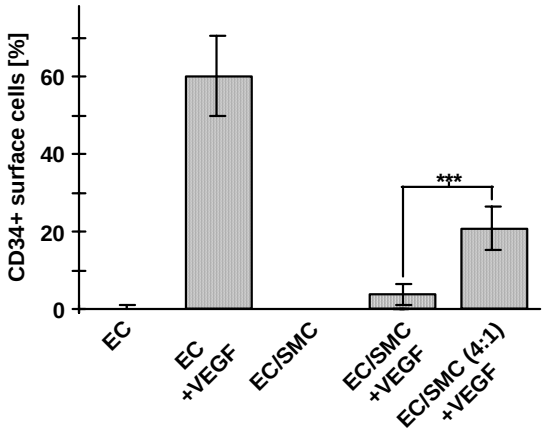


Fig. 3: Analysis of CD34 expression in surface spheroid EC as a readout of VEGF responsiveness. A quantitative analysis of CD34 expression was performed by counting the number of CD34 positive surface EC in relation to the total number of surface EC. EC in solo EC spheroids do not express CD34. Stimulation of EC spheroids induces strong CD34 expression of the surface EC. Likewise, EC in EC/SMC coculture spheroids do not express CD34. Stimulation of EC/SMC coculture spheroids with VEGF leads to CD34 expression in very few surface EC. Some VEGF responsiveness can be restored by shifting the quantitative ration of EC to SMC from 1:1 to 4:1 (***, $p<0.01$).

b.) Effect of SMC on endothelial sprouting angiogenesis upon VEGF stimulation

Spheroids were embedded in collagen gels and stimulated with VEGF in order to analyze the effects of SMC on VEGF mediated sprouting angiogenesis. The cumulative length of outgrowing capillary like sprouts (measured by automated image analysis) was calculated as a readout of *in vitro* angiogenesis (Fig. 4). VEGF acts as a potent inducer of sprouting angiogenesis originating from gel embedded EC spheroids (approximately 4-fold higher cumulative sprout length; $p<0.01$). In contrast, VEGF stimulation of SMC embedded as spheroids does not induce sprouting of SMC into the collagen gel within 24 hours. There was no sprouting of cells from EC/SMC coculture spheroids. Corresponding to the nonresponsiveness of EC in EC/SMC coculture spheroids in the CD34 induction experiments, VEGF had no effect on sprouting of EC into the collagen gel originating from EC/SMC coculture experiments. However, when changing the ratio of EC to SMC to 4:1, we observed a significant induction of EC sprouting angiogenesis by VEGF originating from EC/SMC coculture spheroids (compared to 1:1 EC/SMC spheroids; $p<0.01$).

Assessment of the role of agonists or antagonists of the angiogenic cascade towards VEGF in the EC/SMC coculture spheroid model

Another cytokine that may be able to mediate VEGF responses is Ang-2. Ang-2 has been identified as a functional antagonist of the Tie-2 ligand Ang-1 (Maisonpierre et al., 1997). Presumably, Ang-2

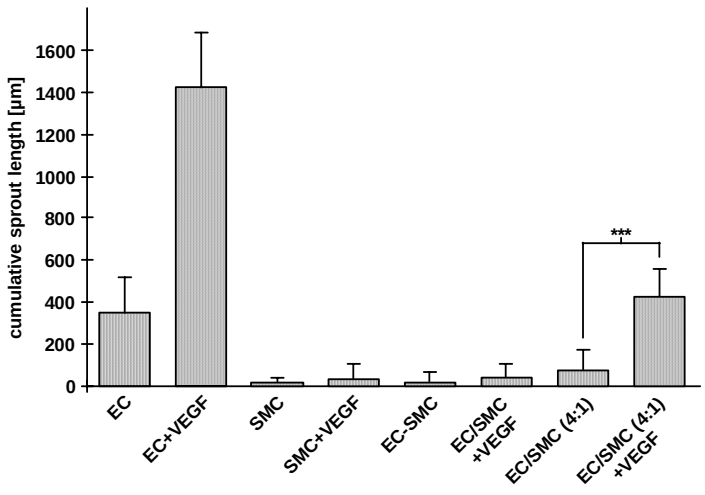


Fig. 4: Effect of VEGF in a 3D angiogenesis assay with collagen gel embedded spheroids of EC and SMC (mean±SD of two independent experiments measuring the average cumulative sprout length of 10 individual spheroids per experimental group). EC spheroids have a low baseline sprouting activity which can be strongly stimulated by exogenous VEGF. SMC originating from SMC spheroids have a very low sprouting activity and do not respond to VEGF. Likewise, there was hardly any sprouting originating from EC/SMC coculture spheroids in the absence as well as in the presence of VEGF. Shifting the ratio of EC to SMC from 1:1 to 4:1 restored some VEGF responsiveness (***, $p<0.01$).

destabilizes interactions of EC with mural cells by competitively binding Tie-2 without transducing an activating signal. Correspondingly, Ang-2 functions are considered context dependent, either facilitating angiogenesis (in the presence of angiogenic activity) or inducing vessel regression (in the absence of angiogenic activity) (Gale and Yancopoulos, 1999; Hanahan, 1997). Ang-2 is produced primarily by endothelial cells and thus seems to act as an autocrine regulator of vessel destabilization (Mandriota and Pepper, 1998; Stratmann et al., 1998). In line with the antagonistic model of Ang-2 function, costimulation of EC/SMC coculture spheroids with VEGF and Ang-2 led to the induction of sprouting angiogenesis in collagen gels, whereas neither VEGF nor Ang-2 was able to induce sprouting angiogenesis on its own. These findings demonstrate for the first time an *in vitro* function of Ang-2 on EC and can be interpreted as reflecting a facilitating role of Ang-2 for VEGF responsiveness in the presence of Ang-1-expressing SMC. When analyzing the effects of Ang-2 on solo EC populations, however, we made the puzzling observation that Ang-2 stimulates lateral sheet migration of EC as well as sprouting angiogenesis of gel-embedded EC spheroids. Collectively, these findings support a hypothetical model whereby Ang-2 function is context dependent in a way that it may act as an antagonistic molecule in the presence of Ang-1 and as an agonistic molecule in the absence of Ang-1.

Xenotransplantation of EC spheroids in SCID mice

In order to develop novel approaches to study EC functional properties during angiogenesis, we developed an *in vivo* angiogenesis assay by transferring our in collagen gel angiogenesis assay into SCID mice. Following injection, the gels are recovered after 14 days and processed for morphological analysis. Numerous crossections of small capillaries can be identified in the gel that form anastomoses with the invading mouse vasculature. This observation is remarkable for several reasons. First, it shows that EC spheroids can be used as a highly efficient system to transplant EC. Secondly, the experiment emphasizes that EC phenotypic properties are microenvironmentally controlled considering the remarkable phenomenon that human umbilical vein EC can be used to grow into perfect capillaries in the mouse. We are currently in the process to use this model for different applications to pursue manipulatory experiments in a humanized vascular network in mice.

2. Functional analysis of the propulsive vs. repulsive functions of EphB4 and EphrinB2 that influence EC differentiation and vascular morphogenesis.

The phenotype of EphB4 and ephrinB2 knockout mice has indicated that EphB/ephrinB interactions mediate distinct cellular guidance cues to EC. Yet, the cellular consequences of EphB forward and ephrinB reverse signaling are largely unknown. We employed a battery of functional assays to elucidate EphB4 and ephrinB2 mediated effector functions.

EphrinB2-Fc acts antiadhesive on endothelial cells

In order to define propulsive and repulsive effector functions of ephrinB2 ligands and EphB4 receptors on EC, HUVEC were seeded on ephrinB2-Fc or EphB4-Fc coated adhesive and non-adhesive tissue culture dishes. Coating with ephrinB2-Fc completely blocks adhesion of HUVE cells to adhesive culture dishes, whereas EphB4-Fc does not interfere with EC adhesion. In turn, when using EphB4-Fc coated non-adhesive tissue culture dishes, a subset of HUVE cells quantitatively corresponding to the ephrinB2-positive HUVEC subpopulation is able to adhere. Based on the observed proadhesive and antiadhesive effects of EphB4-Fc and ephrinB2-Fc, we next studied adhesion-modulating effects of EphB4 and ephrinB2 receptor bodies in our EC/SMC co-culture spheroid model as well as in explanted fragments of umbilical vein. Exposure of differentiated

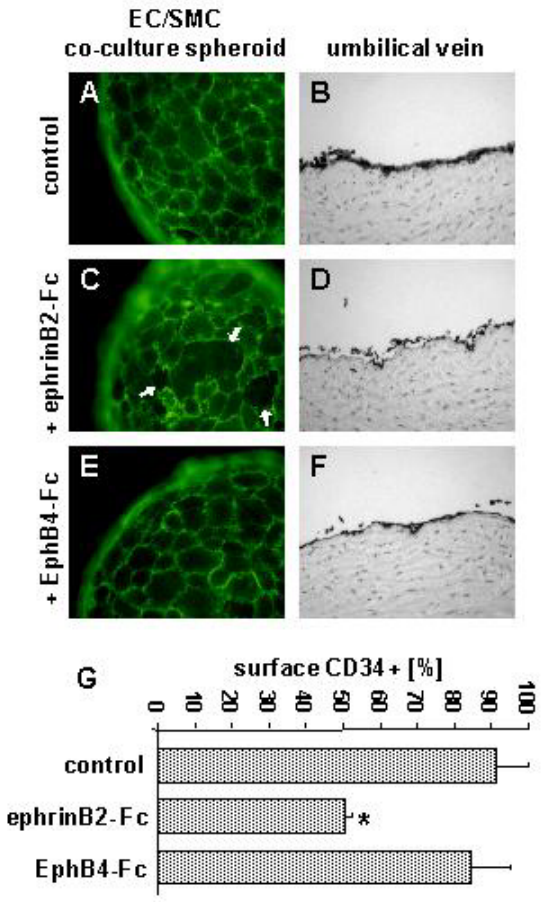


Fig. 5: EphrinB2-Fc induced detachment of endothelial cells from three dimensional co-culture spheroids of human umbilical vein endothelial cells (EC) and human umbilical artery smooth muscle cells (SMC) (left) and from explanted fragments of umbilical vein (right). (**A,C,E**) EC/SMC co-culture spheroids were treated for 24 hours with ephrinB2-Fc or EphB4-Fc. Control spheroids (**A**) and EphB4-Fc treated spheroids (**E**) have an intact surface monolayer of CD31 positive endothelial cells. EphrinB2-Fc (**C**) disintegrates the surface endothelial monolayer and induces EC detachment (arrowheads). (**B,D,F**) Explanted fragments of freshly isolated human umbilical cords were cultured for 24 hours in the presence of ephrinB2-Fc or EphB4-Fc. Control (**B**) and EphB4-Fc (**F**) treated umbilical veins have an intact monolayer of CD34 positive endothelial cells. In contrast, ephrinB2-Fc **(D**) induces detachment and denudation of umbilical veins. (**G**) Quantitation of umbilical vein denudation induced by ephrinB2-Fc. Umbilical vein integrity was assessed by automated image analysis quantitating the relative SMC surface area that is covered by CD34 positive endothelial cells. *, $p<0.05$ compared to control.

EC/SMC co-culture spheroids to ephrinB2-Fc disrupts the integrity of the continuous layer of surface EC and induces detachment of EC (Fig. 5). Likewise, treatment of fragments of explanted umbilical vein with ephrinB2-Fc led to a detachment of EC from their underlying extracellular matrix which could be visualized and quantitated after CD31 staining (Fig. 5D,G). EphB4-Fc did not affect EC in co-culture spheroids or in *in situ* explanted fragments of the umbilical cord (Fig. 5E,F). Together, the *in vitro* and the *in situ* experiments indicate that ephrinB2-Fc, binding to EphB2, EphB3, and/or EphB4 is capable to act antiadhesive on endothelial cells which are in their proper organotypic context with SMC.

EphrinB2-Fc inhibits EC migration and sprouting angiogenesis, whereas EphB4-Fc stimulates migration and sprouting angiogenesis

Based on the observed anti-adhesive capacity of ephrinB2-Fc, we set out experiments aimed at functionally manipulating Eph/ephrin interactions during specific steps of the angiogenic cascade. Two-dimensional lateral cell migration assays as well as the vertical gradient-driven Boyden chamber were used to study EC migration and chemotaxis. Baseline migration as well as VEGF-induced migration of EC was inhibited by ephrinB2-Fc. In contrast, EphB4-Fc stimulated lateral EC migration. Correspondingly, ephrinB2-Fc, but not EphB4-Fc inhibited VEGF mediated chemoattraction of HUVEC in a modified Boyden chamber assay. We next utilized our three dimensional collagen gel assay to quantitate the effect of soluble dimeric ephrin ligands and Eph receptors on sprouting angiogenesis. EphrinB2-Fc inhibited baseline as well as VEGF-induced sprouting angiogenesis which was prominently stimulated by EphB4-Fc. Alignment of EC cultured on top of Matrigel reflects some morphogenic properties of EC during angiogenesis. EphrinB2-Fc strongly inhibits alignment of EC grown on Matrigel which is not affected by EphB4-Fc.

Repulsive forward EphB4 signaling is sufficient to induce endothelial cell segregation

In order to study the functional consequences of cell-cell contact dependent Eph-ephrin signaling, we studied the properties of constitutively EphB4 and ephrinB2 overexpressing endothelial cell lines (PAEC). Transfected PAEC essentially show the same responses to receptor body activation as the endogenous EphB4 and ephrinB2 expressing HUVE cells. Yet, PAE cells have a high baseline sprouting activity. As a consequence, sprouting of collagen gel embedded spheroids of ephrinB2 transfected PAE cells cannot be further enhanced by EphB4-Fc. In turn, ephrinB2-Fc strongly and selectively inhibits sprouting of EphB4 overexpressing PAE cells (and not sprouting of mock transfected cells) (Fig. 6A). EphrinB2-Fc stimulation of EphB4 expressing PAE cells is associated with strong tyrosine phosphorylation of EphB4 (Fig. 6B).

We performed planar co-cultures of either ephrinB2, ΔephrinB2, or EphB4 with mock transfected PAE cells that led to uniform mixing of the cells as evidenced by the even distribution of fluorescent labeled cells upon adhesion in tissue culture dishes. In contrast, mixing of ephrinB2 and EphB4 expressing PAE cells led to the segregation of the cells upon adhesion, and to the formation of clusters of ephrinB2 and EphB4 positive cells. Unidirectional forward EphB4 signaling (intense tyrosine phosphorylation detected) was sufficient for the cellular segregation as evidenced by the segregation of EphB4 expressing cells from ΔephrinB2 PAE cells upon mixing.

Preferential adhesion of EphB4 tumor cells to ephrin B2 expressing endothelial cells

The EphB adhesive-repulsive phenotype to ephrinB2 expressing EC prompted us to hypothesize that ephrinB/EphB interactions may also play a role in mediating tumor cell (TC) adhesive interactions with EC as they occur during metastatic dissemination. We have consequently performed TC/EC adhesion experiments with different tumor and endothelial cell populations that were engineered to express EphB4, ephrinB2 and different truncation mutants of these molecules. These experiments have shown that EphB4 and cytoplasmic truncated EphB4 (ΔEphB4) expressing TC adhere

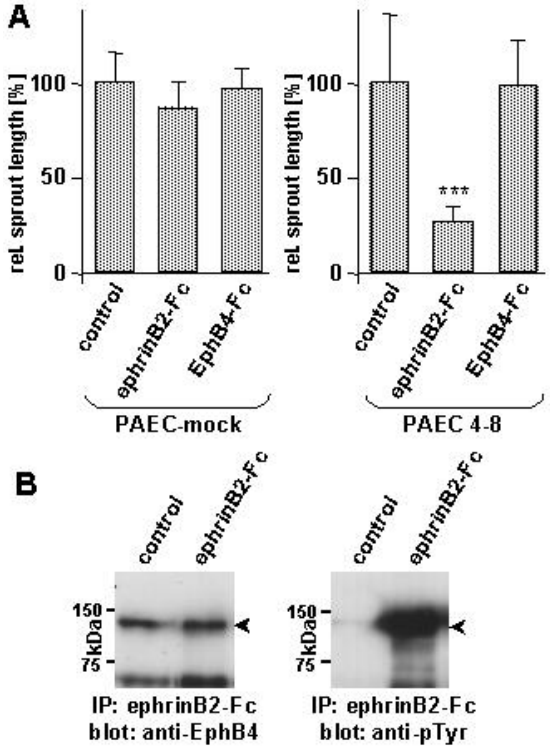


Fig. 6: Effect of EphB4-Fc and ephrinB2-Fc on sprouting angiogenesis of mock and EphB4 transfected PAEC. (**A**) PAEC spheroids were embedded in collagen gels and stimulated with ephrinB2-Fc or EphB4-Fc for 24 h. PAEC have a high baseline sprouting which was set to 100%. The sprouting activity of EphB4-transfected PAEC (4-8) was significantly inhibited upon treatment with ephrinB2-Fc ($p<0.001$) but not upon EphB4-Fc treatment. In contrast, mock-transfected PAEC do not respond to either ephrinB2-Fc or EphB4-Fc treatment. (**B**) EphB4 overexpressing PAEC were stimulated with ephrinB2-Fc for 30 min and analyzed for EphB4-phosphorylation by immunoprecipitating EphB4 using ephrinB2-Fc. Blots were probed with an anti-EphB4 antibody (left) and reprobed with an anti-pTyr antibody (right). EphrinB2-Fc stimulation leads to prominent phosphorylation of EphB4 in PAEC overexpressing EphB4.

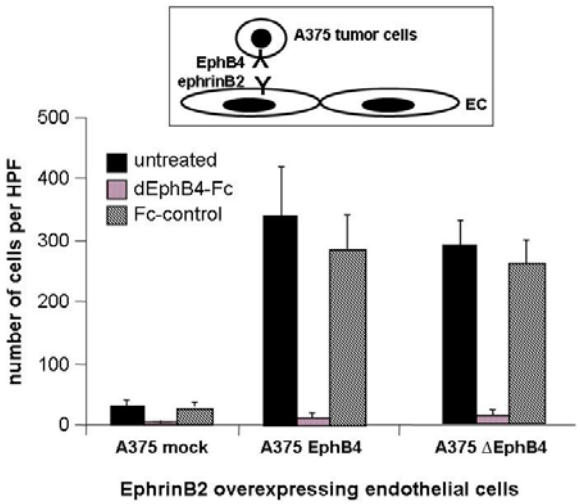


Fig. 7: EphB4 expressing A375 melanoma cells (full length as well as cytoplasmically truncated ΔEphB4]) adhere preferentially to ephrinB2 expressing HUVEC. EphB4-Fc blocks adhesion. Control Fc does not block adhesion.

preferentially to ephrinB2 expressing EC. The specificity of this interaction was demonstrated by a total blocking of the interaction by soluble EphB4-Fc (Fig. 7). We therefore concluded that adhesion of EphB4 overexpressing TC to ephrinB2 overexpressing EC is independent of forward signaling *in vitro*.

3. To assess endothelial ephrinB2 expression and microenvironmental cues that influence its expression and cellular localization.

EphB4 is preferentially expressed by venous endothelial cells and ephrinB2 is almost exclusively expressed by arterial endothelial cells and by angiogenically activated endothelial cells. This asymmetric arteriovenous expression pattern was deduced from the phenotype of the respective knockout mice and some lacZ expression experiments in the heterozygous mice. However, few other expression data are available which is largely due to the limited availability of suitable antibodies. Specifically, little information is available with respect to the following questions: 1.) Does the asymmetric arteriovenous EphB4/ephrinB2 expression pattern persist in the quiescent resting vasculature in the adult? 2.) Is the arteriovenous expression of EphB4/ephrinB2 stably fixed in the respective endothelial cells or is it under microenvironmental control? and 3.) What is the relationship between arterial and angiogenic expression of ephrinB2?

Microenvironmental control of ephrinB2 expression

We have RT-PCR screened a number of different arterial, venous, and microvascular endothelial cell populations and found that EphB4 and ephrinB2 are essentially expressed by all cultured EC. Cytokine activation experiments identified little regulation of EphB4, prompting us to focus on the regulation of ephrinB2 expression. A comparative *in situ* hybridization analysis of ephrinB2 *in vivo* and *in vitro* confirmed the arterial-selective expression of ephrinB2 (human aorta is ephrinB2-positive, whereas human saphenous vein is ephrinB2-negative [Fig 8]). Intriguingly, the embryo-derived EC of the umbilical artery and the umbilical vein are both uniformly ephrinB2-positive (Fig. 8). When these cell populations are cultured *in vitro*, they lose their distinct ephrinB2 expression status. Collectively, these data show that EC ephrinB2 expression is not an inherent property of arterial EC, but rather controlled by microenvironmental factors which are at least in part lost upon the cells transfer in culture.

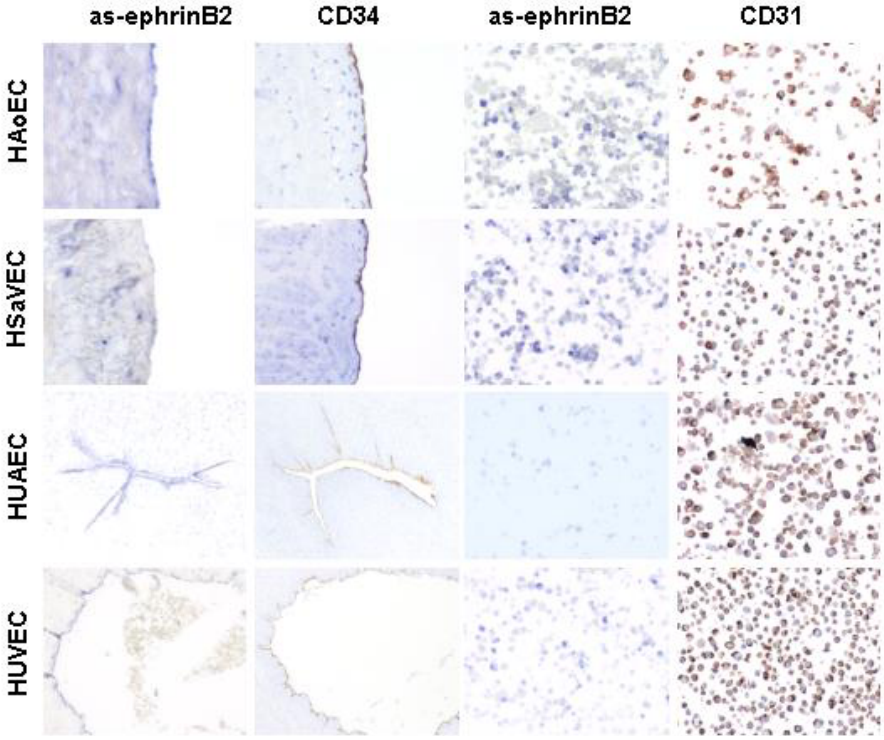


Fig. 8: Comparative *in situ* hybridization analysis *in vivo* and *in vitro* of ephrinB2 expression in human aortic EC (HAoEC), human saphenous vein EC (HSaVEC), human umbilical artery EC (HUAEC), and human umbilical vein EC (HUVEC). Section of human tissue (rows 1 and 2) or sections of embedded monolayer cultured cells (rows 3 and 4) were hybridized with antisense probes against ephrinB2 (rows 1 and 3) or control-stained with CD34 (row 2) or CD31 (row 4), respectively. The asymmetric AV expression pattern between HAoEC and HSaVEC is lost when the cells are transferred in tissue culture. See text for details.

VEGF and contact with SMC positively regulate EC ephrinB2 expression

Based on the above observations, we screened a number of cytokines and microenvironmental milieu factors for their ability to modulate ephrinB2 expression of cultured HUVEC. Stimulation of monolayer cultured HUVEC identified VEGF as an important positive regulator of endothelial ephrinB2 expression. This finding may correspond to the angiogenic expression of ephrinB2 and the arterializing function of VEGF. In order to identify other factors which may be able to regulate endothelial ephrinB2 expression, we employed our spheroidal EC/SMC co-culture system. Co-culture of HUVEC with HUASMC induced a prominent upregulation of EC ephrinB2 expression as evidenced by a uniformly ephrinB2 positive *in situ* hybridization signal of the surface monolayer EC.

Differential cellular localization of ephrinB2 in quiescent and activated EC

We next analyzed the subcellular localization of ephrinB2 on EC *in vitro* and *in vivo*. High-resolution double labeling immunofluorescence analysis of ephrinB2 expression with CD31 staining of umbilical vein EC *in vivo* revealed that quiescent EC express ephrinB2 uniformly in a polarized manner on their luminal aspect (Fig. 9).

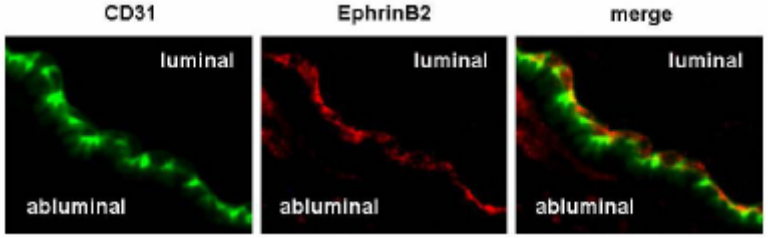


Fig. 9: Subcellular localization of endothelial ephrinB2 in the umbilical vein. EphrinB2 (red) is expressed on the luminal surface of HUVE cells *in vivo* (green: CD31 being localized abluminally and at intercellular contacts).

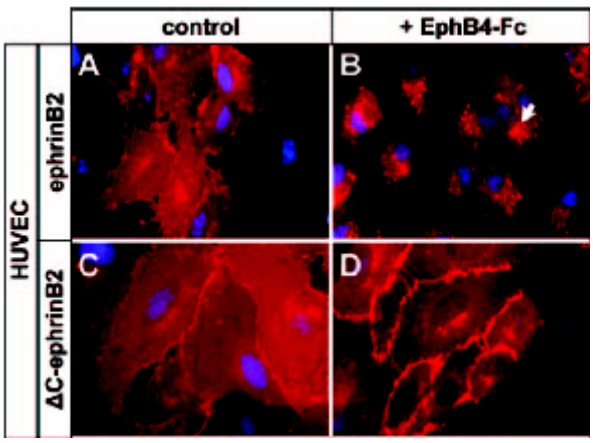


Fig. 10: Interendothelial junctional accumulation of ephrinB2 following EphB4-Fc–mediated receptor body stimulation. HUVEC expressing full-length ephrinB2 (ephrinB2) or cytoplasmically truncated ephrinB2 (ΔC-ephrinB2) were stimulated for 30 min with EphB4-Fc after which monolayers were fixed and the cells stained for ephrinB2. Unstimulated HUVEC express ephrinB2 on their cell surface and at cell–cell contacts (A, C). Stimulation of full-length ephrinB2-expressing HUVEC with EphB4-Fc leads to endocytosis of the receptor-ligand complex (B, arrow). In contrast, ΔC-ephrinB2–expressing cells are not capable of endocytosing the receptor-ligand complex, but translocate ephrinB2 to interendothelial junctions (D).

To probe the requirement of reverse ephrinB2 signaling for receptor body-mediated ligand endocytosis, we stimulated HUVEC expressing cytoplasmically truncated ephrinB2 with EphB4-Fc receptor bodies. Deletion of the cytoplasmic domain of ephrinB2 prevents endocytosis of the EphB4/ephrinB2 complex. Instead, the resulting EphB4/ΔC-ephrinB2 complex translocates to cellular junctions (Fig. 10D vs. 10B).

Based on the observed junctional translocation of ephrinB2 in confluent and particularly EphB4 receptor body-stimulated EC, we performed co-localization experiments of ephrinB2 with the junctional molecule CD31 which is expressed abluminally and at intercellular contacts. In contrast to the luminal expression of ephrinB2 in quiescent EC, subconfluent or angiogenically activated EC display a very different ephrinB2 expression pattern. Based on these findings, we performed co-immunoprecipitation experiments to examine if ephrinB2 and CD31 interact directly in endothelial cells. Immunoprecipitation of ephrinB2 from VEGF-stimulated HUVEC leads to prominent association of CD31 suggesting that both molecules could be part of a junctional complex.

Modulation of ephrinB2 expression upon inflammatory stimulus

A putative role of ephrinB2 in regulating trafficking of circulating cells upon inflammatory conditions is supported by the observation that junctional ephrinB2 is luminally expressed and associates with CD31. Preliminary experiments have allowed us to determine that TNFα leads to transient transcriptional upregulation of endothelial cell ephrinB2 expression (Fig. 11). This finding is actually under investigation in our laboratory in which we have created a set of ephrin transfectants TC in order to determine if endothelial cell ephrinB2 may be involved in recruiting metastasizing tumor cells to sites of inflammation.

4. Characterization of the involvement of the EphB/ephrinB interaction during tumor growth and angiogenesis

Inhibition of tumor growth and angiogenesis by soluble EphB4

Matched pair Northern-blot expression profiling experiments had identified prominently upregulated levels of EphB4 expression in human colon carcinomas compared to adjacent normal tissue. EphB4 expression of tumor cells has in the meantime been reported by a number of other groups. We consequently hypothesized that EphB/ephrinB interactions may regulate multiple tumor cell and endothelial cell interactions that may play a role during tumor progression (including, but not limited to tumor angiogenesis). We consequently studied the role of soluble EphB4 receptors to interfere with

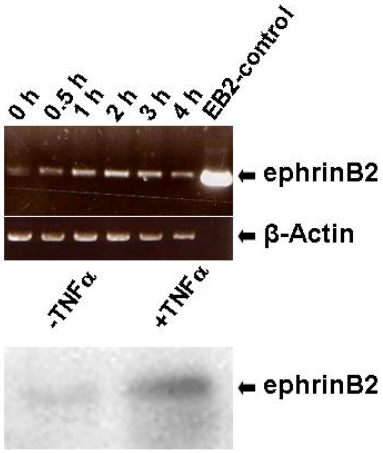


Fig. 11: Induction of EC ephrinB2 expression by inflammatory stimulation with TNFα. Top: TNFα stimulation leads to rapid transcriptional upregulation of ephrinB2 with maximal induction after 2 h (RT-PCR analysis). Bottom: EphrinB2 immunoprecipitation demonstrating upregulation of ephrinB2 protein after 4 h of TNFα stimulation.

tumor growth and angiogenesis. We opted to explore monomeric receptors for this purpose in consideration of the fact that dimeric soluble EphB4 receptors act as inhibitors of forward EphB4 signaling but may also act as stimulators of reverse ephrinB2 signaling, which may enhance rather than inhibit tumor angiogenesis.

EphB4 induced capillary sprout formation is inhibited by soluble monomeric EphB4

We employed our lab's spheroidal three dimensional *in vitro* angiogenesis assay in order to assess the effect of monomeric and dimeric soluble EphB4 receptors. Dimeric EphB4-Fc does not just stimulate capillary sprouting on its own, but is also capable of enhancing VEGF induced in gel sprouting angiogenesis (Fig. 12). In turn, monomeric soluble EphB4 does not affect basal or VEGF induced sprouting angiogenesis, but completely blocks EphB4-Fc induced enhancement of VEGF induced sprouting angiogenesis (Fig 12). These experiments demonstrate the ability of excess monomeric soluble EphB4 (sEphB4) to functionally neutralize ephrinB2 signaling mediated proangiogenic effects.

Soluble EphB4 overexpressing A375 melanoma cells can not form proper adhesive contacts

Based on the observed overexpression of EphB4 in human colon cancers and the *in vitro* angioinhibitory effects of sEphB4, we generated constitutively sEphB4 overexpressing A375 melanoma cells (EphB4 negative and ephrinB2 positive cells) in order to study the effect of sEphB4

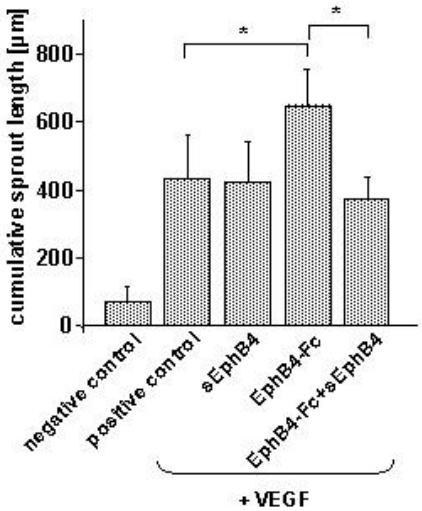


Fig. 12: Effect of monomeric and dimeric soluble EphB4 on in gel sprouting angiogenesis. Concentrations as low as 2 ng/ml VEGF induce a robust sprouting angiogenesis effect originating from collagen gel embedded HUVEC spheroids (negative control vs. positive control). Dimeric EphB4-Fc (5 µg/ml) significantly enhances VEGF induced sprouting angiogenesis (*p<0.05). In contrast, soluble monomeric EphB4 (5 µg/ml) does not affect VEGF induced sprouting angiogenesis. An excess of monomeric sEphB4 (25 µg/ml) however, is capable to completely abrogate EphB4-Fc induced sprouting angiogenesis (*p<0.05).

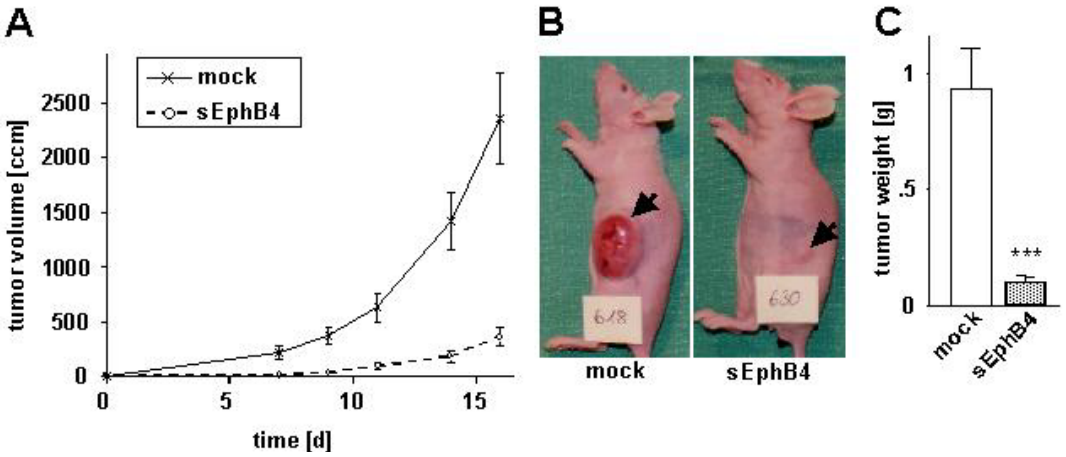


Fig. 13: Growth of sEphB4 overexpressing and mock transfected A375 melanomas. A375 cells (10^6 each) were injected s.c. into nude mice and grown for up to 3 weeks. Tumor growth was monitored by caliper measurement (**A**) and measuring the tumor weight at the end of the experiment (**C**). Mock transfected tumors grow rapidly and form reddish tumors indicative of intense vascularization (**B**). In contrast, sEphB4 overexpressing A375 melanomas form small subcutaneous nodules (**B**) with strongly reduced total tumor weight (**C**, ***p<0.001).

on tumor growth and angiogenesis. Transfected A375 cells express abundant amounts of sEphB4 as evidenced by an intense Northern blot signal. Tumorigenicity (soft agar assay) and tumor cell proliferation (BrdU labeling experiment) collectively are not affected by sEphB4. Yet, cell-cell interactions relating to tumor progression (adhesion, spheroidal organization and proliferation in spheroids) are affected by sEphB4.

Growth of sEphB4 overexpressing A375 tumors is dramatically impaired

sEphB4 overexpressing and mock transfected A375 melanoma cells were s.c. implanted into nude mice. Mock transfected A375 cells show an exponential growth curve (Fig. 13A) and grow to a final tumor weight of about 1 g (Fig. 13B, C). In contrast, sEphB4 overexpressing tumors grow significantly slower (Fig. 13A) which leads to the formation of small tumors with a final tumor weight below 0.2 g (Fig. 13B, C). Microscopic analysis of the tumors revealed no obvious difference in tumor and tumor stroma architecture (data not shown).

Microvessel density is moderately reduced in sEphB4 overexpressing tumors.

Vessel density of sEphB4 and mock transfected A375 tumors was assessed by counting CD34-positive microvessels. A375 melanomas have a prominent proangiogenic phenotype as evidenced by a dense network of microvessels. Total microvessel density in sEphB4 overexpressing tumors is moderately reduced by 19% ($p<0.05$).

5. Establishment of functional *in vivo* experiments in different models of tumor cells dissemination and metastasis in order to characterize the involvement of ephrin molecules.

Because of the increasing roles attributed to Eph receptors and ephrin ligands in tumor growth and progression, we set out experiments aimed at studying the role of EphB4 in its ability to control the metastatic distribution of A375 melanoma cells. Based on the observed preferential adhesion of EphB4 TC to ephrinB2 EC, we have generated luciferase-expressing A375 melanoma cells that

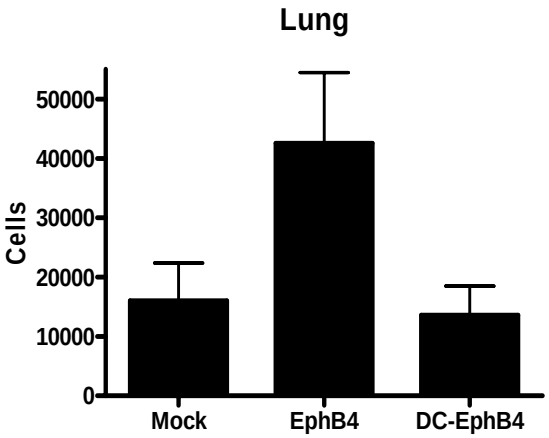


Fig. 14: Biodistribution experiments of mock-transfected, EphB4, and ΔEphB4 expressing A375 melanoma cells one hour following left ventricular injection into the heart. EphB4 expressing A375 cells "home" in higher frequency to the lungs than mock or ΔEphB4 A375 cells.

overexpress EphB4 or cytoplasmic truncated EphB4. To study the biodistribution of these cells in the peripheral circulation, we have established the left ventricular injection of TC into the heart. Direct injection into the heart should lead to biodistribution according to blood flow, if tumor cell dissemination was stochastic. Mice were sacrificed 1h after injection and biodistribution of injected cells was determined by quantitation of luciferase activity in different organs. Injection of 10^5 EphB4 A375 cells into the heart leads to an increased number of cells in the lung compared to mock and ΔEphB4 cells (Fig. 14). It is known that ephrinB2 is expressed by the lung vasculature and we have showed that ephrinB2 is luminally expressed, supporting the idea of a direct interaction between EphB4 and ephrinB2.

DISCUSSION

Three-dimensional tissue culture systems have been used widely in the context of tumor cell biology (Bates et al., 1994; Lincz et al., 1997; Mueller-Klieser, 1997; Sutherland, 1988). Thus, we adapted spheroid cultures to non-transformed EC that provide a suitable differentiation model to study differentiated endothelial cell functions. Bidirectional cross-talk between EC and SMC is a critical regulator of vascular homeostasis, contributing to the control of vascular functions including vasotonus, coagulation, and the trafficking of circulating blood cells. Numerous co-culture systems of EC and SMC have been developed to study paracrine interactions in the vessel wall. These include planar co-culture models of cells cultured together in the same dish, bilayer co-cultures, two-compartment filter systems, and agarose co-cultures (Delia et al., 1993; Fina et al., 1990; Ito et al., 1995; Jaffe et al., 1973). To study paracrine interactions between EC and SMC in a defined *in vitro* model, we developed a co-culture system of EC and SMC that mimics the physiological assembly of the normal vasculature so as to allow analysis of paracrine cellular interactions that regulate vessel assembly, maturation, maintenance, and vessel destabilization. These co-culture spheroids can be regarded as an inside-out assembly of a resting vessel wall in which SMC control the quiescent phenotype of the EC monolayer. Using this model, we have shown that co-stimulation of EC/SMC co-culture spheroids with VEGF and Ang-2 led to the induction of sprouting angiogenesis in collagen gels, whereas neither VEGF nor Ang-2 was able to induce sprouting angiogenesis on its own. These findings demonstrate an *in vitro* function of Ang-2 on EC for the first time and can be interpreted as reflecting a facilitating role of Ang-2 for VEGF responsiveness in the presence of Ang-1-expressing SMC. Taken together, the experiments in this study have established an EC/SMC co-culture system as an *in vitro* representation of the physiological assembly of a normal blood vessel that offers a unique experimental system for the analysis of paracrine interactions of EC and SMC.

Given the potential of our *in vitro* assays, we emphasized our work on the Eph/ephrin system which, through gene inactivation experiments, was identified as a novel regulatory system that is involved in vascular morphogenetic events – though not so much at early steps of the angiogenic cascade, but rather somewhat later during vascular maturation and remodeling.

The EphB/ephrinB system acts as an emerging versatile cell-cell adhesion and communication system. Eph receptors and their ephrin ligands comprise the largest family of receptor tyrosine kinases (Heroult et al., 2006; Kullander and Klein, 2002). In contrast to classical transmembrane receptors and their corresponding secreted ligand signaling systems, both Eph receptors and ephrin ligands are membrane molecules. EphB/ephrinB receptor/ligand interactions are, thus, capable to mediate bi-directional signaling events upon direct cell-cell contact (Holland et al., 1996; Kullander and Klein, 2002; Pasquale, 2005). These provide positive (attractive) and negative (repulsive) positional guidance cues to EphB/ephrinB expressing cells and regulate adhesive, migratory, and invasive cellular functions (Kullander and Klein, 2002; Pasquale, 2005). In order to shed further light on these complex mechanisms, we have utilized a panel of established EC culture assays including specialized 3D spheroidal differentiation and angiogenesis assays (Korff and Augustin, 1999). Collectively, these experiments revealed that EC EphB4 activation transduces anti-adhesive, anti-migratory and anti-angiogenic signals. The findings of our study support a model of endothelial propulsive and repulsive activities that mediate endothelial guidance signals during invasive angiogenesis as well as the positional control of EphB receptor- and ephrinB ligand expressing cells towards each other (Fig. 15).

This model is based on our functional data and takes into account published data on the repulsive guidance of EC and neural crest cells by surrounding cells (Helbling et al., 2000; Krull et al., 1997; Oike et al., 2002; Wang and Anderson, 1997).

Sustained arteriovenous asymmetrical expression of ephrinB2 in the adult vasculature could point to important homeostatic maintenance functions, for example by supporting the cross-talk between EC and SMC. Alternatively, endothelial ephrinB2 in adults could be involved in controlling interactions of circulating cells with the vessel wall. For example, several EphB receptors and ephrinB ligands have been shown to be involved in leukocyte and lymphocyte function (Freywald et al., 2003; Yu et al., 2003; Yu et al., 2004). Similarly, EphB/ephrinB interactions have been shown to regulate platelet aggregation (Prevost et al., 2002).

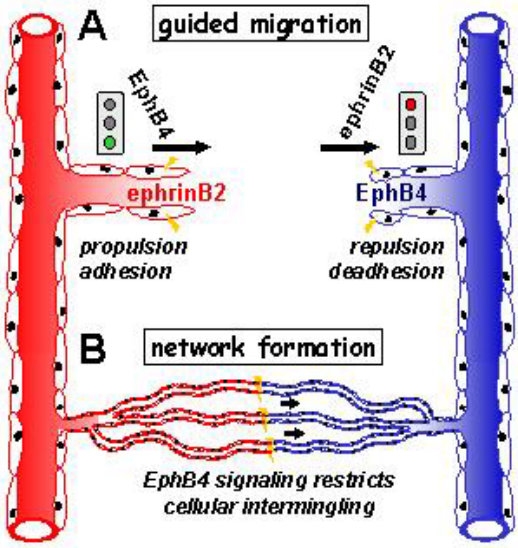


Fig. 15: Proposed model of the functional consequences of endothelial cell ephrinB2 and EphB4 signaling during guided migration (**A**) and capillary network formation (**B**). Similar to guided nerve cell outgrowth, forward EphB4 signals may direct endothelial cells in a repulsive manner upon activation by surrounding cells avoiding areas where ephrinB2 is expressed (guided migration, **A**, stop signal). The opposite, promotion of EC migration may occur if ephrinB2 expressing angiogenic EC are activated by EphB4 (**A**, go signal). Additionally, these effects may segregate endothelial cells from each other to limit cellular intermingling and control arterio-venous positioning of cells (network formation, **B**). Propulsive and repulsive endothelial cell forces during capillary morphogenesis indicate an arterio-venous push and pull situation that supports an artery to vein model of invasive angiogenesis.

The experiments of this study have shown that: (1) the asymmetrical arteriovenous expression of ephrinB2 *in vivo* is lost upon transfer into culture; (2) contact with SMCs (quiescent phenotype) and stimulation with VEGF (angiogenic activation) upregulate ephrinB2 expression in ECs; (3) quiescent resting ECs express ephrinB2 on their luminal cell surface; (4) ephrinB2 accumulates at interendothelial cell junctions on EphB4 receptor body activation; and (5) junctional ephrinB2 associates with CD31. These findings are likely to have important functional and conceptual implications. First, adding to the complexity of intrinsic versus microenvironmental endothelial cell phenotypic regulation, the present study has shown that ECs ephrinB2 expression is not an intrinsic determinant of the arterial EC phenotype, but rather needs to be maintained by local microenvironmental cues. The ephrinB2 luminal expression together with junctional ephrinB2 associates with CD31 are critical findings strongly implying EphB/ephrinB2 interactions in the control of circulating cell interactions with the vessel wall, as has recently been shown for the interaction of lymphocyte-expressed EphA receptors with high endothelial venule-expressed ephrinA1 (Aasheim et al., 2005). Correspondingly, we are currently investigating the regulatory trafficking aspect and were able to show that EphB-expressing monocytes adhere preferentially to ephrinB2-expressing EC (Dennis Pfaff, unpublished results).

Our Matched pair Northern-blot expression profiling experiments had identified prominently upregulated levels of EphB4 expression in human colon carcinomas compared to adjacent normal tissue. EphB4 expression of tumor cells has in the meantime been reported by a number of other groups. We consequently studied the role of soluble EphB4 receptors to interfere with tumor growth and angiogenesis. Collectively, we demonstrated that 1.) EphB4 is upregulated in human colon cancers, 2.) sEphB4 is capable to inhibit in gel sprouting angiogenesis induced by dimeric EphB4-Fc, 3.) sEphB4 dramatically inhibits growth of sEphB4 expressing A375 melanomas, and 4.) sEphB4 interferes negatively with tumor angiogenesis and vessel organization. The experiments suggest that the EphB/ephrinB system may offer an attractive molecular system as a therapeutic target. They also show that EphB/ephrinB interactions regulate multiple tumor progression related steps that are not just restricted to tumor angiogenesis.

This broader view of Eph/ephrin molecules as key regulators of cellular guidance and trafficking is also supported by recent experiments demonstrating that Eph/ephrin interactions transduce positional guidance cues in epithelial cells, most notably during colonic epithelial cell differentiation (Batlle et al., 2002). Intriguingly, EphB receptors have recently also been characterized as tumor suppressors in colorectal tumors which are downregulated during tumor progression (Batlle et al., 2005; Jubb et al., 2005). Yet, these puzzling and provocative findings are in conflict with an increasing list of publications suggesting that EphB receptors are upregulated during tumorigenesis in several types of human tumors (Dodelet and Pasquale, 2000) including among others colon tumors (Liu et al., 2002; Martiny-Baron et al., 2004; Stephenson et al., 2001), mammary tumors (Wu et al., 2004), lung tumors (Tang et al., 1999), endometrial carcinomas (Takai et al., 2001), prostate tumors (Xia et al., 2005b) and mesotheliomas (Xia et al., 2005a). Likewise, functional manipulation of EphB4 signaling through a soluble receptor approach (Martiny-Baron et al., 2004) or of EphB2 through an antibody approach (Mao et al., 2004) was shown to negatively interfere with tumor growth. The data also provide a strong rationale for an important role of the EphB/ephrinB system in the control of metastatic cell dissemination. Consequently, we are currently following experiments aiming at systematically studying the role of adhesive and angiogenic EphB/ephrinB crosstalk in controlling interactions between TC and EC that are involved in tumor progression and metastasis.

Our preliminary results have shown that EphB4 expressing A375 cells adhere to ephrinB2 expressing HUVEC and that intracardially injected EphB4 A375 cells preferentially colonize the lungs. We hypothesize that the EphB4/ephrinB2 system plays a critical role in the adhesion of

metastatic TC to the vasculature (blood vessels and lymphatics). We have developed sensitive luciferase-based TC tracing techniques, which enable us to invasively detect as few as 100 cells in an organ and to non-invasively detect micrometastases containing only a few thousand cells. Moreover, knowing the inflammatory conditions of certain tumors, our preliminary data have shown that TNFα induces the transient upregulation of EC ephrinB2 expression. We therefore hypothesize that inflammation may play a role in redirecting TC to sites of injury/inflammation.

REFERENCES

Aasheim HC, Delabie J, Finne EF: Ephrin-A1 binding to CD4+ T lymphocytes stimulates migration and induces tyrosine phosphorylation of PYK2. *Blood* 105: 2869-2876, 2005.

Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R: Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* 13: 295-306, 1999.

Bates RC, Buret A, van Helden DF, Horton MA, Burns GF: Apoptosis induced by inhibition of intercellular contact. *J Cell Biol* 125: 403-415, 1994.

Batlle E, Bacani J, Begthel H, Jonkheer S, Gregorieff A, van de Born M, Malats N, Sancho E, Boon E, Pawson T, et al.: EphB receptor activity suppresses colorectal cancer progression. *Nature* 435: 1126-1130, 2005.

Batlle E, Henderson JT, Beghtel H, van den Born MM, Sancho E, Huls G, Meeldijk J, Robertson J, van de Wetering M, Pawson T, Clevers H: Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 111: 251-263, 2002.

Delia D, Lampugnani MG, Resnati M, Dejana E, Aiello A, Fontanella E, Soligo D, Pierotti MA, Greaves MF: CD34 expression is regulated reciprocally with adhesion molecules in vascular endothelial cells in vitro. *Blood* 81: 1001-1008, 1993.

Dodelet VC, Pasquale, EB: Eph receptors and ephrin ligands: embryogenesis to tumorigenesis. *Oncogene* 19: 5614-5619, 2000.

Fina L, Molgaard HV, Robertson D, Bradley NJ, Monaghan P, Delia D, Sutherland DR, Baker MA, Greaves MF: Expression of the CD34 gene in vascular endothelial cells. *Blood* 75: 2417-2426, 1990.

Freywald A, Sharfe N, Rashotte C, Grunberger T, Roifman CM: The EphB6 receptor inhibits JNK activation in T lymphocytes and modulates T cell receptor-mediated responses. *J Biol Chem* 278: 10150-10156, 2003.

Füller T, Korff T, Kilian A, Dandekar G, Augustin HG: Forward EphB4 signaling in endothelial cells controls cellular repulsion and segregation from ephrinB2 positive cells. *J Cell Sci* 116: 2461-2470, 2003.

Gale NW, Yancopoulos GD: Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev* 13: 1055-1066, 1999.

Gerety SS, Wang HU, Chen ZF, Anderson DJ: Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol Cell* 4: 403-414, 1999.

Hafner C, Schmitz G, Meyer S, Bataille F, Hau P, Langmann T, Dietmaier W, Landthaler M, Vogt T: Differential gene expression of Eph receptors and ephrins in benign human tissues and cancers. *Clin Chem* 50: 490-499, 2004.

Hanahan D: Signaling vascular morphogenesis and maintenance. *Science* 277: 48-50, 1997.

Helbling PM, Saulnier DM, Brandli AW: The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in Xenopus laevis. *Development* 127: 269-278, 2000.

Heroult M, Schaffner F, Augustin HG: Eph receptor and ephrin ligand-mediated interactions during angiogenesis and tumor progressions. *Exp Cell Res*: in press, 2005.

Holland SJ, Gale NW, Mbamalu G, Yancopoulos GD, Henkemeyer M, Pawson, T: Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. *Nature* 383: 722-725, 1996.

Ito A, Nomura S, Hirota S, Suda J, Suda T, Kitamura Y: Enhanced expression of CD34 messenger RNA by developing endothelial cells of mice. *Lab Invest* 72: 532-538, 1995.

Jaffe EA, Nachman RL, Becker CG, Minick, CR: Culture of human endothelial cells derived from umbilical veinsIdentification by morphologic and immunologic criteria. *J Clin Invest* 52: 2745-2756, 1973.

Jubb AM, Zhong F, Bheddah S, Grabsch HI, Frantz GD, Mueller W, Kavi V, Quirke P, Polakis P, Koeppen H: EphB2 is a prognostic factor in colorectal cancer. *Clin Cancer Res* 11: 5181-5187, 2005.

Korff T, Augustin HG: Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J Cell Biol* 143: 1341-1352, 1998.

Korff T, Augustin HG: Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. *J Cell Sci* 112: 3249-3258, 1999.

Korff T, Dandekar G, Pfaff D, Füller T, Goettsch W, Morawietz H, Schaffner F, Augustin, HG: Endothelial ephrinB2 is controlled by microenvironmental determinants and associates context-dependently with CD31. *Arterioscler Thromb Vasc Biol* 26, 468-474, 2006.

Korff T, Kimmina S, Martiny-Baron G, Augustin, HG: Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J* 15: 447-457, 2001.

Krull CE, Lansford R, Gale NW, Collazo A, Marcelle C, Yancopoulos GD, Fraser SE, Bronner-Fraser M: Interactions of Eph-related receptors and ligands confer rostrocaudal pattern to trunk neural crest migration. *Curr Biol* 7: 571-580, 1997.

Kullander K, Klein R: Mechanisms and functions of Eph and ephrin signalling. *Nat Rev Mol Cell Biol* 3: 475-486, 2002.

Lincz LF, Buret A, Burns GF: Formation of spheroid structures in a human colon carcinoma cell line involves a complex series of intercellular rearrangements. *Differentiation* 61: 261-274, 1997.

Liu W, Ahmad SA, Jung YD, Reinmuth N, Fan F, Bucana CD, Ellis, LM: Coexpression of ephrin-Bs and their receptors in colon carcinoma. *Cancer* 94: 934-939, 2002.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, et al: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277: 55-60, 1997.

Mandriota SJ, Pepper MS: Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ Res* 83: 852-859, 1998.

Mao W, Luis E, Ross S, Silva J, Tan C, Crowley C, Chui C, Franz G, Senter P, Koeppen H, Polakis P: EphB2 as a therapeutic antibody drug target for the treatment of colorectal cancer. *Cancer Res* 64: 781-788, 2004.

Martiny-Baron G, Korff T, Schaffner F, Esser N, Eggstein S, Marmé D, Augustin HG: Inhibition of tumor growth and angiogenesis by soluble EphB4. *Neoplasia* 6: 248-257, 2004.

Mueller-Klieser W: Three-dimensional cell cultures: from molecular mechanisms to clinical applications. *Am J Physiol* 273: C1109-1123, 1997.

Oike Y, Ito Y, Hamada K, Zhang XQ, Miyata K, Arai F, Inada T, Araki K, Nakagata N, Takeya M, et al.: Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells. *Blood* 100, 1326-1333, 2002.

Pasquale EB: Eph-ephrin promiscuity is now crystal clear. *Nat Neurosci* 7: 417-418, 2004.

Pasquale EB: Eph receptor signalling casts a wide net on cell behaviour. *Nat Rev Mol Cell Biol* 6: 462-475, 2005.

Prevost N, Woulfe D, Tanaka T, Brass LF: Interactions between Eph kinases and ephrins provide a mechanism to support platelet aggregation once cell-to-cell contact has occurred. *Proc Natl Acad Sci USA* 99: 9219-9224, 2002.

Augustin HG, Reiss Y: EphB receptors and ephrinB ligands: regulators of vascular assembly and homeostasis. Cell Tissue Res 314: 25-31, 2003.

Stephenson SA, Slomka S, Douglas EL, Hewett PJ, Hardingham JE: Receptor protein tyrosine kinase EphB4 is up-regulated in colon cancer. *BMC Mol Biol* 2: 15, 2001.

Stratmann A, Risau W, Plate KH: Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am J Pathol* 153: 1459-1466, 1998.

Sutherland RM: Cell and environment interactions in tumor microregions: the multicell spheroid model. *Science* 240: 177-184, 1988.

Takai N, Miyazaki T, Fujisawa K, Nasu K, Miyakawa I: Expression of receptor tyrosine kinase EphB4 and its ligand ephrin-B2 is associated with malignant potential in endometrial cancer. *Oncol Rep* 8: 567-573, 2001.

Tang XX, Evans AE, Zhao H, Cnaan A, London W, Cohn SL, Brodeur GM, Ikegaki N: High-level expression of EPHB6, EFNB2, and EFNB3 is associated with low tumor stage and high TrkA expression in human neuroblastomas. *Clin Cancer Res* 5: 1491-1496, 1999.

Wang HU, Anderson DJ: Eph family transmembrane ligands can mediate repulsive guidance of trunk neural crest migration and motor axon outgrowth. *Neuron* 18: 383-396, 1997.

Wang HU, Chen ZF, Anderson DJ: Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* 93: 741-753, 1998.

Wu, Q, Suo, Z, Risberg, B, Karlsson, MG, Villman, K, Nesland, JM. Expression of EphB2 and EphB4 in breast carcinoma. *Pathol Oncol Res* 10: 26-33, 2004.

Xia, G, Kumar, SR, Masood, R, Koss, M, Templeman, C, Quinn, D, Zhu, S, Reddy, R, Krasnoperov, V, Gill, PS: Up-regulation of EphB4 in mesothelioma and its biological significance. *Clin Cancer Res* 11: 4305-4315, 2005a.

Xia, G, Kumar, SR, Masood, R, Zhu, S, Reddy, R, Krasnoperov, V, Quinn, DI, Henshall, SM, Sutherland, RL, Pinski, JK, et al.: EphB4 expression and biological significance in prostate cancer. *Cancer Res* 65, 4623-4632, 2005b.

Yu, G, Luo, H, Wu, Y, Wu, J: Ephrin B2 induces T cell costimulation. *J Immunol* 171: 106-114, 2003.

Yu, G, Luo, H, Wu, Y, Wu, J: EphrinB1 is essential in T-cell-T-cell co-operation during T-cell activation. *J Biol Chem* 279: 55531-55539, 2004.

Mechanism of ephrinB2 Reverse Signaling during Angiogenesis

Dr. Rüdiger Klein
Dr. Amparo Acker-Palmer

Dept. of Molecular Neurobiology
Max Planck Institute of Neurobiology
Am Klopferspitz 18, 82152 Martinsried, Germany

Member of the SPP 1069 from 1999 until 2005

SUMMARY

EphB receptor tyrosine kinases and one of their transmembrane ligands, ephrinB2, are expressed in and regulate cell-to-cell communication in the developing vascular network. We have shown that the cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis in vivo. This suggests that endothelial cell communication via the ephrin/Eph system is bi-directional, including forward signaling by the EphB tyrosine kinase and reverse signaling by ephrinB2. In our project over the last six years we have subdivided our studies in two major topics: the genetic and the biochemical analysis of ephrinB2 ligand signaling. We generated signaling mutants of ephrinB2 in vivo and studied the importance of ephrinB2 in lymphangiogenesis. On the other hand, at the molecular level we have investigated positive and negative intracellular regulators of ephrinB2 signaling to establish their roles in angiogenic remodeling. We demonstrated that ephrinB engagement with the corresponding EphB receptor induces the rapid co-clustering of ephrinB and Src family kinases (SFKs), causing SFK activation and ephrinB phosphorylation. Tyrosine phosphorylation of ephrinB is downregulated by the tyrosine phosphatase PTP-BL, which interacts with ephrinB via one of its PDZ-domains. We studied cell-to-cell communication and the process of cell repulsion mediated by Eph and ephrins and identified endocytosis of EphB/ephrinB complexes as a required step for termination of cell adhesion and promotion of cell repulsion. Finally, our work using modern proteomics approaches have identified novel interaction partners of ephrinB2 and we are investigating at the moment their relevance for reverse signaling.

INTRODUCTION

Blood vessels and nerves run in parallel in the body, they physically associate and use similar signals and mechanisms to differentiate, grow and navigate towards their targets. Recent studies have implicated an increasing number of molecular cues which excitingly have similar roles in the development of both nerves and blood vessels (reviewed in Carmeliet and Tessier-Lavigne, 2005). The same as a growth cone navigates and senses the surrounding tissue for specific cues which direct its movement towards its target, vessels show a specialized “tip” cell which has been suggested to guide the extension of vessel sprouts (Gerhardt et al., 2003). Four families of axon guidance molecules have been described: the netrins, semaphorins, ephrins and Slits. These molecules were extensively studied in the context of nervous system development and some years after all have turned out to be essential for the formation and maturation of the vasculature (reviewed in Carmeliet and Tessier-Lavigne, 2005

and Eichmann et al., 2005). The first example of a neural guidance cue involved in vessel navigation were the Eph receptors and their ligands, the ephrins.

EphrinB ligands are transmembrane proteins and not only induce signaling downstream of the Eph receptors (forward signaling), but also signal themselves into the cell that expresses them (reverse signaling) (reviewed in Palmer and Klein, 2003). The diverse biological roles of ephrins and Ephs in embryonic development include patterning and morphogenetic processes of the nervous and vascular systems, and in the adult, processes as synaptic plasticity, neurogenesis, nervous system regeneration and tumorgenesis. In vivo evidence that members of the Eph-ephrin family are involved in the development of the vasculature came from studies in mouse embryos bearing targeted null mutations in the genes for EphB4 (Gerety et al, 1999) and ephrinB2 (Wang et al. 1998; Adams et al. 1999). These mice showed defects in early angiogenic remodeling which led to embryonic lethality around E10.0. Moreover, these studies revealed an interesting reciprocal pattern of distribution within the developing vasculature in which ephrinB2 is expressed in the endothelium of primordial arterial vessels while EphB4 marks the endothelium of primordial venous vessels, suggesting the involvement of ephrinB2 and EphB4 in establishing arterial versus venous identity in the developing embryo. EphrinB2 is required specifically in the endothelial and endocardial cells for angiogenesis since endothelial specific knockout of ephrinB2 leads to angiogenic remodeling and cardiac defects that are indistinguishable from those of the conventional ephrinB2 knockout (Gerety and Anderson, 2002).

On the other hand, Eph/ephrin signaling was also shown to be important for the interactions that blood vessels establish with the surrounding mesenchyme. In addition to ephrinB2 and EphB4, other ephrins and Ephs are coexpressed on both arteries and veins and on the mesenchyme surrounding blood vessels suggesting a complicated scenario of Eph/ephrin interaction between arteries, veins and mesenchymal cells (reviewed in Adams 2002). Studies in mouse (Adams et al., 1999) and Xenopus (Helbling et al., 2000) attributed a role for mesenchyme-derived ephrinB2 signals in restricting blood vessel growth to the intersomitic space. Expression of ephrinB2 in the adult extended progressively to the smooth muscles cells surrounding the vessels suggesting a role for this ligand in smooth muscle layer assembly (Gale et al., 2001; Shin et al., 2001). The recruitment of supporting pericytes and vascular smooth muscle cells (mural cells) ensures the formation of a mature and stable vascular network. It has been recently shown that mural cells require ephrin-B2 for normal association with small-diameter blood vessels (microvessels). Selective deletion of ephrinB2 in pericytes and vascular smooth muscle cells leads to perinatal lethality, vascular defects in skin, lung, gastrointestinal tract, and kidney glomeruli and abnormal migration of smooth muscle cells to lymphatic capillaries (Foo et la., 2006).

As expected from their role during developmental angiogenesis, Eph receptors and ephrin ligands have turned out to be also important molecules at the sites of pathological angiogenesis in the adult like the new vessels induced by tumors. The process of neovascularization provides solid tumors with growth factors and oxygen is necessary to assure tumor survival, growth and malignancy. Moreover, vessels are an entry point for tumor cells to metastasise. Eph receptors and ephrins have emerged in the last years as a major class of RTKs regulating neovascularization. Brantley et al. (2002) provided the first functional evidence for EphA class receptor regulation of pathogenic angiogenesis in tumors. Treatment with soluble EphA2-Fc chimeric receptors in a vascular window assay resulted in decreased neovascularization in two different tumor models and in impaired tumor progression in vivo. With respect to EphB class receptors, several studies indicate that EphB receptors and ephrinB ligands are upregulated as blood vessels invade tumors (Gale et al., 2001; Shin et al., 2001). Moreover, EphB4 has

been shown to play a role in tumor progression by binding to ephrinB2 expressed in the endothelial cells and provide an attractive signal that promotes angiogenesis (Noren et al., 2004).

METHODS

The genetic analysis of ephrinB2 reverse signaling require the use of modern mouse molecular techniques including the knock-in of mutated cDNAs into a defined locus of the mouse genome and conditional gene targeting procedures involving the use of different recombinases. The laboratory of Rüdiger Klein has a solid background on mouse genetics and an excellent record of modern gene targeting studies over the last ten years which was crucial for the successful completion of the first part of this project.

To study the molecular mechanisms of Eph/ephrin signaling in the context of two important processes, axon guidance and angiogenesis, the laboratory of Signal Transduction headed by Amparo Acker-Palmer at the Max Planck Institute of Neurobiology employs different cellular models including primary mouse cortical neurons, endothelial and endothelial precursor cells as well as different transgenic mouse techniques. Studies include biochemical and cell biology methods, including in vitro kinase assays, immunoprecipitation, immunofluorescence, tissue culture with different migration, repulsion/adhesion and proliferation assays, generation of cell lines stably expressing different proteins of interest, etc. We also use innovative techniques like time lapse imaging for the study of cell adhesion/repulsion induced by Eph and ephrin signaling. In the last four years the laboratory of Amparo Acker-Palmer lab has focused in applying modern proteomic techniques to study ephrinB reverse signaling. The TAP tag technology was established by the laboratory of Bertrand Seraphin at EMBL, Heidelberg in 1999 being successfully used for mapping of protein-protein interactions in yeast. The TAP tag consists of two immunoglobulin-binding domains of proteinA from *Staphylococcus aureus*, a cleavage site for the tobacco etch virus (TEV) protease, and the calmodulin-binding peptide (CBP). Two different affinity steps allow the purification of complexes under native conditions. The purified complexes can be then used for protein identification by mass spectrometry. Cells stably expressing the TAP proteins or tissues from transgenic mice expressing the TAP-tagged proteins, have both served as starting sources of material to perform the complex purification in our laboratory. We have a scientific collaboration with the Biotech Company CellZome, GmbH, located on the campus of the EMBL (www.cellzome.de). CellZome provides the mass spectrometry know-how and service for efficient identification of complexes.

RESULTS AND DISCUSSION

1. Genetic analysis of ephrinB2 ligand signaling

1.1 The cytoplasmic domain of ephrinB2 is required for vascular morphogenesis

In order to identify functions of ephrinB2 that required “reverse” signaling, we first generated a gene targeted mouse expressing a mutated version of ephrinB2, which consisted of the extracellular and transmembrane domains of ephrinB2, lacking the cytoplasmic domain (ephrinB2ΔC). Therefore, homozyogous ephrinB2ΔC mutant mice express ephrinB2, which is functionally restricted to induce forward signaling by Eph receptors on adjacent cells, but is not longer able to exert reverse signaling via its cytoplasmic domain. In this study, which was the first publication from this project (Adams et al., 2001), we presented genetic evidence that ephrinB2 exerts a dual role in mouse development. Besides

being an important regulator of vascular development (Wang et al., 1998; Adams et al., 1999), ephrinB2 mediated branchial arch morphogenesis and aortic arch formation by guiding migrating neural crest cells. In this process, ephrinB2 served primarily as a ligand for Eph receptors expressed in neural crest cells, since carboxyterminal truncated ephrinB2 rescued the migration defect of branchial neural crest cells founded in the ephrinB2 null mouse. Expression of truncated ephrinB2ΔC protein did not restore the lethal angiogenic phenotype described for the ephrinB2 null mouse. Endothelial cells were arranged in a loose, irregular network and did not form a tubular structure, resembling the phenotype of the ephrinB2 null mutants. These findings provide genetic proof that ephrinB2 requires a cytoplasmic domain to mediate vascular morphogenesis during mouse embryogenesis. We also looked if the expression of different proteins involved in vascular development changed in the ephrinB2ΔC mouse, in order to look for putative downstream effects of Eph/ephrinB2 signaling. Expression of both Ang-1 and Tie-2 was specifically reduced in mesenchymal cells in ephrinB2 mutant animals, whereas other vascular markers were not significantly altered, implying that reverse ephrin or bidirectional ephrin/Eph signaling is required for proper communication between endothelial and mesenchymal cells.

1.2. Knock-in of mutant isoforms of ephrinB2

Using the knock-in strategy, the endogenous ephrinB2 allele was replaced by the ephrinB2 cDNA in which all five tyrosine residues in the cytoplasmic domain had been mutated into phenylalanine (ephrinB2-5F) or in which the carboxyterminal valine residue had been deleted (ephrinB2ΔV). Thus, ephrinB2 tyrosine phosphorylation (5F) and interaction with PDZ domain-containing proteins (ΔV) were abolished, respectively, allowing *in vivo* characterization of the mechanisms of ephrinB2 reverse signaling.

1.2.1. EphrinB2ΔV mutants show defects in lymphangiogenesis

Homozygous *ephrinB2*$^{\Delta V/\Delta V}$ and *ephrinB2*$^{5F/5F}$ mice undergo normal embryonic development, suggesting that the mice survive the requirement of ephrinB2 in embryonic vascular remodeling. While *ephrinB2*$^{5F/5F}$ mutant mice survived to adulthood, *ephrinB2*$^{\Delta V/\Delta V}$ mutants died during the first three weeks after birth. *ephrinB2*$^{\Delta V/\Delta V}$ mice developed chylothorax, a condition characterized by effusion of chylous lymphatic fluid from the thoracic duct into the pleural space, suggesting that they had defects in the lymphatic vascular system.

Lymphatic vessels are important for the maintenance of normal tissue fluid balance, for immune surveillance and for the adsorption of digested fats. Recent studies have also demonstrated the essential role of lymphatic vessels in certain pathological conditions, such as lymphedema and lymphatic metastasis (reviewed in Alitalo et al., 2005). Lymphatic vessels arise in the peripheral connective tissue as blind-ended capillaries which collect the excess of extravasated tissue fluid, originated as capillary infiltration from the blood serum, and drain into larger lymphatic vessels. These vessels converge and unite, pass to the lymph nodes, and return the fluid to the venous circulation via the final collecting trunk, the thoracic duct. Different types of lymphatic vessels can be distinguished morphologically; the lymph capillaries consist of valveless endothelial tubes which have discontinuous basement membrane, overlapping endothelial cell junctions and lack pericytes and smooth muscle cells, making them highly permeable to large macromolecules. In contrast, collecting lymphatic vessels have a sparse smooth muscle coverage, which helps in propelling lymph forward, and numerous, irregularly located valves, which prevent the backflow.

To begin addressing the role of ephrinB2 in lymphatic vessel development, we first investigated the expression of ephrinB2 and its cognate receptors on lymphatic endothelium, because no data was available from previous studies. Within the vasculature, EphrinB2 expression was reported to occur in arterial endothelium while its receptor, EphB4, is expressed in the venous endothelium. We found that ephrinB2 was also strongly expressed in the endothelium of collecting lymphatic vessels, and EphB4 was found to be expressed both in lymphatic capillaries and collecting lymphatic vessels. Analyses of the collecting lymphatic vessels in the *ephrinB2*$^{\Delta V/\Delta V}$ mutant mice revealed that they were hyperplastic and devoid of luminal valves. Consistent with the lack of lymphatic valves, we observed retrograde lymphatic flow, which presumably also contributed to the functional failure of the lymphatic system in these mice. More detailed analyses of the *ephrinB2*$^{\Delta V/\Delta V}$ mice demonstrated that the initial lymphatic development was normal. However, the primary plexus appears to be remodeled postnatally via generation of new vessel sprouts (Fig. 1), and as a result a hierarchically organized vessel network consisting of lymphatic capillaries and collecting lymphatic vessels is formed. This remodeling process was defective in the *ephrinB2*$^{\Delta V/\Delta V}$ mice (Fig. 1), and therefore the lymphatic vasculature remained morphologically and functionally immature. Finally, we found that a subset of known ephrinB2 interacting PDZ effector proteins, including PDZ-RGS3 and Dvl2, was mislocalized in the lymphatic endothelium in *ephrinB2*$^{\Delta V/\Delta V}$ mice. A proper subcellular localisation of RGS3 and Dvl2 therefore depends on the presence of an intact PDZ target site in ephrinB2, suggesting that these molecules are candidate effectors of ephrinB2 reverse signaling during lymphatic development. The function of RGS3 and Dvl2 in lymphangiogenesis has not been explored, however, since they are expressed and likely to play important roles in several tissues and cell types, further work would require generation of mice which would allow inactivation of these genes specifically in lymphatic endothelium. Further functional work would be also required to identify other ephrinB2 interacting PDZ domain proteins, which would have specific functions and perhaps specific expression in lymphatic endothelial cells. With more than 400 genes encoding PDZ domain proteins in the mouse genome, the identification of these molecules may require high-throughput approaches such as affinity purification or protein arrays.

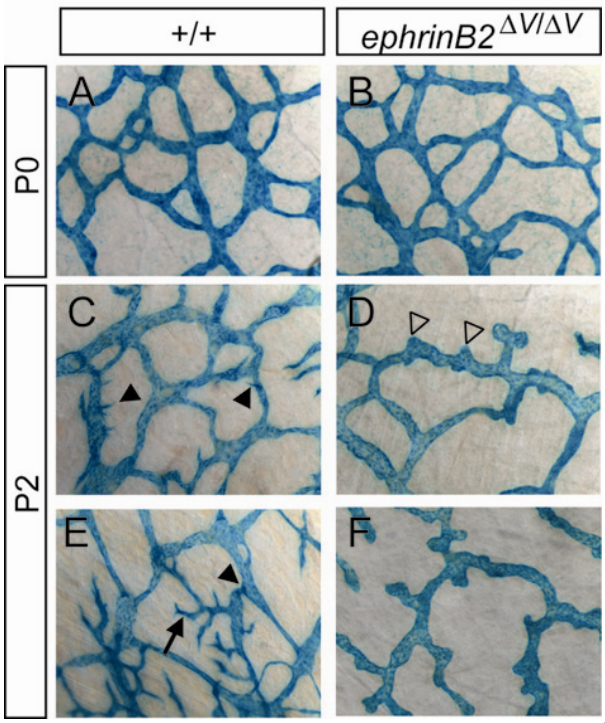


Fig. 1: Defective remodelling of the lymphatic capillary plexus in *ephrinB2*$^{\Delta V/\Delta V}$ mutant skin. Wholemount X-gal staining of lymphatic vessels in the *VEGFR3*$^{LZ/+}$ background of wild type (+/+) (A, C, E) and *ephrinB2*$^{\Delta V/\Delta V}$ (B, D, F) mutant skin. Ventral skin biopsies were taken from mice at the indicated ages (P0 and P2). The primary lymphatic capillary plexus is remodelled via formation of endothelial sprouts (filled arrowheads in panel C), which elongate to upper dermal layers (black arrow in panel E) and form a superficial lymphatic capillary plexus. In the mutants, the sprouting is disturbed (open arrowheads in D), which leads to failure in the formation of the superficial capillary plexus.

In contrast to the *ephrinB2*$^{\Delta V/\Delta V}$ mice, the morphology of the lymphatic capillaries and collecting lymphatic vessels of *ephrinB2*$^{5F/5F}$ mice were normal, and only a mild lymphatic phenotype, characterized by abnormal drainage in the skin, was detected. The lack of a marked phenotype in *ephrinB2*$^{5F/5F}$ mutants was surprising due to the fact that regulated tyrosine phosphorylation of the ephrinB1 cytoplasmic tail was viewed as the first strong evidence for ephrinB reverse signaling. It is possible that phosphotyrosine-dependent signaling is more important downstream of ephrinB1 than ephrinB2. On the other hand, there are additional functions of ephrinB2, which remain to be investigated and may depend on phosphotyrosine signaling, including pathological vascular remodeling in the adult, and axon guidance and neuronal plasticity in the nervous system.

In conclusion, our results demonstrate the requirement for ephrinB2 reverse signaling mediated via interaction with downstream PDZ effectors in lymphatic endothelium and establish ephrinB2 as an essential component in postnatal lymphatic development. In contrast, phosphotyrosine-mediated signaling was dispensable for ephrinB2 mediated functions in the development of lymphatic, as well as blood vasculature. The functional failure of the lymphatic system in ephrinB2 mutant mice further demonstrates that the previously uncharacterized postnatal remodeling is a critical process during the establishment of a normal lymphatic vascular network, and failure in this process may contribute to pathological conditions of the lymphatic vasculature, such as lymphedema (Mäkinen et al., 2005).

2. Biochemical analysis of ephrinB2 ligand signaling

2.1 Phosphotyrosine-dependent signaling

Although ephrinB ligand phosphorylation is thought to be an important event in reverse signaling, the identity of the kinase that phosphorylates the carboxyterminal domain of ephrinB ligands and its mechanism of activation remained to be elucidated at the early stages of this project. We identified Src family kinases as the kinases responsible for the phosphorylation of ephrinB ligands (Palmer et al., 2002). To identify these kinases we established an in vitro kinase assay using as the substrate the purified ephrinB1 cytoplasmic domain fused to GST. Both NIH3T3 cells overexpressing ephrinB1 and primary human arterial endothelial cells (HUAECs) endogenously expressing ephrinB2, upregulated a kinase activity upon stimulation with EphB2 and EphB4 receptors, respectively. The kinase was activated 10 min after stimulation with the Eph receptor and rapidly downregulated by 30 min, suggesting the existence of a downregulation mechanism for ephrinB ligand signaling (see below). In-gel kinase assays demonstrated that this kinase activity was around 60KDa in size, which corresponded to the molecular weight of Src family kinases (SFKs). Consistent with this observation, independent methods, i.e. use of a Src inhibitor and anti-Src immunodepletion, demonstrated a requirement for SFKs in ephrinB phosphorylation in vitro. Having established this strong causal link between SFKs and ephrinB phosphorylation, we asked if SFKs were also required for ephrinB reverse signaling. Adrenal-cortex derived microvascular endothelial (ACE) cells co-express ephrinB ligands and EphB receptors and can be induced *in vitro* to form small capillary sprouts (Koblizek et al., 1998; Adams et al., 1999). Sprout formation can by soluble, preclustered EphB4-Fc, suggesting that ephrinB ligands signaling can trigger this response (Fig. 2). The sprouting activity induced by EphB4-Fc was comparable to potent angiogenic factors such as vascular-endothelial growth factor (VEGF) (Koblizek et al., 1998). We examined whether in this assay SFKs were required for the angiogenic response mediated by ephrinB ligands. We showed that Src family kinases are required for sprouting angiogenesis induced by Eph receptors (Fig. 2). The response of endothelial cells to other potent angiogenic factors such as SDF-1 and Ang-1 was not

affected by inhibition of SFKs, suggesting that different signaling cascades are involved in the angiogenic process regulated by these different molecules. In summary, our findings identified SFKs as important mediators of ephrinB phosphorylation and implicated SFKs in ephrinB reverse signaling.

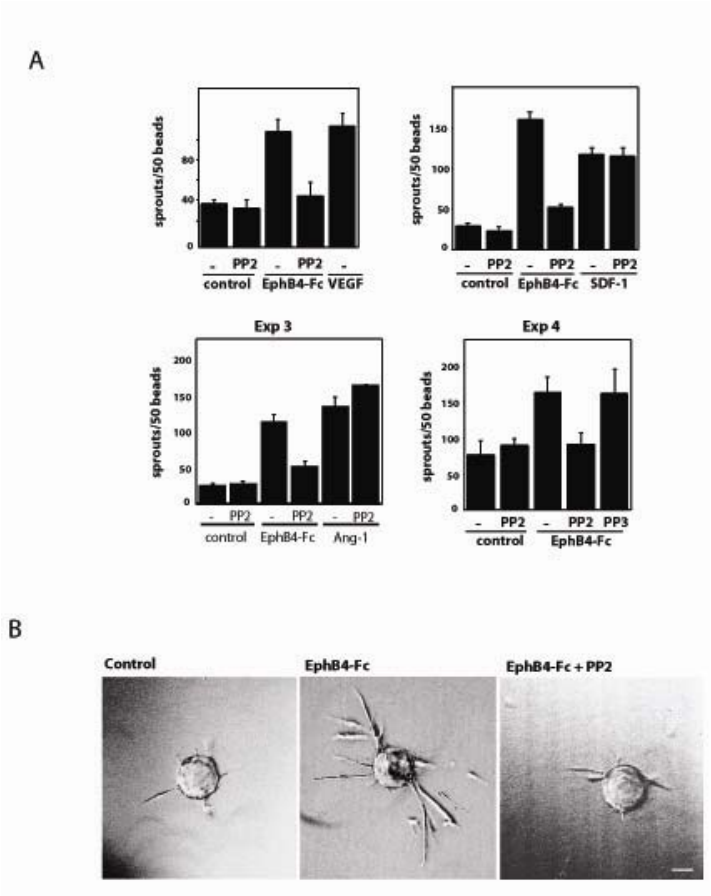


Fig. 2: Src family kinases are required for the angiogenic sprouting response mediated by ephrinB ligands. Adrenal-cortex derived microvascular endothelial (ACE) cells were seeded on MC beads and incubated in three-dimensional fibrin gels with different angiogenic factors in the presence or absence of the Src inhibitor PP2. (A) Quantitative analysis of sprout formation expressed as the number of capillary sprouts with lengths exceeding the diameter of the MC bead for every 50 MC beads counted. (B) Phase-contrast photomicrographs of angiogenic sprouts induced by EphB4-Fc in the presence and absence of PP2. Scale bars represent 25 µm.

We next asked, if SFKs were required for ephrinB phosphorylation in vivo and whether this pathway was active in other cell types as well. EphrinB reverse signaling is also thought to occur in forebrain cortical neurons, in particular during commissural axon tract formation during development (Henkemeyer et al., 1996; Kullander et al., 2001). Most of the evidence came from genetic studies, however, Eph receptor-induced ephrinB phosphorylation in vivo had never been demonstrated. Stimulation of primary mouse cortical neurons with preclustered EphB1-Fc led to a marked increase in phosphorylation of the endogenous ephrinB ligand, compared to the stimulation with preclustered, unfused Fc control protein. In the presence of the SFK inhibitor, PP2, this increase in phosphorylation was completely abolished. Therefore, in living neurons, EphB receptor-induced ephrinB tyrosine phosphorylation required the activity of SFKs. Moreover, short-term stimulation of neurons with EphB1-Fc activated both Src and Fyn kinases in cortical neurons. The activation was quick a transient and returned to baseline by 30 min.

In order to elucidate the mechanism by which SFKs are activated upon stimulation of cells with EphB receptors we looked at the subcellular localization of these proteins. Lipid microdomains, so-called rafts, are enriched in signaling molecules and are thought to act as platforms for signaling events. Membrane raft microdomains are characterized by detergent-insolubility at low temperatures and low buoyant

density. In order to investigate if ephrinB ligands and Src colocalize in membrane rafts, Triton X-100 flotation gradients were prepared from E12.5 mouse embryo heads. A large fraction of ephrinB was found in the top fractions of the flotation gradient, indicating raft localization. Probing the same fractions with the SFK antibody anti-cst1 revealed the colocalization of SFKs in the rafts fraction. We next attempted to visualize if ephrinB1 and Src colocalize in living cells. EphB2-Fc, but not unfused Fc, stimulation of NIH3T3-ephrinB1 cells induced the formation of receptor clusters in the membrane. Immunofluorescence using anti-ephrinB antibodies showed the redistribution in vivo of ephrinB1 into these receptor patches. Double immunostainings with anti-human Fc and anti-Src monoclonal antibodies revealed a redistribution of endogenous Src protein into patches that essentially overlapped with EphB-ephrinB complexes, indicating regulated colocalization of these proteins in the living cell. EphB-induced recruitment of Src into membrane patches was also confirmed in primary cultures of cortical neurons, expressing endogenous ephrinB ligands and Src kinases.

The fact that Src is only transiently activated downstream of ephrinB ligands made us think about the existence of a negative regulator that could silence the signaling downstream of ephrinB ligands. Our preliminary data suggested that PTP-BL, a cytoplasmic protein tyrosine phosphatase with 5 PDZ domains that binds in vitro to the PDZ binding domain of ephrinB ligands (Lin et al., 1999), was involved in the negative regulation of SFK activity. In our study published in 2002 (Palmer et al., 2002) we implicated PTP-BL as an important component of the signaling apparatus downstream of ephrinB ligands. Interestingly, not only SFK activity was transient and regulated by PTP-BL, but also ephrinB ligands were progressively dephosphorylated by the recruitment of PTP-BL to membrane patches containing the ligands and the receptors. In vitro phosphatase assays on ephrinB immunoprecipitated from mouse embryos showed that PTP-BL is able to dephosphorylate the native phosphorylation sites of ephrinB ligands. Co-expression studies in HeLa cells demonstrate that PTP-BL interacts with and dephosphorylates ephrinB in living cells. Based on these data we suggested the following model for

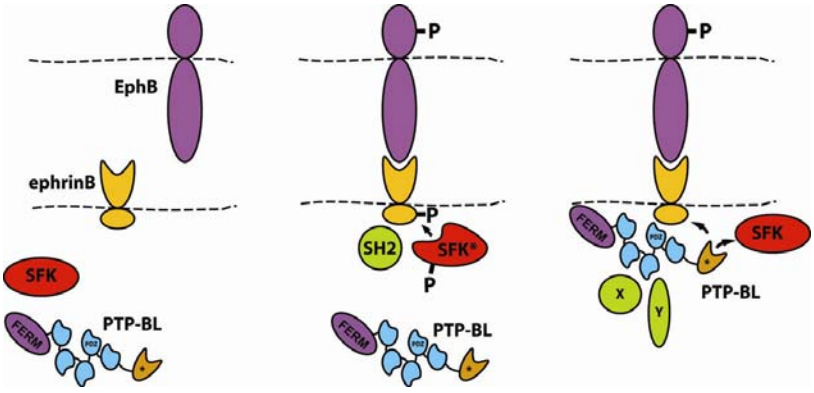


Fig. 3: Switch model for ephrinB-reverse signaling. (A) EphrinB ligand in the unbound state. EphrinB, SFKs and PTB-BL are localized to different compartments in the cell. The reverse signaling pathways are switched off. (B) Binding of EphB-receptors to ephrinB-ligands recruits SFKs and ephrinB to the same compartments in the membrane. SFKs are activated and subsequently phosphorylate ephrinB ligands on tyrosine. The cytoplasmic domain of ephrinB now serves as a docking site for adaptor-molecules like SH2-domain containing proteins, which transduce a “phospho-tyrosine dependent signal” to the interior of the cell. (C) PTP-BL is recruited with delayed kinetics to the signaling complexes containing ephrinB and SFKs. PTP-BL dephosphorylates ephrinB ligands and inactivates Src. Phosphotyrosine-dependent signaling is switched off and the multiple PDZ-domains allow the interaction with other cytoplasmic effectors (X,Y). “PDZ-dependent signaling” is prolonged and supersedes the “phospho-tyrosine dependent” signaling.

ephrinB reverse signaling: EphrinB engagement with its EphB receptors induces the rapid co-clustering of ephrinB and SFKs, causing SFK activation and ephrinB phosphorylation. Both active SFKs and phosphorylated ephrinB activate signaling pathways, either independently or in concert with each other, involving phosphotyrosine/SH2 interactions. With delayed kinetics, EphrinB clusters recruit PTP-BL, which dephosphorylates both Src and ephrinB, effectively turning off signaling by ephrinB and Src via phosphotyrosine. The recruitment of PTP-BL to ephrinB may not terminate ephrinB signaling completely, but rather shifts signaling from phosphotyrosine-dependent to PDZ-domain-dependent signaling (see below) (Fig. 3).

2.2 PDZ-dependent signaling

An important domain in the cytoplasmic tails of ephrinB ligands is a PDZ binding domain. Some multi-PDZ-domain-containing proteins have been shown to bind to the carboxy-terminal (YKV) domain of ephrinB ligands. Based on our studies we have proposed the presence of a switch mechanism that allows a shift from phosphotyrosine-dependent signaling to PDZ-domain-dependent signaling (Palmer et al., 2002). This part of the proposal was designed to dissect the nature of protein complexes that are involved in the PDZ-dependent signaling downstream of ephrinB ligands. We choose as a target the glutamate receptor interacting protein GRIP1, a 7 PDZ-domain containing protein that was shown to be specifically recruited into rafts through association with the cytoplasmic domain of ephrinB (Brückner et al., 1999).

We tagged the GRIP1 protein at the C-terminus with a double tag that allowed us to purify protein complexes using the tandem affinity purification method (TAP) (Rigaut et al., 1999). The tag consists of two protein A immunoglobulin (Ig)G-binding domains plus a calmodulin binding peptide tag, separated by a spacer containing the specific recognition site for the tobacco etch virus (TEV) protease. In

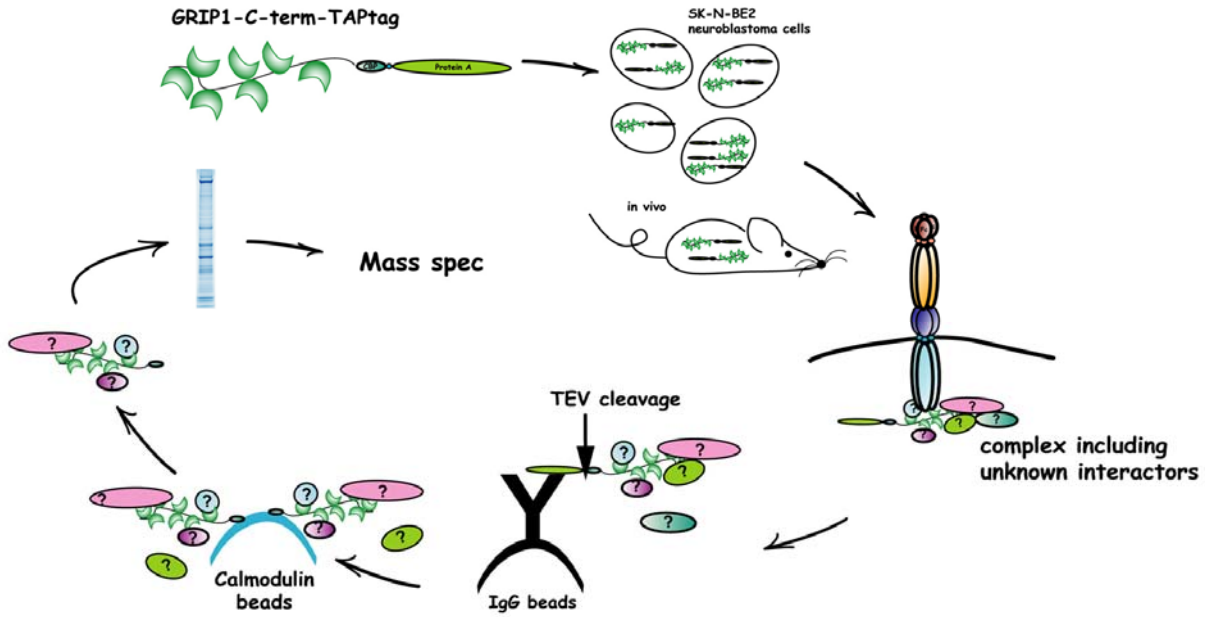


Fig. 4: Tandem affinity purification (TAP) method. This technique combines a first high-affinity purification, mild elution using a site-specific protease, and a second affinity purification to obtain protein complexes with high efficiency and specificity. The purified protein assemblies are separated by denaturing gel electrophoresis and bands are digested by trypsin. Analysis was done in collaboration with Cellzome employing matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF MS) and identification by database search algorithms.

collaboration with Cellzome GmbH, we generated a neuroblastoma cell line (SKN-BE2) stably expressing the GRIP1-CTAP protein by viral infection. After expansion of the cells, purifications of complexes were carried out. The purification consisted of four specific steps (Fig. 4): first, high affinity binding to IgG beads; second, elution using TEV protease; third, high-affinity binding to calmodulin beads in the presence of Ca^{2+}; and finally, elution using Ca^{2+}-chelating agents. GRIP1 interactors were identified by peptide sequencing using tandem mass spectrometry (LC-MS/MS). In total, the raw data set contained 134 non-redundant protein hits. Among those 132 interactors in total, 73 (54%) were considered to be sticky due to their frequent identification as interactors of various TAP-tagged molecules by Cellzome. The remaining 59 interactors were selected for further validation by literature screening on the basis of potential relevance in ephrinB reverse signaling. One specific binding partner has been selected for biochemical and functional validation. The function of such interactor in the context of ephrinB reverse signaling is being investigated at the moment.

We have also applied the TAP technology *in vivo*, by generating transgenic mice expressing GRIP1-CTAP under the control of the ubiquitin promoter. The mice did not show any overt phenotype and several founders have been tested for GRIP1-CTAP protein expression. The founders express different levels of GRIP1-TAP, but in general the expression was moderate, possibly not more than 2-3 fold above endogenous levels. The TAP approach in transgenic mice has several advantages over other methods for identification of protein-protein interactions: (a) the use of an *in vivo* model; (b) the purification of a protein complex that has been established in its natural environment, and (c) the possibility of comparison of protein complexes derived from different tissues and at different developmental stages.

2.3. Mechanisms of cell to cell adhesion/detachment: endocytosis of EphB/ephrinB

Repulsion of migrating cells and pathfinding axons represents an important mechanism for tissue patterning and morphogenesis mediated by ephrins. Interactions of ephrins with their receptors are of high affinity, contrasting with the highly dynamic and rapid process of contact-mediated repulsion. This apparently results in a paradox: Although complex formation between ligand and receptor per se is an adhesive event, it results in detachment and retraction of cells and their cellular processes. Therefore, mechanisms have to be in place that overcome adhesion immediately after cell-cell contact.

An important contribution of our studies has been the identification of endocytosis of EphB-ephrinB complexes as a novel mechanism for termination of adhesion and promotion of cell repulsion after intercellular (trans) interaction between two transmembrane proteins.

This work was published in September 2003 in *Nature Cell Biology* (Zimmer et al., 2003) and had a high novelty impact in the field (Wilkinson, 2003; Bussel, 2003; Halford et al., 2003). We have shown that both EphB2 and ephrinB1 are endocytosed upon binding their respective partners, when presented as soluble Fc fusion proteins. More importantly, bi-directional endocytosis of ephrinB-EphB complexes is observed when EphB2-expressing cells encounter ephrinB1-expressing cells in culture. Initially, clusters of EphB2 and ephrinB1 are present locally at sites of cell-cell contact and are subsequently found in internal vesicles in both cells. C-terminal truncation of EphB2 abolishes EphB2-mediated forward endocytosis, but not ephrinB1-mediated reverse endocytosis occurring in the ligand-expressing cell. Likewise, C-terminal truncation of ephrinB1 abolishes reverse, but not forward endocytosis. We further show that endocytosis is both sufficient and necessary for cell detachment, which we define as termination of adhesion between two cells (Fig. 5). Interestingly, intercellular (trans) interaction of truncated forms of

ephrinB and EphB promotes strong adhesion. This response is terminated, i.e. converted into detachment, when one of the interaction partners is full-length and competent for endocytosis. Growth cone collapse mediated by ephrinB reverse signaling in primary neurons still occurs when neurons encounter cells expressing an EphB2 mutant deficient in forward endocytosis. However, in this situation axon detachment is delayed, indicating the requirement of bidirectional endocytosis for efficient growth cone detachment. Our results suggest that bi-directional endocytosis of ephrinB-EphB complexes represents a novel mechanism for contact-mediated repulsion. A challenging question raised by these findings was to understand the unresolved issue of how the choice between Eph-ephrin-mediated contact repulsion and stable adhesion is made in vivo at the molecular level. We have now shown that the process of bidirectional endocytosis occurs also in endothelial cells. We have used endothelial cells and mouse embryonic endothelial precursor cells (eEPCs, obtained in collaboration with Antonis Hatzopolous from the SPP1069 Program, Hatzopoulos et al., 1998). Time lapse imaging of eEPCs showed active movement and repulsion accompanied by endocytosis of complexes in both Eph- as well as ephrinB-expressing endothelial cells. We are currently investigating endothelial cell communication through the Eph and ephrin system and how repulsion versus adhesion is modulated in the process of tubule formation.

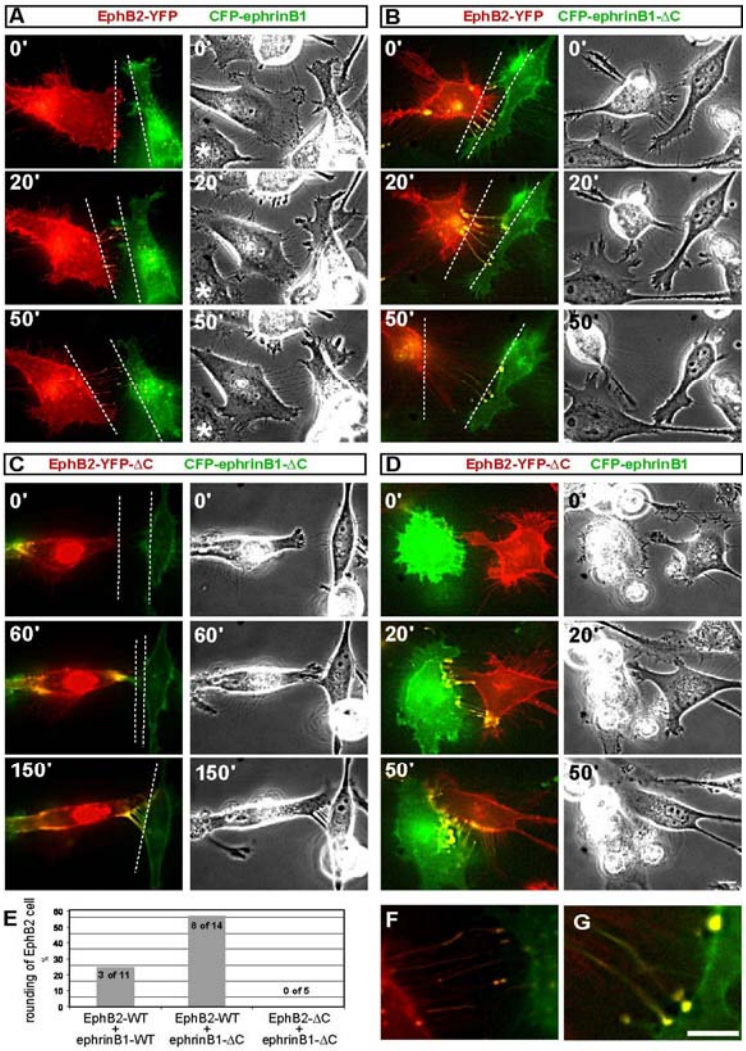


Fig. 5: Block of ephrinB1 endocytosis enhances cell repulsion of EphB2-expressing cells. (A) HeLa cells transfected with full-length EphB2-YFP (red) and full-length CFP-ephrinB1 (green), and co-cultured on laminin-coated glass cover slips. Time points of imaging are indicated in upper left corner. Left panels: Selected fluorescence images Right panels: phase contrast images. Intense clustering of EphB2 and ephrinB1 is seen at the contact site between the two cells at 20', the EphB2-YFP cell retracts a lammellipodium from the ephrinB1 cell (indicated by the distance between the two stippled lines). (B) Similar experiment as shown in panels A, except that C-terminally truncated CFP-ephrinB1ΔC was used. (C) C-terminally truncated CFP-ephrinB1ΔC and EphB2-YFP-ΔC were used. (D) Wild-type CFP-ephrinB1 and EphB2-YFP-ΔC were used. (E) Quantification of cell rounding. Numbers of time lapse recordings used for the analysis are indicated. (F,G) Higher power images of 50 min-panels of A and B are shown.

REFERENCES

Adams RH, Wilkinson GA, Weiss C, Diella F, Gale NW, Deutsch U, Risau W, Klein R: Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis and sprouting angiogenesis. *Genes Dev* 13: 295-306, 1999.

Adams RH, Diella F, Hennig S, Helmbacher F, Deutsch U, Klein R: The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* 104: 57-69, 2001.

Adams, RH: Vascular patterning by Eph receptor tyrosine kinases and ephrins. *Semin Cell Dev Biol* 13: 55-60, 2002

Alitalo K, Tammela T, Petrova TV: Lymphangiogenesis in development and human disease. *Nature* 438: 946-53, 2005.

Brantley DM, Cheng N, Thompson EJ, Lin Q, Brekken RA, Thorpe PE, Muraoka RS, Cerretti DP, Pozzi A, Jackson D, Lin C, Chen J: Soluble Eph A receptors inhibit tumor angiogenesis and progression in vivo. *Oncogene* 21: 7011-26, 2002.

Bussel K: Kiss and Break up. Highlights Signalling. *Nat Rev Mol Cell Biol* 4: 753, 2003.

Brückner K, Labrador JP, Scheiffele P, Herb A, Bradke F, Seeburg PH, Klein R: EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains*. Neuron* 22: 511-524, 1999.

Carmeliet P, Tessier-Lavigne M: Common mechanisms of nerve and blood vessel wiring. *Nature* 436: 193-200, 2005.

Eichmann A, Mäkinen T, Alitalo K: Neural guidance molecules regulate vascular remodeling and vessel navigation. *Genes Dev*. 19: 1013-21, 2005.

Foo SS, Turner CJ, Adams S, Compagni A, Aubyn D, Kogata N, Lindblom P, Shani M, Zicha D, Adams RH: Ephrin-B2 controls cell motility and adhesion during blood-vessel-wall assembly. *Cell* 124: 161-73, 2006.

Gale NW, Baluk P, Pan L, Kwan M, Holash J, DeChiara T, McDonald DM, Yancopoulos GD: Ephrin-B2 selectively marks arterial vessels and neovascularization sites in the adult, with expression in both endothelial and smooth-muscle cells. *Dev Biol* 230, 151-160, 2001.

Gerety SS, Wang HU, Chen ZF, Anderson DJ: Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol Cell* 4: 403-14, 1999.

Gerety SS, Anderson DJ: Cardiovascular ephrinB2 function is essential for embryonic angiogenesis. *Development* 129: 1397-410, 2002.

Gerhardt, H, Golding, M, Fruttiger, M, Ruhrberg, C, Lundkvist, A, Abramsson, A, Jeltsch, M, Mitchell, C, Alitalo, K, Shima, D, Betsholtz, C: VEGF guides angiogenic sprouting utilizing endothelial tip cell filopodia. *J Cell Biol* 16:1163-77, 2003.

Halford MM, Chumley MJ, Henkemeyer M: Ephective endocytosis. Previews. *Dev Cell* 4: 536-7, 2003.

Hatzopoulos AK, Folkman J, Vasile E, Eiselen GK, Rosenberg RD: Isolation and characterization of endothelial progenitor cells from mouse embryos. *Development* 125: 1457–1468, 1998.

Helbling PM, Saulnier DM, Brandli AW: The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in Xenopus laevis. *Development* 127: 269-78, 2000.

Henkemeyer M, Orioli D, Henderson JT, Saxton TM, Roder J, Pawson T, Klein R: Nuk controls pathfinding of commissural axons in the mammalian central nervous system. *Cell* 86: 35-46, 1996.

Koblizek TI, Weiss C, Yancopoulos GD, Deutsch U, Risau W: Angiopoietin-1 induces sprouting angiogenesis in vitro. *Curr Biol*, 8: 529-532, 1998.

Kullander K, Mather NK, Diella F, Dottori M, Boyd AW, Klein R: Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. *Neuron* 29: 73-84, 2001.

Lin D, Gish GD, Songyang Z, Pawson T: The carboxyl terminus of B class ephrins constitutes a PDZ domain binding motif. *J Biol Chem* 274: 3726-3733, 1999.

Mäkinen T, Adams RH, Bailey J, Lu Q, Ziemiecki A, Alitalo K, Klein R, Wilkinson GA: PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature *Genes Dev* 19: 397-410, 2005.

Noren NK, Lu M, Freeman AL, Koolpe M, Pasquale EB: Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth. *Proc Natl Acad Sci USA* 101: 5583-8, 2004

Palmer A, Zimmer M, Erdmann KS, Eulenburg V, Porthin A, Heumann R, Deutsch U, Klein R: EphrinB phosphorylation and reverse signaling: regulation by Src kinases and PTP-BL phosphatase. *Mol Cell* 9: 725-37, 2002.

Palmer A, Klein R: Multiple roles of ephrins in morphogenesis, neuronal networking, and brain function. *Genes Dev* 17: 1429-1450, 2003

Rigaut G, Shevchenko A, Rutz B, Wilm M, Mann M, Seraphin BA: generic protein purification method for protein complex characterization and proteome exploration. *Nat Biotechnol* 17:1030-2, 1999.

Shin D, Garcia-Cardena G, Hayashi SI, Gerety S, Ashara T, Stavrakis G, Isner J, Folkman J, Gimbrone MA Jr, Anderson DJ: Expression of EphrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. *Dev Biol,* 230: 139-150, 2001.

Wang HU, Chen ZF, Anderson DJ: Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* 93: 741-753, 1998.

Wilkinson DG: How attraction turns to repulsion. News and Views. *Nat Cell Biol* 5: 851-53, 2003.

Zimmer M, Palmer A, Köhler J, Klein R: Contact-mediated repulsion by ephrinB ligands and EphB receptors requires bi-directional endocytosis. *Nature Cell Biol* 5: 869-878, 2003.

Pathophysiologic and Therapeutic Significance of Protein Tyrosine Kinases and Eph Family Receptors for Angiogenesis, Microcirculation, and Growth of Malignant Brain Tumors

PD Dr. Peter, Vajkoczy
Neurosurgical Clinic
University Clinic Mannheim
University of Heidelberg-Mannheim
Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

Professor Dr. Axel, Ullrich
Dept. of Molecular Biology
Max-Planck-Institute for Biochemistry
Am Klopferspitz 18A, 82152 München, Germany

Members of the SPP 1069 from 1999 until 2005

SUMMARY

Part I: "Angiogenesis, microcirculation, and growth of high-grade gliomas: In vivo analysis of the pathophysiological and therapeutic significance of protein tyrosine kinases"

The concept of treating tumors by interfering with their vascularization is based on the premise that tumor growth is dependent on angiogenesis and that tumor progression is suppressed if neovascularization is prevented. Vascular endothelial growth factor (VEGF) has been shown to be the central positive regulator of tumor angiogenesis and, therefore, has become the primary target when exploiting anti-angiogenic strategies. As a result, both antibodies and inhibitors specifically directed against VEGF or its receptor VEGFR-2 have been raised and have been demonstrated to potently prevent vascularization and growth of a large number of experimental tumor types. Successful treatment of established human tumors, however, might require not only prevention of further angiogenesis but also destruction of already existing tumor blood vessels in order to reduce the already existing tumor mass.

Recently, however, it has been realized that the susceptibility of established tumor blood vessels to an interference with VEGF/VEGFR-2 signalling may be restricted to a fraction of immature vessels which lack co-localization with pericytes. Contact of endothelial cells with pericytes or smooth muscle cells, stabilizes new blood vessels, promotes endothelial survival and inhibits endothelial cell proliferation. Although the detailed molecular basis of how pericytes confer vessel stabilization remains unknown, studies of embryogenesis have highlighted the involvement of the angiopoetin (Ang)-1/Tie2 system and the platelet-derived growth factor (PDGF)-B/PDGF receptor (PDGFR)-β system. To test our hypothesis that simultaneous targeting of endothelial cells and pericytes in tumors may enhance enforced tumor vessel regression, we compared the effects of selective VEGFR-2 inhibition versus VEGFR-2 plus PDGFR-β inhibition on the established microvasculature of C6 tumor xenografts. We show that VEGFR-2 targeting results in activation of endothelial cell survival mechanisms by pericytes, thereby, providing escape strategies from enforced tumor vessel regression. However, this resistance to tumor vessel regression can be overcome when both VEGFR-2 plus PDGFR-β are simultaneously inhibited, resulting in tumor specific endothelial cell apoptosis, blood vessel destabilization and regression. These results suggest that a successful intervention in advanced tumors and long-term disease control with anti-

angiogenic compounds can be best achieved with a combination therapy, targeting not only endothelial cells but also pericytes. Therefore, besides selectively targeting VEGF/VEGFR-2 as the pivotal pathway for angiogenesis, additional pathways involved in vascular stabilization and maturation should be included into the target profile, and, finally, tissue hypoxia.

Part II: "Pathophysiologic and therapeutic significance of EphrinB2/EphB4 for angiogenesis, microcirculation, and growth of malignant brain tumors

Only little is known about the role of Ephrin ligands and Eph receptors in tumor angiogenesis. We could show that Ephrin ligands and Eph receptors are differentially expressed by both tumor cells and blood vessels during brain tumor progression. EphrinB2/EphB4 represent the sole molecules of this family whose vascular expression positively correlates with the activation of brain tumor angiogenesis. In line with this, we have demonstrated that ephrinB2 and EphB4 are overexpressed in human and experimental malignant brain tumors. As a first approach to investigate ephrinB2 and EphB4 function in malignant brain tumors, we set out to study the function of endothelial EphB4 signaling in tumor angiogenesis. The fact that EphB4 was expressed by both tumor cells and endothelial cells necessitated a vessel specific transgenic approach. Endothelial overexpression of EphB4wt did not affect initial angiogenesis, as indicated by a regular vascular initiation of the tumors, but markedly affected vascular morphogenesis, as indicated by a switch from angiogenic sprouting to circumferential vessel growth. Furthermore, overexpression of EphB4wt reduced the leakiness of tumor blood vessels. The fact that overexpression of EphB4dn phenocopied the EphB4wt induced vascular changes demonstrated that these effects were independent of the EphB4 tyrosine kinase activity. Consequently, EphB4 reverse signaling through ephrinB2 represents the predominant signaling pathway in this context.

Moreover, the results of our study not only reveal a novel function for EphB4 during tumor vascularization, but also establish a novel concept in tumor biology by demonstrating that morphogenesis and organization of the postnatal vascular system are regulated by guidance molecules. If this holds true, vascularization of a tumor would be driven by two distinct molecular vascularization programs acting hand-in-hand, the vascular initiation program (driven by VEGF and other endothelial cell mitogens) and the vascular patterning program (driven by EphB4 and putatively other vascular guidance molecules). Although interference with the latter program may not directly translate into a successful anti-tumor strategy, a better understanding of this program and the underlying mechanisms may provide novel opportunities for the development of therapeutic strategies aimed at a ´vascular reprogramming` of tumors.

INTRODUCTION

Pathology of malignant brain tumors

Supratentorial glial neoplasms represent the majority of primary tumors of the central nervous system in adults. Based on histopathological criteria they are graded into low-grade (astrocytoma WHO grade I and II) and malignant glioma (anaplastic astrocytoma, WHO grade III), and glioblastoma multiforme (WHO grade IV)). Despite various therapeutic and diagnostic efforts, malignant glioma still represent an unsolved problem. 5-year survival rates are currently 10% to 35% and near 0% for anaplastic astrocytomas and glioblastoma multiforme, respectively (Nelson 1999); (Stupp et al., 2005). Among the most prominent pathological hallmarks of malignant glioma are a high activity of tumor angiogenesis as well as a high degree of local invasiveness into the adjacent brain tissue.

Brem and coworkers (Brem et al. 1992) have shown that malignant gliomas are among the best vascularized tumors in humans. A strong line of experimental evidence supports the concept that solid

glioma growth is critically dependent on angiogenesis (Kim et al. 1993); (Millauer et al. 1994); (Stan et al. 1995). The infiltrative tumor component, in contrast, is characterized by an aggressive infiltration of single tumor cells into the surrounding, yet morphologically and functionally intact brain tissue. To date, it remains unknown to what extent the diffuse, infiltrative growth of gliomas is, similar to solid tumor growth, angiogenesis-dependent as well.

Brain tumor angiogenesis

A large number of angiogenic growth factors has been implicated in glioma-associated angiogenesis. Among those, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) are ascribed a central role in directly (VEGF, bFGF, PDGF) or indirectly (PDGF) mediating glioma-associated angiogenesis.

A pivotal role for VEGF in glioma angiogenesis has been demonstrated (Plate 1996) but, together with the principal factor VEGF, other growth factors, like Fibroblast-Growth Factor (FGF) and Plateled-Derived–Growth-Factor (PDGF) and their corresponding receptors may also contribute to glioma angiogenesis, either by directly stimulating endothelial cells or regulating the expression of VEGF. The growth factors mentioned above have in common that they exert their effects through binding to specific receptors which have tyrosine kinase activity, which in turn stimulates a cascade of downstream events.

FGF-1, also called acidic FGF (aFGF) and FGF-2, or basic FGF (bFGF) were proposed to have roles in glioma angiogenesis, since high levels of FGF-2 expression could be demonstrated in glioma cell lines (Murphy et al. 1988) and elavated FGF-1 expression was shown in human glioma (Stefanik et al. 1991). Especially FGF-2 has been linked directly with endothelial cell- proliferation and migration (Thomas 1987) and tube formation in vitro (Montesano et al. 1986). FGF-2 immunoreactivity on endothelial cells has been reported to correlate with tumor grade (Zagzag et al. 1990). Apart from directly stimulating endothelial cells it is proposed that FGF-2 may contribute to angiogenesis by participating to the degradation of the ECM by mediating the activity of plasmin, through the induction of the urokinase-type plasminogen activator (uPA) (Moscatelli et al. 1986) (Mignatti et al. 1991), and collagenase. Furthermore in glioma cell lines FGF-2 has been shown to stimulate VEGF expression in a dose dependent manner and thereby contributing to angiogenesis (Tsai et al. 1995). Studies further supporting a role for FGF-2 in glioma angiogenesis comprise an intracranially implanted tumor, which showed a lower degree of neovascularization after treatment with anti-FGF-2 antibodies (Stan et al. 1995). Higher levels of FGF receptors have also been reported for glioma cells (Morrison et al. 1994) but there is still conflicting evidence concerning the expression of FGF receptors on intratumoral endothelial cells.

PDGF, initially discovered as a mitogen for fibroblasts, has also been shown to contribute to angiogenesis. Glioma express both, PDGF-B and PDGF-A and the coressponding receptor subtypes (Guha 1991), with the exception of the PDGFR-ß receptor which is exclusively expressed on tumor vessels (Hermansson et al. 1988), (Plate et al. 1992). The expression of PDGF-A and PDGF-B increases with tumor stage, supporting the possible importance of functional autocrine/paracrine loops involved in glioma tumorigenesis/angiogenesis. PDGF-BB and PDGFR-ß are also involved in vascular remodeling and endothelial-pericyte association. Benjamin et al. showed that the pericyte recruitment to vascular endothelial cells could be disrupted by injection of ecotopic PDGF-BB in the retina and that the obstruction of pericyte-endothelial association resulted in an abnormal remodeling of the vasculature. Interestingly only a subset of pericytes, those involved in ongoing migration was responsive, while pericytes already completely covering endothelial cells remained unaffected (Benjamin et al. 1998). In addition it was shown that in xenografted and primary human tumors vessels that had not yet recruited pericytes (immature vessels) were highly dependent on VEGF; withdrawal of VEGF led to endothelial cell apoptosis of these immature vessels (Benjamin et al. 1999). Thereby the PDFG-BB/PDGFR-ß dependent recruitment of pericytes to endothelial cells defines a marker for the maturity of vessels and

implices that immature vessels are highly dependent on VEGF as a survival factor. Therefore it would be a prerequisite for an anti-VEGF therapy that vessels are in an immature state and therefore sensitive to an impaired VEGF signaling.

Although VEGF ist the key angiogenic growth factor in the regulation of glioma angiogenesis, the exact roles played by VEGF, bFGF, PDGF and their cognate receptors in mediating glioma-associated angiogenesis in vivo is still controversial. Also, it is not well understood whether angiogenic growth factors can compensate for the loss (e.g. induced therapeutically) of individual signal transduction systems, thus allowing tumors to switch to another molecule by clonal selection.

The Ephrin ligands and Eph receptors

Only recently, the largest group of known families of receptor tyrosine kinases (RTK's) and their lignads, the Eph receptors and their ligands the ephrins have been implicated in vascular development.

Ephrin ligands and Eph receptors have been most studied in the developing central nervous system, where they have been shown to function in contact-mediated axon guidance, axon fasciculation and guided cell migration. Moreover, they have been shown to be expressed outside the central nervous system, being involved in neural crest migration, somitogenesis, and epithelial morphogenesis (Flanagan and Vanderhaeghen 1998); (Holder and Klein 1999).

Their role in the vascular development has been recently summarized in detail (Gale and Yancopoulos 1999); (Yancopoulos et al. 2000). Taken together, (i) Ephrin ligands and Eph receptors are expressed on the developing vasculature and the adjacent mesenchyme (Helbling et al. 2000); (Daniel et al. 1996); (McBride and Ruiz 1998); (Pandey et al. 1995), (ii) ephrin B2 and EphB4 mark arterial and venous endothelial cells during vascular development suggesting a role in early vascular differentiation (Adams et al. 1999); (Wang et al. 1998), (iii) in addition, other ephrin-B ligands and EphB receptors are involved in endothelial cell-endothelial cell as well as endothelial cell-mesenchymal cell interactions during vascular development (Adams et al. 1999); (iv) angiogenesis and vascular modelling (in contrast to nerual crest migration) require bidirectional signaling between ephrin B ligands and their cognate receptors (Adams et al. 2001); (v) the ephrin ligand/eph receptor systems are part of a cross talk between angiogenic signaling pathways potentially influencing or being influenced by VEGF/VEGFR or Ang1/Tie-2 actvity (Adams et al. 2001). With respect to postnatal angiogenesis the significance, of Ephrin ligands and Eph receptors is less well defined. Using the cornea pocket assay and an in vitro sprouting angiogenesis assay several ephrin ligands (ephrin-A1, ephrin-B1 and ephrin-B2) as well as eph receptors (ephA2, ephB1-4) have been implicated in endothelial cell migration, endothelial cell assembly and capillary sprouting (Stein et al. 1998); (Pandey et al. 1995); (Daniel et al. 1996); (Adams et al. 1999); (Adams et al. 2001). Obviously, the significance of the individual ligands and receptors for the angiogenic process depends on binding specificities and the type of angiogenic assay applied.

Whether Ephrin ligands and Eph receptors also regulate tumor angiogenesis remains speculative, so far. In two recent reports, Ephrin-A1/EphA2 (Ogawa et al. 2000) and Ephrin-B2 (Shin et al. 2001) expression were shown in experimental tumor xenografts and surgically removed human cancers. Furthermore, Ephrin-B2 expression in a subset of tumor vessels suggested continued arterial differentiation which contrasts the believe that a categorization into arterioles, capillaries, and venules based on morphological and physiological criteria is not applicable to tumor microvasculature and that tumor vessels arise from the venular segments of the host mircrovasculature. The molecular and cellular mechanisms how these signaling systems might contribute to tumor angiogenesis, however, are currently not understood. This is further complicated by the fact, that despite the general notion that Eph receptors respresent specific endothelial RTKs, Eph receptors are not soley expressed by endothelial cells but also by periendothelial cells and tumor cells which is in line with previous reports on the

potential role of Ephrin ligands and Eph receptors in cellular transformation (Easty et al. 1999); (Tang et al. 1999).

METHODS

Intravital microscopic approaches to study tumor angiogenesis and microcirculation

The applicants are experienced in the use of intravital multi-fluorescence microscopic techniques and transparent chamber preparations to study angiogenesis, vascularization, and microcirculation of experimental brain tumors. Furthermore, they have repeatedly applied the same techniques to study microvascular cell-cell interactions during angiogenesis, graft rejection, and inflammation. Using a multi-fluorescence labeling technique, these models allow for the repetitive and simultaneous identification of individual glioma cells and precise quantitative measurement of tumor growth, the visualization of individual glioma microvessels as well as quantitative analysis of microcirculatory parameters. In addition, the applicants have established intravital microscopic techniques to non-invasively assess tumor tissue oxygenation (Vajkoczy et al. 1998), (Vajkoczy et al. 1999), (Vajkoczy et al. 2000), (Vajkoczy et al. 2003).

Techniques to study gene expression

The applicants have extensively studied the expression and function of angiogenic signaling systems in experimental and human tumors. Both laboratories are familiar with current expression analysis techniques both on the mRNA and protein level. Furthermore, the applicants are experienced with the cDNA array hybridization technology which they have recently applied to study the role of FGFR4 in the pathogenesis of mammary carcinomas and to study the expression pattern of Eph receptors in human glioma. During our recent project we have also developed an armentarium of species specific primers for Ephrin ligands and Eph receptors which allow us to seperately assess the expression of these molecules by tumor cells and vascular cells in our human xenograft models.

Histomorphological and histoanalytical techniques

The applicants are familiar with current histomorphological and immunohistochemical techniques to detect endothelial cells (by staining for CD31, vWF), angiogenic growth factors and their receptors, pericytes (by staining for PDGFR-β, NG2, and SMA), cell proliferation (Ki67) and cell apoptosis (TUNEL staining). These analyses are performed in tight collaboration with Hans-Peter Hammes (Mannheim/Germany). As demonstrated recently the applicants have also established radioacive and non-radioactive in situ hybridization techniques for human and experimental tumor tissue to study the expression of mRNA for VEGF/VEGFR-2, Angiopoietins/Ties, as well as Ephrin ligands and EpH receptors and Progress Report). Furthermore they have experience in the use of laser scanning confocal microscopy and electron microscopy to assess blood vessel morphology. To further Valentin Djonov (Bern/Switzerland).

RESULTS

Part I

Angiogenesis, microcirculation, and growth of high-grade gliomas: In vivo analysis of the pathophysiological and therapeutic significance of protein tyrosine kinases.

Expression of RTKs in glioma cell lines and human biopsy material

First, we attempted to identify relevant angiogenic and oncogenic RTK targets using the cDNA microarray hybridization technique on glioma cell lines in vitro and glioma xenografts in vivo as well as on human biopsies of malignant gliomas. While the increased expression of several angiogenic signal transduction systems in gliomas has been previously demonstrated (e.g. VEGF/VEGFR, bFGF/FGFR, PDGF/PDGFR), the present analysis was aimed to concentrate on identifying additionally overexpressed RTKs in both glioma cell cultures and human biopsy material of astroglial tumors.

Human (SF767, SF126, U118, U-373) and rat (C6) glioma cell lines were analysed after growth in culture and after subcutaneous implantation into nude mice and demonstrated consistent high in vivo expression patterns for EGFR, PDGFR-ß, VEGFRs, FGFR-1, as well as Tie-1/Tie-2. In addition CSF-1R was highly expressed indicating a strong inflammatory component of these tumors. Interestingly, EphA (EphA2) and EphB (EphB2-4) receptors were as well overexpressed in our analyses. Remarkably, however, Eph receptors were already expressed by tumor cells in vitro with their expression further increasing by 3 to 4-fold in established tumor grafts.

Relevance of individual signal transduction systems in mediating tumor angiogenesis and maintenance of tumor microcirculation/perfusion in vivo

The role of distinct signal transduction systems in the regulation of glioma angiogenesis in vivo has been analysed by selective targeting of individual tyrosine kinase receptors and analysing glioma growth, angiogenesis, and microcirculation by means of intravital fluorescence videomicroscopy. Based on various studies in experimental glioma models (Plate et al. 1993); (Stan et al. 1995); (Strawn et al. 1996) and human glioma specimens (Samoto et al. 1995); (Stefanik et al. 1991) as well as the detailed characterization of the C6 glioma model in terms of angiogenic growth factor/receptor expression (Millauer et al. 1994); (Plate et al. 1993) our analyses has been focused on the three most prominent signal transduction systems discussed to be involved in glioma angiogenesis: VEGF/VEGFRs, FGF-2/FGFR1 and PDGF/PDGFR-β. For selective and specific targeting of the receptors we have used small molecule RTK inhibitors with distinct target profiles (Table 1).

	VEGFRs	FGFR	PDGFR	EGFR	Her2
SU5416	**1.0**	> 50	20	> 100	> 100
SU5402	**0.4**	**0.1**	8.1	> 100	> 100
SU6668	**2.6**	**3.7**	**0.5**	> 100	> 100

Table 1. Target profile of tyrosin kinase inhibitors IC50s for the different compounds and targets.

Selective VEGFR-2 inhibition fails to induce regression of tumor vessels

Using dorsal skinfold preparations the growth and angiogenesis of C6 glioma was studied by intravital multi-fluorescence video microscopy. Targeting the VEGF-receptors with SU5416 demonstrated an anti-angiogenic activity by a significant growth suppression of the tumor. In fact, we did not observe changes in blood vessel density, blood vessel diameter, permeability, or microvascular architecture of SU5416-treated tumors. In contrast, SU5416 significantly suppressed further neovascularization and, concomitantly, tumor expansion. These results clearly indicate that selective targeting of VEGFR-2 represents a potent means to prevent new blood vessel formation, but fails to force already established tumor vessels into regression.

Pericytes protect tumor vessels from enforced regression

To address the mechanisms underlying the resistance of established tumor blood vessels to regression after interference with VEGF signalling, we first determined the fraction of mature tumor blood vessels, i.e. blood vessels associated with pericytes, using double fluorescence immunostaining for the endothelial cell marker CD31 and the pericyte marker desmin. As shown in Figs. 1A and C, the majority of blood vessels in the control tumors was associated with pericytes, resulting in a maturation index of 80%. Treatment with SU5416 slightly further increased this fraction of mature vessels (Figs. 1B and C). This abundant association between pericytes and endothelial cells was confirmed by electron microscopy (Fig. 1D and E). Furthermore, electron microscopy revealed an important difference between control and treated tumors. While blood vessels of control tumors were characterized by a loose and improper coverage of blood vessels by pericytes, blood vessels of SU5416-treated tumors demonstrated an intimate pericyte-endothelial cell association. In view of the fact that SU5416 treatment did not induce regression of immature vessels (≈ 20% in control tumors) these results indicate that, as a consequence of VEGFR-2 targeting, pericytes were recruited to the tumor and intensified their coverage and, thus stability, to tumor blood vessels. With the aim to further characterize these pericytes and to identify a tyrosine kinase-based target for a selective anti-pericyte intervention we performed an immunostaining for PDGFR-β which confirmed expression of PDGFR-ß in perivascular cells, but not in tumor cells (Fig. 1F).

To gain insight into the signalling mechanisms underlying tumor blood vessel stabilization and pericyte recruitment, the expression of angiogenic growth factors and their cognate receptors in control and SU5416-treated tumors was analysed by RT-PCR and Northern blotting. While expression of the VEGF and FGF systems remained largely unchanged, expression of PDGFR-β and Tie2 were nearly doubled and expression of Ang-1 was increased by 9-fold in SU5416-treated tumors (Figs. 2A - C). This change in the angiogenic expression profile is also reflected in the Ang-1:Ang-2 ratio which was 1:11 in control tumors and turned into 8:1 in SU5416-treated tumors. By using species-specific primers 1, the increased expression of PDGFR-β and Tie2 was clearly attributable to pericytes and endothelial cells (i.e. cells of mouse origin), respectively. To further localize the increased Ang-1 expression, we performed in situ hybridizations of control and SU5416-treated tumors. These experiments demonstrated that Ang-1 was predominately expressed by perivascular cells, and only to a lower extent by tumor cells (Figs. 2D and E). Consequently, pericytes not only stabilized tumor blood vessels by cell-to-cell contact but also protected tumor blood vessels from regression by providing Ang-1 signalling which mediates endothelial cell survival.

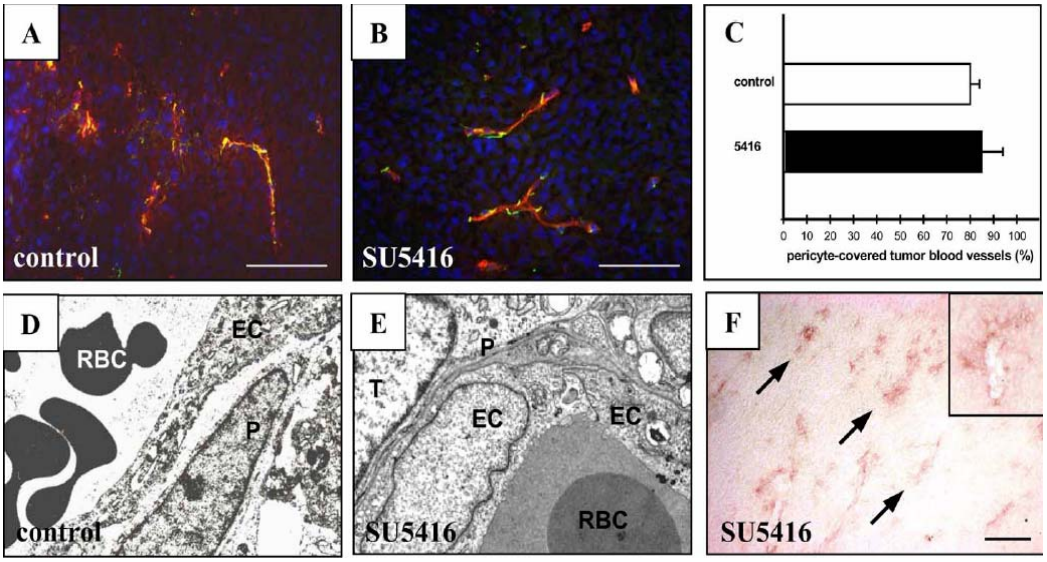


Fig. 1: Control and SU5416-treated tumors demonstrate a large fraction of mature blood vessels. (A and B) Double fluoresecence immunostaining for CD31 (red) and desmin (green) in control (A) and SU5416-treated C6 tumors(B). Counterstain of tumor cell nuclei with DAPI (blue). Bars = 100 µm. C) Quantitative analysis of C6 tumor vesselmaturation index, defined as the fraction of vessels (identified by CD31 staining) that are associated with pericytes(identified by desmin staining).(D and E) Ultrastructural analysis of pericyte-endothelial cell association in control (D) andSU5416-treated (E) C6 tumors. P, pericyte; EC, endothelial cell; T, tumor cell; RBC, red blood cell. F) Immunohistochemistry demonstrates expression of PDGFR-β by pericytes inSU5416-treated tumors. Inset shows tumor blood vessels associated with PDGFR-β-positive pericytes at highermagnification. Arrows indicate blood vessels with positive staining for PDGFR-β. Bar = 50 µm.

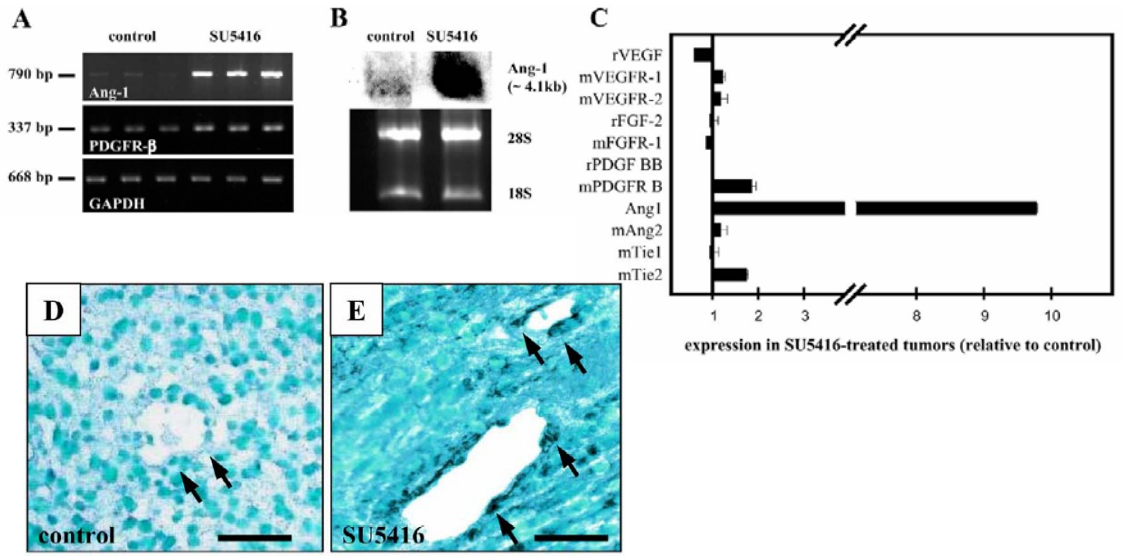


Fig. 2: Selective targeting of VEGFR-2 leads to upregulation of ANG-1 and PDGFR-β by pericytes. (A) RT-PCRfor Ang-1, PDGFR-b and GAPDH mRNAs from C6 tumors. Representative gel image of a control and SU5416-treated tumor. Experiments were performed in triplicate. B) Northern blot for Ang-1 mRNA from C6 tumors. Representative image of a control and SU5416-treated tumor. C) Angiogenic expression profile of SU5416-treated tumors relative tocontrol tumors as assessed by RT-PCR using species-specific primers (m, mouse; r, rat. Graphical representations are given as relative changes to the expression observedin untreated tumors. D and E) In situ hybridization of control (D) and SU5416-treated C6 tumors (E) for Ang-1 mRNA demonstrates an increase in signal in pericytes after SU5416 treatment. In contrast, expression of Ang-1 in tumor cells is less pronounced. Arrows indicate detection of Ang-1 mRNA expression in perivascular cells covering two tumor blood vessels. Bars = 20 µm.

Inhibition of VEGFR-2 plus PDGFR-β induces tumor specific blood vessel regression

At this point, we hypothesized that a simultaneous targeting of endothelial cells and pericytes may provide more effective means to enforce regression of mature tumor blood vessels. To test this, we treated C6 xenografts with SU6668, a tyrosine kinase inhibitor that not only potently targets VEGFR-2 (like SU5416) but also PDGFR-β. Under these conditions, tumor blood vessels rapidly regressed within 24 hours. As demonstrated by sequential intravital fluorescence microscopy, vascular sprouts were most susceptible to SU6668 treatment, showing disintegration and destruction within a few hours following initiation of treatment. This was followed by destabilization and regression of already established and perfused tumor blood vessels. Regression of tumor blood vessels was accompanied by an increase in microvascular permeability and by microvascular hemorrhage, characterized by the extravasation of the high molecular weight FITC-Dextran and the escape of red blood cells from blood vessels, respectively. In contrast to the tumor blood vessels undergoing rapid regression, blood vessels of the host tissue were unaffected by the treatment with SU6668. The quantitative assessment of tumor vessel density revealed that inhibition of VEGFR-2 plus PDGFR-β forced 40% of tumor blood vessels into regression without changing the morphology of the surviving vessels.

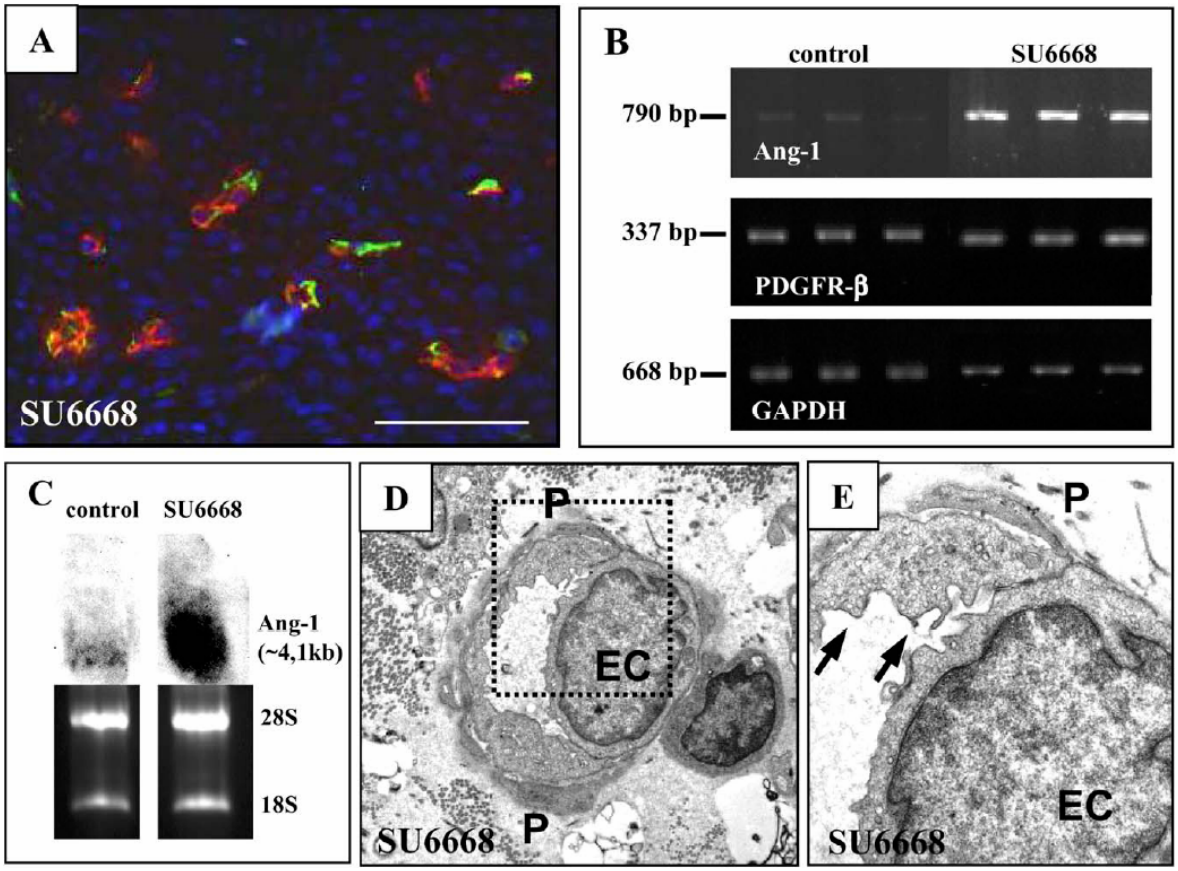


Fig. 3: Targeting of VEGFR-2 plus PDGFR-b interferes with pericyte-mediated endothelial cell survival mechanisms (A) Double fluoresecence immunostaing for CD31 (red) and desmin (green) in SU6668-treated C6 tumorsreveals a preserved high maturation index of tumor blood vessels. Counterstain of tumor cell nuclei with DAPI (blue). Bars = 100 µm. (B) rt-PCR for Ang-1, PDGFR-b, and GAPDH mRNAs from control and SU6668-treated C6 tumors. Gel image illustrates experiments performed in triplicate. (C) Northern blot for ANG-1 mRNA from C6 tumors. Representativegel image of a control and SU6668-treated tumor. (D and E) Ultrastructural analysis of pericyte-endothelial cellassociation in a SU6668-treated tumor reveals loose cell-to-cell contact as an indicator of disturbed pericyte-endothelialcell interaction. Also, note hyperplasia of endothelial cells and increased number of luminal folds (indicated by arrows).

Inhibition of VEGFR-2 plus PDGFR-β interferes with pericyte-mediated endothelial cell survival mechanisms

In order to understand the distinct effects of selective VEGFR-2 targeting versus VEGFR-2 plus PDGFR-β targeting, we next studied the pericyte-endothelial cell interaction in SU6668-treated tumors. Double fluorescence immunostaining for CD31 and desmin showed that the majority of SU6668-treated tumor blood vessels (93±6 %, i.e. slightly increased compared to SU5416-treated tumors) remained in association with pericytes (Fig. 3A). Noteworthy, our experiments did not reveal a pericyte drop off from tumor blood vessels as previously suggested as a mechanism of vessel destabilization following SU6668 treatment. Immunohistochemistry further cornfirmed that these pericytes expressed PDGFR-β at a level comparable to SU5416-treated tumors. Also, the angiogenic expression profile following SU6668 treatment was comparable to SU5416-treated tumors (Fig. 3B and C vs. Fig. 2A-C). Again, RT-PCR analysis and Northern blotting revealed that expression of PDGFR-β and Tie2 were nearly doubled and expression of Ang-1 was increased by 7-fold. These results suggest that the amount of PDGFR-β-positive perivascular cells was comparable in SU5416-treated and SU6668-treated tumors, and that perivascular cells continued to express increasing amounts of Ang-1 mRNA despite the additional inhibition of PDGFR-β by SU6668. The latter was confirmed by in situ hybridization results.

Finally, the clue of how VEGFR-2 plus PDGFR-β targeting forced tumor blood vessels into regression came from our electron microscopic studies. As shown in Figs. 3D and E, these studies demonstrated that although pericytes were still present their association with endothelial cells in SU6668-treated tumors was less intimate than in the SU5416 group suggesting that SU6668 negatively influenced the pericyte-endothelial cell interaction. Further evidence for a negative influence of VEGFR-2 plus PDGFR-β targeting on the pericyte-endothelial cell interaction could be derived from studying the endothelial cell morphology in these tumors. Similar as previously demonstrated for transgenic mouse embryos that lack pericyte-endothelial cell interaction during vascular development due to a defect in PDGF/PDGFRR-β signalling, tumor blood vessels in SU6668-treated tumors presented with endothelial hyperplasia and an increased number of luminal folds (Figs. 3C and D; compare to Fig. 1D). Ultrastructural anaylsis of normal blood vessels in the non-tumor bearing skinfold confirmed that SU6668 had not effect on the physiological pericyte/endothelial cell association.

During Part I “Angiogenesis, microcirculation, and growth of high-grade gliomas: In vivo analysis of the pathophysiological and therapeutic significance of protein tyrosine kinases” of the project, we could demonstrate that selective interference with VEGFR-2 signalling fails to enforce regression of established tumor blood vessels. This resistance of tumor blood vessels to selective VEGFR-2 inhbition was confered by pericytes that (i) initially stabilize matured vessels, (ii) are secondarly recruited to immature vessels upon therapy, and (iii) compensatorily express endothelial cell survival factors (e.g. Ang-1). In contrast, inhibition of VEGFR-2 plus PDGFR-β, which simultaneously targeted endothelial cells and pericytes, acts as a potent anti-vascular strategy, inducing endothelial cell apoptosis, tumor vessel destabilization and regression, and finally tumor hypoxia. Beyond these therapeutic aspects, this part of the study provided novel insights into the mechanisms of how pericytes differentially promote blood vessel stabilization and maturation via the Ang-1/Tie2 and PDGF-B/PDGFR-β systems.

Consequently, an extended anti-angiogenic strategy, targeting endothelial cells and pericytes, represents an attractive approach in treating patients presenting with advanced tumors with established blood vessels. Especially because many of the endothelial-specific anti-angiogenic compounds seem to be effective in rather preventing tumor initiation than in successfully interfering with the progressed tumor mass (Herbst et al. 2001), (Gordon 2000). The VEGFR-2 plus PDGFR-β inhibitor SU6668 represents one example of a potent polyvalent tyrosine kinase inhibitor that not only prevents further angiogenesis but also provides a potent anti-vascular efficacy even resulting in regression of selected experimental tumor types (Laird et al. 2000).

This study, however, has not only provided insights into the mechanisms of enforced tumor vessel regression by VEGFR-2 plus PDGFR-β targeting, but has also illustrated the microvascular consequences of its anti-vascular effects. Clearly, an important caveat to using similar inhibitors in a clinical setting is the acute and rapid destabilization of tumor blood vessels which may lead to edema formation and vascular hemorrhaging. This may be especially detrimental in the treatment of brain tumors or in combining VEGFR-2 plus PDGFR-β targeting with radiation therapy which already leads to transient vessel destabilization per se.

In summary, the results of this study suggest that a successful intervention in advanced tumors and long-term disease control with anti-angiogenic compounds can be best achieved with a combination therapy, targeting not only endothelial cells but also pericytes. Therefore, besides selectively targeting VEGF/VEGFR-2 as the pivotal pathway for angiogenesis, additional pathways involved in vascular stabilization and maturation should be included into the target profile.

Part II

Pathophysiologic and therapeutic significance of Eph family receptors for angiogenesis, microcirculation, and growth of malignant gliomas

Upregulation of Eph-receptor expression after xenotransplantation of human glioma cells

The RTK expression profile of human glioma cell lines (SF767, U373, U118) was determined by a RTK cDNA macroarray technique (Bange et al. 2002). Besides EGFR and PDGFR-β, which have been already shown to be involved in glioma biology, the Eph-receptors EphA2 and EphB2-EphB4 were found to be highly expressed in glioma cells in culture. Interestingly, and thereby suggesting an involvement in glioma biology, after subcutaneous implanation of glioma cells into the left flank regions of nude mice and re-examination of the expression profile, EphA2 and EphB2-EphB4 expression had increased about two-fold. These results have initially suggested that the Eph receptors, together with their also signal-propagating ligands, might be involved in brain tumor progression, and further encouraged us to study the expression of Eph receptors and Ephrin ligands in xenotransplants as well as in human glioma in more detail, and to elucidate the origin of the Eph receptor and Ephrin ligand expression, e.g. endothelial cells and/or tumor cells.

EphrinB2 and EphB4 are expressed by endothelial cells of malignant brain tumors

In order to study the relevance of ephrins and Ephs in the context of tumor angiogenesis we have continued our examinations with an expression analysis of ephrin and Eph mRNAs in human brain tumor xenografts (human glioma cell lines SF126 and SF767). Since ephrins and Ephs may be expressed by both mouse endothelial and human tumor cells we have used species-specific RT-PCR in order to discriminate between tumor cell derived and tumor blood vessel derived expression of ephrins and Eph mRNA. All ephrin and Eph mRNAs analyzed, were detected both in tumor cells (human specific primers) and tumor blood vessels (mouse specific primers) (Fig. 1A). Next, we studied ephrin- and Eph mRNA expression in human surgical brain tumor specimens. Here, we analysed mRNA expression of 4 ephrins (ephrinA1, ephrinB1, ephrinB2 and ephrinB3) and 5 Eph receptors (EphA2, EphA5, EphB2, EphB3, and EphB4) in a total of 26 high-grade glioma (all glioblastoma multiforme), 6 low-grade glioma (all astrocytoma WHO grade II), and 3 non-neoplastic brain specimens. Only ephrinB2 and EphB4 mRNAs were consistantly expressed at higher levels in the tumor tissue relative to controls (Fig. 1B and C). In order to localize their expression we used in situ hybridization with DIG-11-UTP labeled probes. In control tissue (n=3), ephrinB2 and EphB4 mRNAs were detected only at low levels in glial cells and

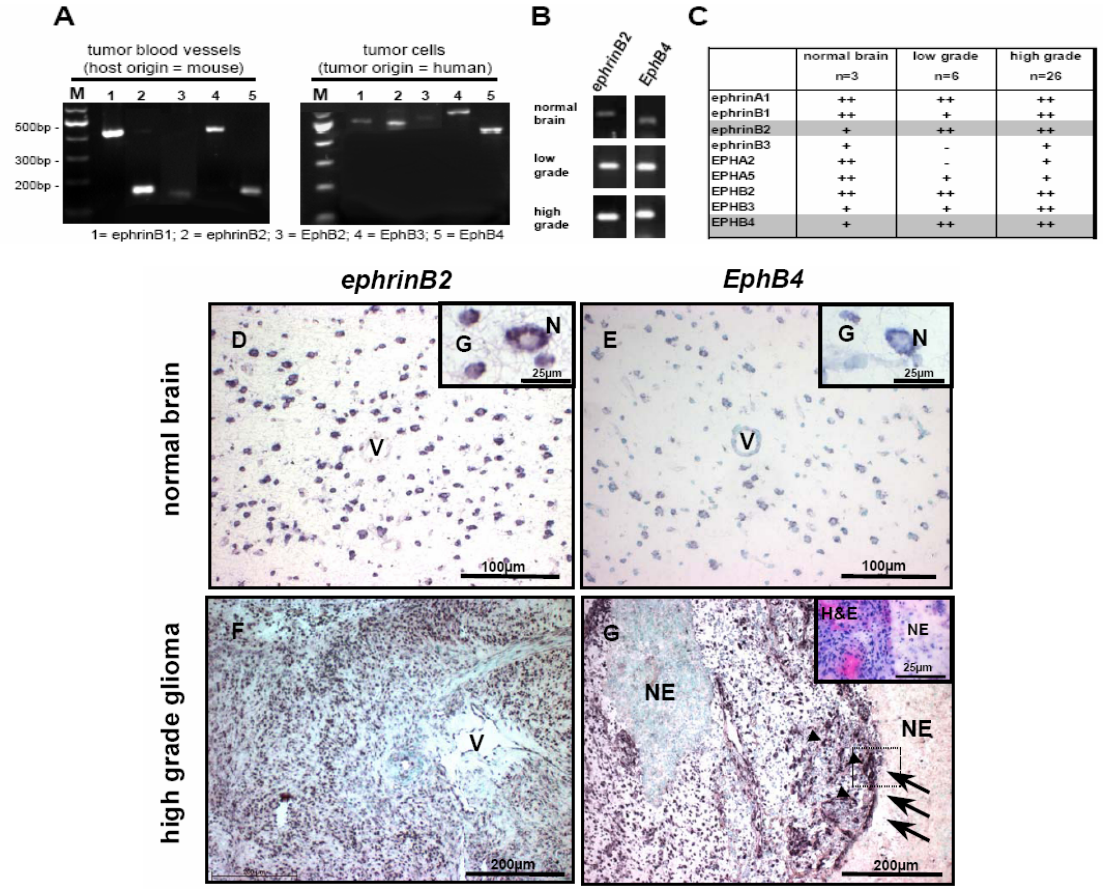


Fig. 1: Ephrin and Eph receptor expression analysis in experimental and human glioma. (A) RT-PCR expression analysis of SF126 human glioma xenografts following transplantation into nude mice. In order to discriminate between tumor cell-derived (human origin) and blood vessel-derived (mouse origin) expression of individual Ephs and ephrins in glioma xenografts, species specific primers were designed. (B) RT-PCR expression analysis for ephrinB2 and EphB4 on human surgical specimens of normal brain, low-grade glioma and high-grade glioma. (C) Summary of results of RT-PCR expression analysis on human astrocytoma. (D-G) In situ hybridization of normal brain (D and E) and high-grade glioma (F, G and H) for ephrinB2 and EphB4 using DIG-labeled cRNA-probes. Higher maginfication (H) showsstrong signal for EphB4 mRNA in tumor vessesl (arrowhead) and tumor cells adjacent to necrosis (arrow denotes margin of necrotic area). Cells are denoted glial cells and neurons on the basis of morphological critereria. G= glial cell, N= neuron, V= vessel, NE = necrosis. Counterstain with methyl green. Bar scale as indicated.

neurons, but not in brain blood vessels (Fig. 1D and E). In high-grade glioma (n=15), ephrinB2 and EphB4 RNA were expressed at high levels in both tumor cells and blood vessels (Fig. 1F and G).

Experimental manipulation of EphB4 signaling in vivo

In order to selectively manipulate EphB4 signaling in vascular cells we decided to implant human tumor cells into nude mice and to apply a ecotropic retroviral strategy (only directed against host cells = mouse cells = vascular cells), similar as previously described for VEGFR, PDGFR, or Tie Receptors, where tumor cells were co-implanted with virus packaging cells that released vectors carrying the genes for the manipulated RTKs (Millauer et al. 1994), (Strawn et al. 1994), (Stratmann et al. 2001)). In order to respect the biderectional signaling capacity of EphB4 we designed four experimental groups: (i) transfection with empty control vector (pLXSN); (ii) transfection with vector encoding for the EphB4 wildtype receptor (EphB4-WT); (iii) transfection with vector encoding for a EphB4 truncated dominant-

negative receptor (EphB4-DN). This would result in unchanged biderectional signaling (group i), enhanced EphB4 biderectional signaling (group ii), and inhibition of EphB4 forward signaling/enhanced reverse EphB4 signaling (group iii), which should allow us to dissect the role of EphB4 signaling in vivo.

Generation of recombinant retrovirus prodrucing cell lines for in vivo EphB4 function analysis

We established stable virus producing clones of Phoenix E producer cells. In a xenograft tumor, an ecotropic retrovirus should selectively infect the mouse endothelial cells leading to an exclusive expression of the transgene in the new vascular system.

Virus production was verified by infection of NIH 3T3 murine fibroblasts with the supernatants of Phoenix E cell cultures. Western Blot analysis of infected NIH 3T3 cells with a polyclonal antibody directed against the extracellular domain of EphB4 detected EphB4wt and EphB4dn at the predicted molecular weights of approx. 120kD and 70kD, respectively (Fig. 2A and B).

Since cell surface expression of EphB4 is essential we examined infected NIH 3T3 cells for the localization of the introduced EphB4 variants. Immunofluorescence staining, using ephrinB2-Fc chimeras, confirmed that EphB4wt and Eph4dn were expressed on the cell surface of virus infected NIH3T3 cells, clustering at their filopodial protrusions (Fig. 2C). The functional activity of virally introduced EphB4wt and EphB4dn was determined by stimulation of infected NIH3T3 cells using ephrinB2-Fc chimeras and assessment of receptor tyrosine phosphorylation by immunoprecipitation of EphB4 and immunoblotting with an anti-phosphotyrosine (PTyr) specific antibody. EphB4wt infected cells showed higher levels of EphB4 protein expression and increased EphB4 tyrosine phosphorylation when compared to control virus-infected cells (Fig. 2D). In contrast, overexpression of EphB4dn protein abolished phosphorylation of the endogenous EphB4 receptor, indicating efficient inhibition of EphB4 forward signaling (Fig. 2D).

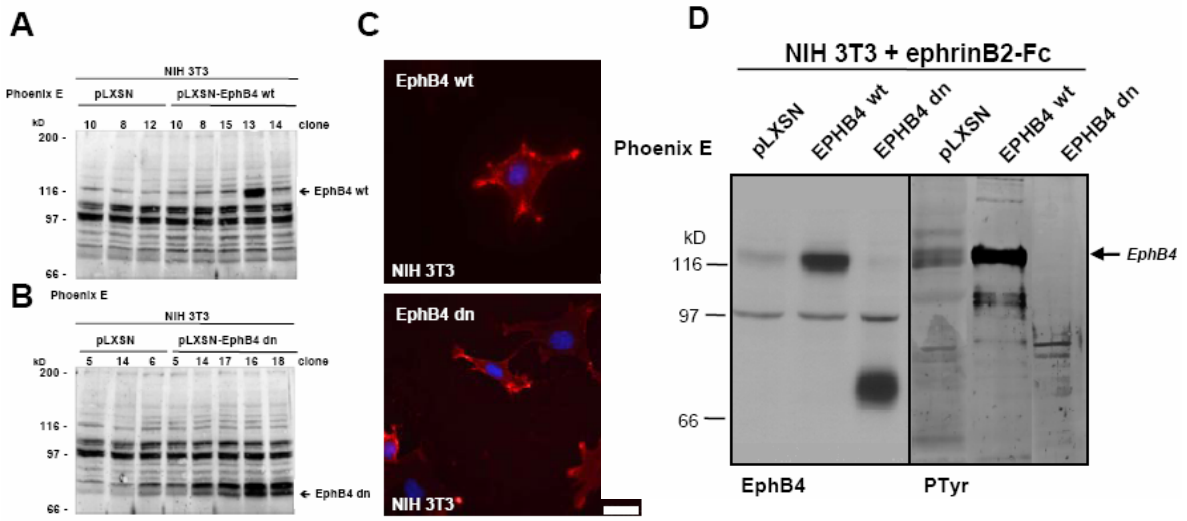


Fig. 2: Generation of retrovirus packaging cell lines to manipulate EphB4 signaling in vivo. (A and B) Stable virus producing clones of Phoenix E producer cells were established. Western Blot analysis of infected NIH 3T3 cells detected EphB4wt (A) and EphB4dn (B) at the predicted molecular weights of approx. 120kD and 70kD, respectively. (C) Immunofluorescence staining of virally transfected NIH 3T3 cells using ephrinB2-Fc chimeras. Scale bar = 10µm. (D) Western blot analysis of virally transfected NIH 3T3 cells for EphB4 and for phosphotyrosine (PTyr) after stimulation with ephrinB2-Fc chimera.

Endothelial EphB4 regulates vascular morphogenesis and permeability, independently of its tyrosine kinase activity

Next we sought to study the consequences of endothelial EphB4 manipulation for tumor angiogenesis. Therefore, Phoenix E virus producing cells were subcutaneously co-implanted with SF126 cells into nude mice (ratio 1:1). The effects of infection with the viruses containing the cDNA for EphB4wt and EphB4dn were compared with the effects elicited by the empty control vector (pLXSN). RT-PCR analysis and immunoblot analysis of tumor xenograft lysates confirmed overexpression of EphB4wt and EphB4dn relative to control tumors (Fig. 3A and B). Probing of tumor lysates with an anti-phosphotyrosine antibody revealed high levels of EphB4 phosphorylation in EphB4wt virus infected tumors, while phosphorylation of endogeneous EphB4 was nearly abolished in EphB4dn expressing tumors (Fig. 3B). In parallel, ephrinB2 expression was unaffected in these tumors (Figs. 3A and B). However, analysis of ephrinB2 phosphorylation demonstrated that overexpression of EphB4wt and EphB4dn activated EphB4 reverse signaling (Fig. 3B).

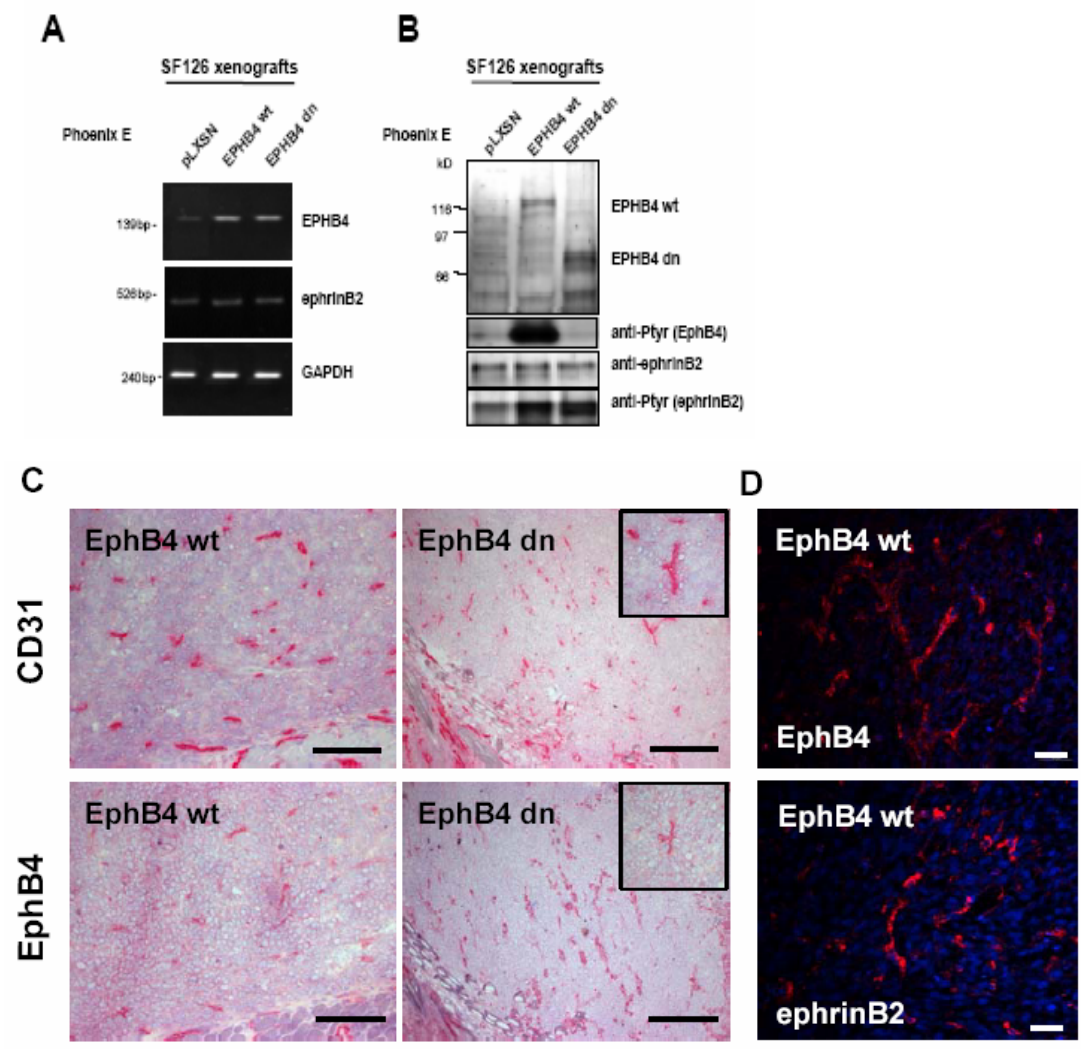


Fig. 3: Interference with endothelial EphB4 signaling in vivo alters tumor blood vessel morphology. (A and B) RT-PCR expression analysis (A) and Western blot analysis (B) of SF126 xenografts following s.c. co-implantation with virus producing Phoenix E cells. (C) Immunohistochemical staining for EphB4 and CD31 on consecutive cryo-fixed sections. Counterstain with hematoxylin. Scale bar = 100µm. (D) Immunofluorescent staining for EphB4 and ephrinB2 on related zinc-fixed sections of e-EphB4 wt tumors. Scale bar = 20µm.

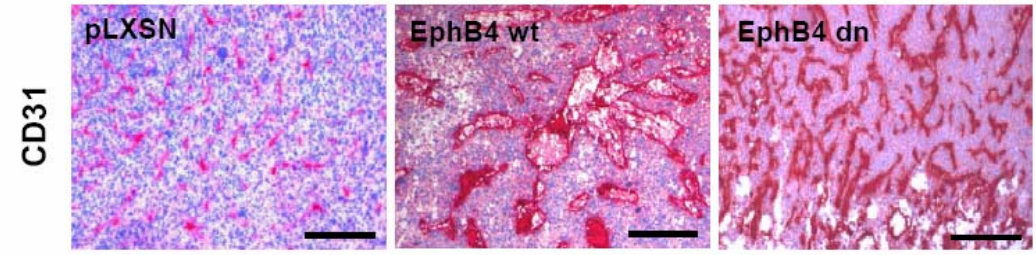


Fig. 4: Immunohistochemical staining for CD31. Counterstain with hematoxylin.
Scale bar = 100µm.

Immunohistochemistry confirmed that in vivo infection and consecutive expression of the EphB4 variants was restricted to tumor blood vessels in our co-transplantation model. (Fig. 3C). The resultant tumors were termed e-EphB4wt and e-EphB4dn tumors. Immunofluorescent stainings on consecutive sections further suggested that EphB4wt and EphB4dn were co-expressed with ephrinB2 in the same blood vessels of these tumors (Fig. 3D).

To further assess the involvement of endothelial EphB4 in tumor angiogenesis and growth, we performed immunohistochemical stainings for CD31 (Fig. 4). The tumors infected with control virus were characterized by a microvascular network with small to medium sized tumor blood vessels. In contrast, e-EphB4wt tumors were characterized by an increase in vessel area density, i.e. the area covered by the CD31 positive blood vessels. A similar increase was observed in e-EphB4dn tumors.

To obtain a detailed insight into this vascular phenotype, we next applied intravital fluorescence videomicroscopy. During the first week after implantation, e-pLXSN, e-EphB4wt and e-EphB4dn tumors were characterized by a similar angiogenic activity and architecture of their microvascular network, suggesting that vascular initiation is independent of EphB4 signaling (Fig. 5A-C). By the second week, the microvascular network of e-EphB4wt and e-EphB4dn tumors started to differ from control tumors, in that their blood vessels were enlarged to giant sizes and extravasation of FITC-Dextran was reduced, indicating a reduced vascular permeability and decreased edema formation (Fig. 5D-F). Also, we observed differences in the tumors´ angioarchitecture characterized by a parallel tumor blood vessel alignment and unidirectional blood flow (Figs. 5D-F). Measurements confirmed the increased vessel area density in e-EphB4wt and e-EphB4dn tumors, which was primarily attributable to an increase in blood vessel diameter (Fig. 5G-I). Thus, these studies clearly demonstrated that EphB4 signaling is involved in morphogenesis and permeability of the tumor vascular system; a function that is independent of the receptor tyrosine kinase activity.

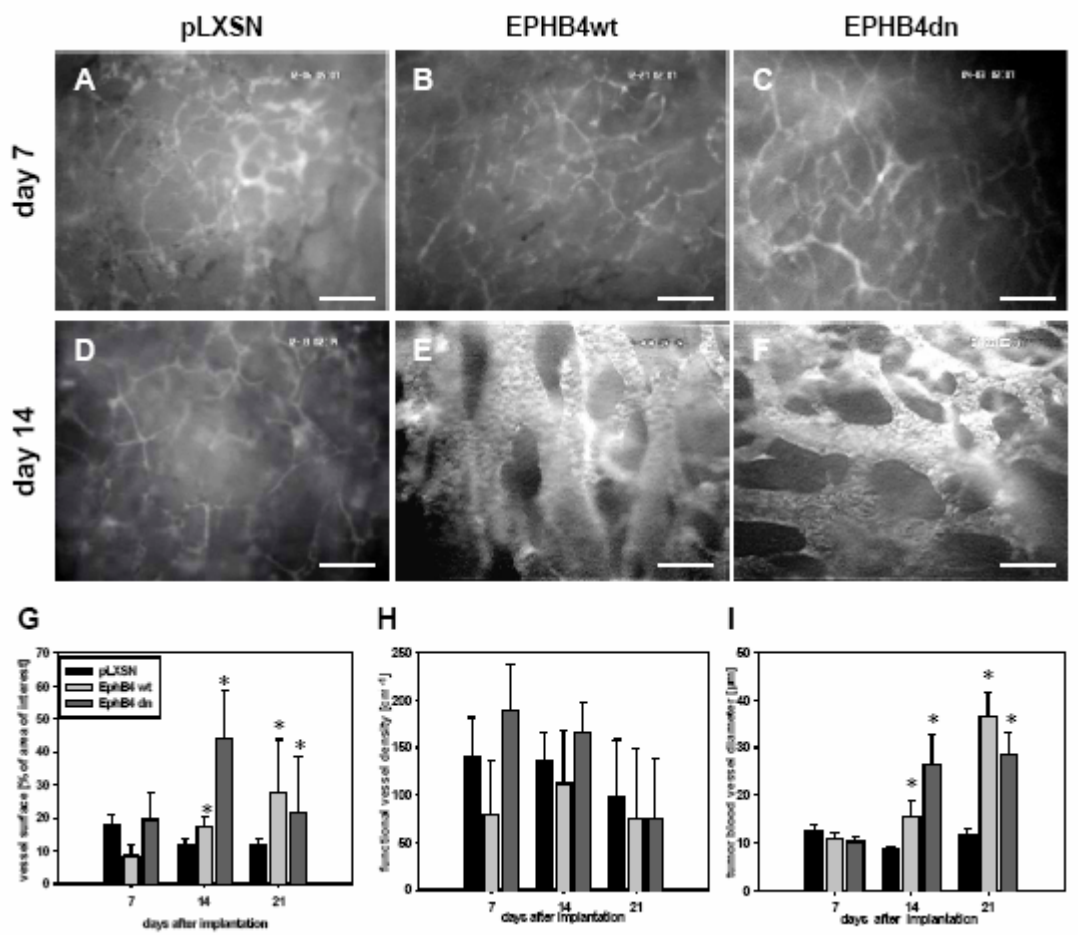


Fig. 5: Endothelial EphB4 regulates vascular morphogenesis and permeability, independently of its tyrosine kinase activity. (A-F) Intravital fluorescence videomicroscopy of SF126 human glioma cells that were co-implanted with virus producing Phoenix E cells von Days 7 (A-C) and 14 (D-F) after implantation. Scale bar = 100µm. (G-I) Quantitative measurements of tumor blood vessel surface (G), functional tumor blood vessel density (H), and tumor blood vessel diameter (I). Values are represented as means ± SD. Number of animals per experimental group: e-pLXSN n=5; e-EphB4wt n=5, e-EphB4dn n=6. ∗ $p<0.05$ versus e-pLXSN.

Expression of endothelial EphB4 results in circumferential blood vessel growth

Next, we sought to determine the nature of vessel enlargement to understand how overexpression of EphB4 in sprouting blood vessels affects tumor blood vessel morphogenesis. Immunohistochemical staining for CD31 revealed that the enlarged tumor vessels were consistently covered by an endothelial lining (Fig. 4). This indicated that endothelial cell proliferation had to be increased in e-EphB4wt and e-EphB4dn tumor blood vessels. Staining for mouse Ki67 revealed a 3-4-fold increase of positively stained blood vessels in e-EphB4wt and e-EphB4dn tumors versus control tumors (Fig. 6D-G). Moreover, while blood vessels of control tumors exhibited only few proliferating endothelial cells (Fig. 6D), e-EphB4wt tumors and e-EphB4dn tumors were characterized by a clustering of proliferating endothelial cells within the wall of enlarged blood vessels (Figs. 6E and 6F). These results led to the hypothesis that the enlargement of EphB4wt and EphB4dn tumor blood vessels was the result of a switch from sprouting angiogenesis to circumferential vessel growth. This was confirmed by using intravital fluorescence videomicroscopy which demonstrated that interconnection of angiogenic sprouts and vascular network formation were indeed impaired in e-EphB4wt and e-EphB4dn tumors resulting in significant reduction of vascular branching points.

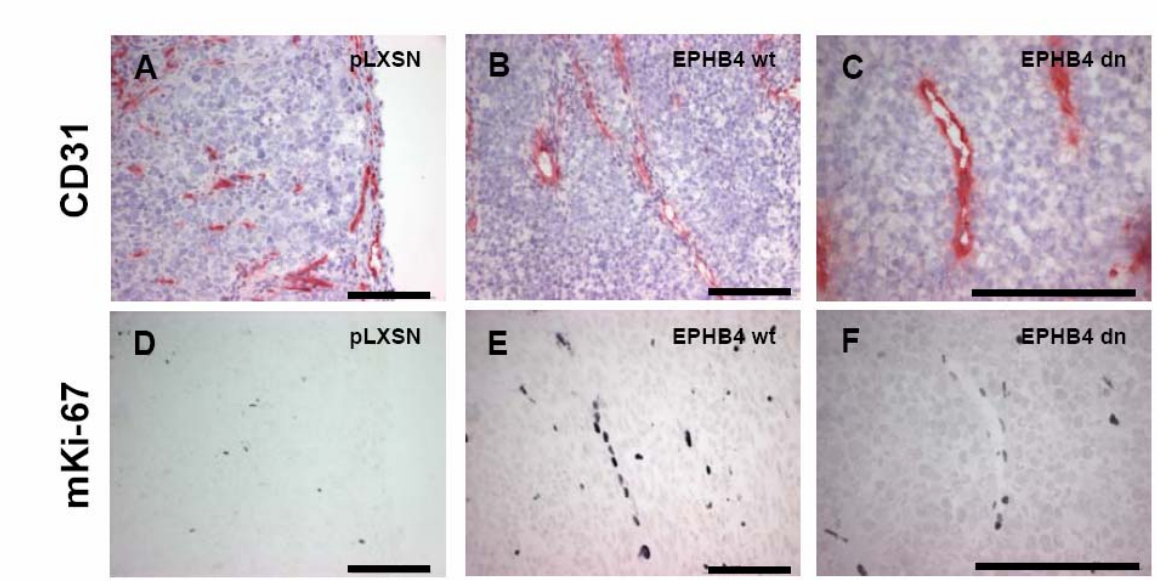


Fig. 6: Manipulation of endothelial EphB4 signaling results in circumferential blood vessel growth. (A-F) Consecutive cryofixed sections of SF126 glioma xenografts were immunohistochemically stained for CD31 (A-C) and the proliferation associated antigen Ki-67 using a murine specific antibody (Tec3) (D-F). Scale bar = 100µm.

Endothelial EphB4 is involved in the regulation of tumor blood vessel permeability

Our intravital microscopic studies also suggested that EphB4wt and EphB4dn infection of tumor blood vessels reduced their permeability (Fig. 5D-F). Measurement of FITC-Dextran extravasation confirmed a 20-30% decrease in tumor blood vessel permeability. Since, apart from cell to cell contacts, transendothelial permeability had been correlated with the microvascular coverage by pericytes (Hellstrom et al. 2001), and EphB4 signaling had been implicated in pericyte recruitment, we next sought to determine pericyte coverage of e-EphB4wt and e-EphB4dn tumor blood vessels. However, double-immunofluorescent staining against desmin and CD31 failed to explain the observed changes in vascular permeability. The phenomenon of reduced vascular leakiness was reminiscent of the Ang-1 overexpression phenotype (Thurston et al. 1999) suggesting a molecular link between EphB4 and the Ang-1/Tie2 system. To further investigate this, we determined the expression of Ang-1, Ang-2, and Tie2 by RT-PCR (Fig. 7A). While control tumors expressed low Ang-1 and high Ang-2 mRNA levels, e-EphB4wt and e-EphB4dn tumors were characterized by an inversed Ang-1/Ang-2 ratio with a prevalence of Ang-1 mRNA expression. Moreover, analyzing the phosphorylation (e.g. activation) of the Tie2 receptor in e-EphB4wt and e-EphB4dn tumors and control tumors (pLXSN) revealed a strong activation of the Tie2 receptor in e-EphB4wt and e-EphB4dn tumors as compared to control tumors (Fig. 7B), thereby further supporting a relation between Ang1 expression, leading to the observed inversed Ang-1/Ang2 ratio in transfected tumors by the activation of ephrinB2 through leading to increased reverse signaling, which is dependent on the extracellular domain of EphB4 and therefore likewise observed in tumors which express either the full-length wt version or the truncated kinase deficient variant of EphB4 in their vascular compartment.

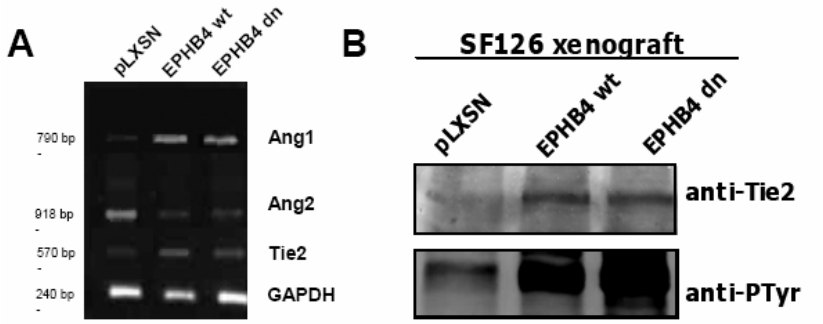


Fig. 7: Manipulation of endothelial EphB4 signaling results in circumferential blood vessel growth and a reduced tumor blood vessel permeability. (A) RT-PCR expresion analysis of SF126 human glioma xenografts for Ang-1 and Ang-2 following s.c. co-implantation with virus producing Phoenix E cells. (B) Western blot analysis for mTie2 (anti-Tie2) and Phosphotyrosine (anti-PTyr) after immunoprecipitation for mTie2 of SF126 human glioma xenografts following s.c. co-implantation with virus producing Phoenix E cells.

Since analysis of the number of pericytes associated to vessels using immunoflurescence staining for CD31 and desmin did not reveal significant changes in e-EphB4wt and e-EphB4dn tumors as compared to controls we next sought to perform ultrastructural analysis of pericyte association to vessels of e-EphB4wt and e-EphB4dn tumors. Transmission electron microscopy revealed, exemplified for e-EphB4 wt tumors a regular endothelium densely packed by pericytes which were closely attached to the endothelium. Even newly formed, seamless capillaries were already tightly covered by pericytes. (Fig. 8). In contrast pLXSN control tumors showed small tumor microvessel rich on endothelial nuclei covered by periendothelial cells, only loosely associated to the endothelium.

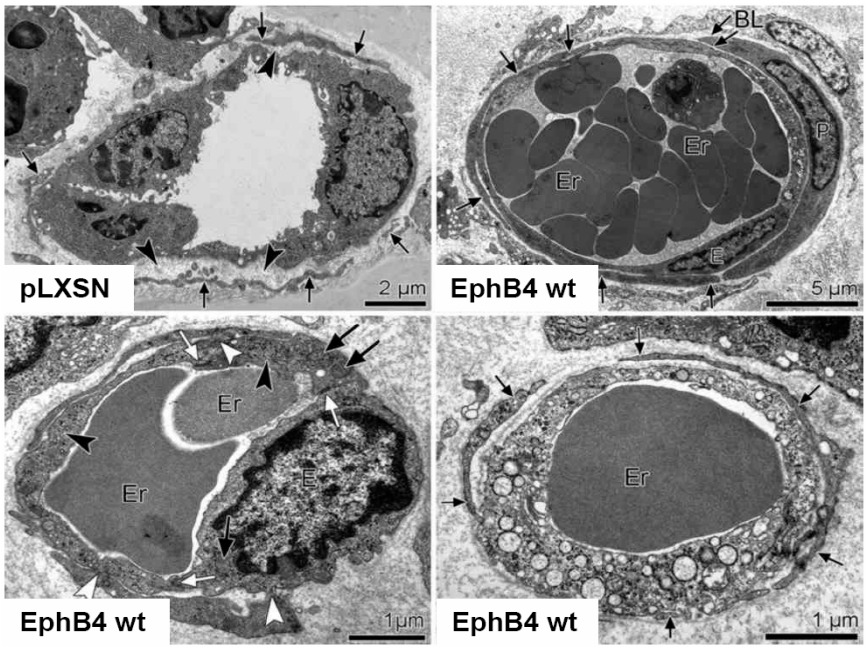


Fig. 8: Manipulation of endothelial EphB4 signaling confers reduced tumor blood vessel permeability via the Tie2/Ang axis. Endothelial – pericytic assembly in SF126 e- pLXSN tumors (top left) compared with SF126 e-EphB4 wt tumors revealed by transmission electron microscopy. Upper panel left: Small tumor microvessel of a SF126 e-pLXSN tumors rich on endothelial nuclei (E) covered by periendothelial cells (arrows). Arrowheads indicate loose association of pericytes to endothelial cells. Upper panel right: Cross section of enlarged tumorvessel demonstrating a regular endothelium (E) densely packed by pericytes (P). Arrows indicated strands of pericytic cytoplasm closely attached to the endothelium. A common basal lamina (BL) for the endothelium and pericytes is well-established. Lower panel left: Small capillaries contained numerous mitochondria (arrows) and profiles of free ribosomes and granular endoplasmic reticulum (arrowheads), referring to activated endothelium. White arrows indicated the endothelial to endothelial cell contacts and white arrowheads the intimate endothelial to pericytes cell contacts.Lower panel right: Newly formed, seamless capillaries contained abundant vesicles indicating enhanced transcytosis. Even these capillaries are tightly covered by pericytes (arrows). Er = Erythrocytes. Scale as indicated

In ephrinB2KO/KO and ephrinBDC/DC embryos reduced expression of angiopoietin-1 and the tie2 gene (while angiopoientin-2 remained unaffected) has been shown by Adams et al. (Adams et al. 2001), suggesting a requirement for Eph/ephrin signaling in endothelial/mesencymal cell interactions. Especially augmenting the cytoplasmic domain of ephrinB2 and reverse signalling. The retroviral transfection of vascular cells with EphB4 in our studies lead to an induction of ephrinB2 phosphorylation, therefore to an increased reverse signalling which might be responsible for the observed effect of increased Ang1 and Tie 2 expression in the tumors. We assume, that ephrinB2 signalling increased the expresson of Tie2 in endothelial cells, wheras the source of the observed increased levels of Ang1, as suggested by in-situ hybridization on e-EphB4 wt and e-EphB4 dn tumors, indicating perivascular localization of the message for Ang1, while Ang2 is restricted to endothelial cells (Fig. 9), will be mural cells or pericytes, also known to express ephrinB2. Ang1 is known to stabilize nascent vessels and make them leak-resistant, presumably by facilitating communication between ECs and mural cells, also the exact mechanisms are unclear yet.

Our study ultrastructural analysis has shown (Fig. 8) a tight association of pericytes to vascular EC in EphB4 transfected tumors, already at the level of capillaries. Suggesting a functional relationship between altered Eph/ephrin signalling the Ang1/Tie2 system and the observed phenotype of reduced vascular leakiness. These results indicate that endothelial EphB4 signaling regulates tumor blood vessel permeability by interfering with the balance between Ang-1 and Ang-2 expression and thereby the activation of the Tie2 receptor, leading to a stabilized vessels characterized by tightly associated pericytes, as hallmarks of mature and tightly sealed vessels.

The effects of EphB4 manipulation on tumor vessel morphogenesis and permeability are reproducible in other tumor models

To exclude that the impact of EphB4wt and EphB4dn expression on the tumor vasculature was specific for SF126 glioma cells, we investigated other human glioma cell lines for their vascular phenotype when co-implanted with our ecotropic EphB4wt and EphB4dn virus producer cell lines. Co-implantation with SF767 human glioma cells resulted in a vascular phenotype that was comparable to the one observed in SF126 tumors. Both, e-EphB4wt and e-EphB4dn tumors demonstrated an increase in vessel area

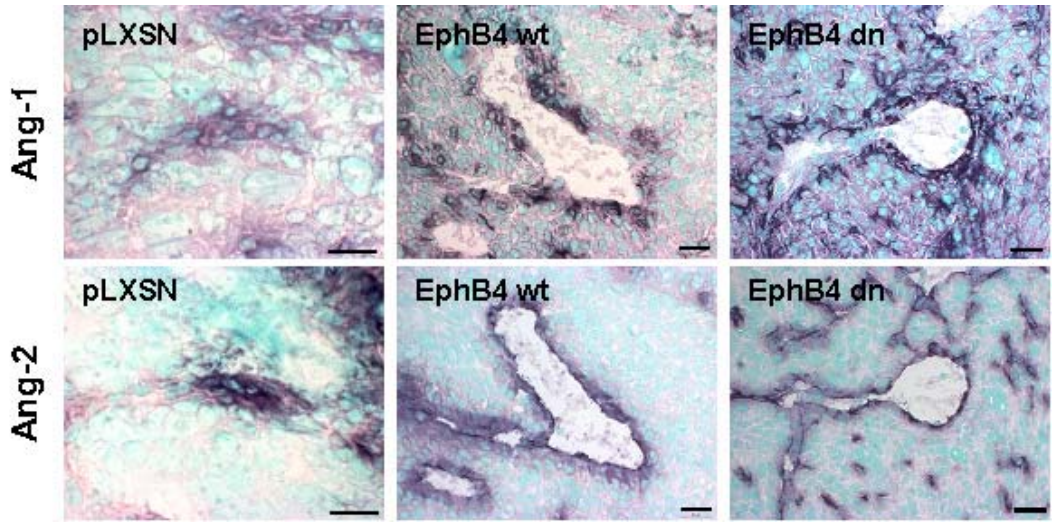


Fig. 9: Manipulation of endothelial EphB4 signaling confers reduced tumor blood vessel permeability via the Tie2/Ang axis. In situ hybridization of SF126 - e-pLXSN, e-EphB4 wt and e-EphB4 dn tumors for Ang-1 mRNA (upper panel) and Ang-2 (lower panel) demonstrating an localizaion of the Ang-1 mRNA increase in signal in perendothelial cells, whereas Ang-2 m-RNA expression was restricted to endothelial cells. Scale bar = 20µm.

densities due to an increase in vessel diameters (both $p<0.05$ versus control). Also, SF767 e-EphB4wt and e-EphB4dn tumors were characterized by a reduced vascular leakiness. Similar results as for the SF126 and SF767 glioma cell lines were obtained when implanting SF188 human glioma cells.

The effects of EphB4 signaling on vascular morphology are independent of tumor angiogenesis

Finally, we addressed the question whether the role for endothelial EphB4 in vessel morphogenesis and permeability is restricted to tumor angiogenesis. Therefore, we analyzed the vascular phenotype following implantation of non-neoplastic producer cell lines alone. In fact, intravital fluorescence videomicroscopy demonstrated that their implantation and vascularization would give rise to a similar vascular phenotype as observed for the xenografts with enlarged blood vessels and reduced vessel permeability.

To further confirm the notion that the relevance of EphB4 signaling for vascular morphogenesis and vascular organization may be generalized from tumor to postnatal vascularization, we examined the retinal vasculature of newborn mice after intravitreal injection of producer cells releasing the EphB4wt and control virus. The formation of the retinal vasculature represents an ideal model to study the postnatal development of blood vessels under physiological conditions.

In order to first characterize the behavior of our producer cells following intravitreal injection, we assessed the distribution of DiI-labeled producer cells. Fluorescence microscopic analysis revealed the presence of DiI-labeled cells superficial of the developing retina indicating an appropriate spatial distribution of the virus releasing cells (data not shown). Next, we assessed the vascular phenotype of the retinas at postnatal Day 15 (p15). While, the microvasculature of retinal digest preparations of eyes that had been injected with control cells appeared normal, large, malformed, and cell-rich vessels composing an irregular vascular patterning had developed in retinas of e-EphB4wt eyes (Fig. 10A and B). Small vessels with capillary-like appearance were absent. Consequently, endothelial overexpression of EphB4wt resulted in a significant enlargement of newly formed retinal blood vessels (Fig. 10C). In addition, e-EphB4wt retinas were characterized by an altered blood vessel branching resulting in a disorganized retinal angioarchitecture (Fig. 10D), thereby, mimicking the vascular phenotype that had been observed in e-EphB4wt tumors.

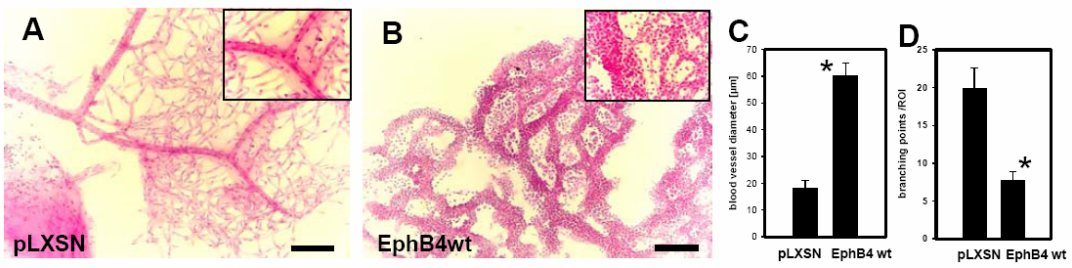


Fig. 10: The effects of EphB4 signaling on vascular morphology are independent of tumor angiogenesis. (A and B) Retinal digest preparations on Day p15, i.e. 10 Days following intravitreal injection of Phoenix E cells. Scale bar = 100µm, or as indicated in the insets of higher magnification. (C and D) Quantitative measurement of retinal blood vessel diameters (C) and vascular branching points (D). Values are represented as means ± SD. Number of animals per experimental group: e-pLXSN n=5; e-EphB4wt n=5. ∗ $p<0.05$ versus e-pLXSN.

In summary, ephrins and Eph receptors represent putative mediators of angiogenesis. In line with this we have demonstrated that ephrinB2 and EphB4 are overexpressed in human and experimental malignant brain tumors. Therefore, we set out to study the function of endothelial EphB4 signaling in tumor angiogenesis first. The fact that EphB4 was expressed by both tumor cells and endothelial cells necessitated a vessel specific transgenic approach, in contrast to previous work where EphB4 signaling was unspecifically inhibited by expression of soluble EphB4 (Martiny-Baron et al. 2004) or its manipulation was restricted to tumor cells (Noren et al. 2004). Endothelial overexpression of EphB4wt did not affect initial angiogenesis, as indicated by a regular vascular initiation of the tumors, but markedly affected vascular morphogenesis, as indicated by a switch from angiogenic sprouting to circumferential vessel growth. Furthermore, overexpression of EphB4wt reduced the leakiness of tumor blood vessels. The fact that overexpression of EphB4dn phenocopied the EphB4wt induced vascular changes demonstrated that these effects were independent of the EphB4 tyrosine kinase activity. Consequently, EphB4 reverse signaling through ephrinB2 represents the predominant signaling pathway in this context.

Moreover, the results of our study not only reveal a novel function for EphB4 during tumor vascularization, but also establish a novel concept in tumor biology by demonstrating that morphogenesis and organization of the postnatal vascular system are regulated by guidance molecules. So far, the vascular phenotype and the angioarchitecture of an individual tumor have been regarded as something re-active, i.e. the indirect result of an unregulated and chaotic tumor growth behavior. In contrast to this current notion, the present study now suggests that morphogenesis and organization of the tumor vascular system follow a pro-active, molecular program with EphB4 representing one mediator that regulates vessel branching and interconnection. If this holds true, vascularization of a tumor would be driven by two distinct molecular vascularization programs acting hand-in-hand, the vascular initiation program (driven by VEGF and other endothelial cell mitogens) and the vascular patterning program (driven by EphB4 and putatively other vascular guidance molecules). Although interference with the latter program may not directly translate into a successful anti-tumor strategy, a better understanding of this program and the underlying mechanisms may provide novel opportunities for the development of therapeutic strategies aimed at a ´vascular reprograming´ of tumors.

DISCUSSION

In Part I of the study we could demonstrate that selective interference with VEGFR-2 signalling fails to enforce regression of established tumor blood vessels. This resistance of tumor blood vessels to selective VEGFR-2 inhbition is confered by pericytes that (i) initially stabilize matured vessels, (ii) are secondarly recruited to immature vessels upon therapy, and (iii) compensatorily express endothelial cell survival factors (e.g. Ang-1). In contrast, inhibition of VEGFR-2 plus PDGFR-ß, which simultaneously targets endothelial cells and pericytes, acts as a potent anti-vascular strategy, inducing endothelial cell apoptosis, tumor vessel destabilization and regression, and finally tumor hypoxia. Beyond these therapeutic aspects, our study provides novel insights into the mechanisms of how pericytes differentially promote blood vessel stabilization and maturation via the Ang-1/Tie2 and PDGF-B/PDGFR-ß systems.

In contrast to the successful prevention of tumor angiogenesis and tumor growth, selective inhibition of VEGF/VEGFR-2 signalling using SU5416 failed to interfere with endothelial cell survival and to enforce regression of already established tumor blood vessels in the present study. This was unexpected, because VEGFR-2 not only mediates endothelial cell proliferation but also endothelial cell survival (Gerber et al. 1998) and VEGF withdrawal has been previously shown to ablate immature tumor vessels using a tetracycline-regulated expression system for VEGF (Benjamin and Keshet 1997). This resistance to vessel regression could in part be explained by the high maturation index of C6 tumor blood vessels (≈ 80%), defined as the fraction of vessels that are associated with pericytes.

The findings of our study are in agreement with a previous report on the beneficial effects of targeting endothelial cells and pericytes using SU6668 and SU5416 (Bergers et al. 2003). In this report, using the spontaneous pancreatic islet tumor model of RIPTag2 mice, the authors have demonstrated that a combined targeting of VEGF and PDGF signalling disrupts the association of pericytes with endothelial cells, reduces the tumor vascularity, and results in improved tumor control compared to targeting of VEGF signalling alone. However, the mechanisms of how pericytes protect endothelial cells from VEGFR-2 targeting have remained unclear.

Our study extended these previous findings and provides the cellular and molecular mechanisms of how pericytes may protect tumor endothelial cells during targeting of VEGF signalling. As demonstrated, vessel maturation at the initiation of treatment is not the sole determinant of resistance to enforced tumor blood vessel regression. Our study revealed at least two further mechanisms of how pericytes may confer resistance to targeted tumor blood vessels *in vivo*. First, pericytes were recruited to immature vessels and formed an intimate association with endothelial cells, thereby stabilizing these vessels through an enhanced cell-to-cell contact. Second, our expression analyses using RT-PCR and in situ hybridization suggested that the pro-apoptotic effect of selective VEGFR-2 targeting may be overwhelmed by the increased activity of endothelial cell survival factors other than VEGF. Interestingly, this strategy has recently been proposed as one potential mechanism of aquired resistance to anti-angiogenic therapies (Kerbel et al. 2001).

The Ang-1/Tie2 system which has been upregulated during SU5416 treatment seems to play a central role in both ways. On the one hand, Ang-1 overexpression enhances the pericyte coverage and increases the vessel maturation index in experimental tumors by a yet unknown mechanism (Hawighorst et al. 2002). On the other hand, Ang-1 dependent activation of Tie2 has been shown to exert similar anti-apoptotic properties as VEGF, acting as an alternative survival factor for endothelial cells via the same signalling cascade, i.e. PI3K and Akt.

Besides Ang-1/Tie2, the PDGF-B/PDGFR-β is the second important signalling system mediating pericyte-endothelial cell interaction. So far, it remains unknown how Ang-1/Tie2 and PDGF-B/PDGFR-β exactly interact in mediating vessel maturation. The phenotypic similarities of transgenic mice lacking either one of these signalling pathways suggests that they most likely act in concert in order to realize stabilization and maturation of newly formed blood vessels during vascular development (Sato et al. 1995), (Hellstrom et al. 2001). The massive vessel regression that we observed following VEGFR-2 plus PDGFR-β targeting indicates that this may also apply to tumor angiogenesis and that Ang-1/Tie2 alone, in the absence of PDGF-B/PDGFR-β signalling, is not sufficient to maintain endothelial cell survival and tumor vessel maturation.

It is important to note that combined targeting of VEGFR-2 and PDGFR-β had no effect on normal blood vessels of the host tissue. Intravital fluorescence videomicroscopy and electron microscopy have demonstrated that in contrast to the tumor vasculature the integrity and function of host blood vessels as well as their pericyte/endothelial cell interaction remained unaffected following treatment with SU6668.

Consequently, an extended anti-angiogenic strategy, targeting endothelial cells and pericytes, represents an attractive approach in treating patients presenting with advanced tumors with established blood vessels. Especially because many of the endothelial-specific anti-angiogenic compounds seem to be effective in rather preventing tumor initiation than in successfully interfering with the progressed tumor mass. The VEGFR-2 plus PDGFR-β inhibitor SU6668 represents one example of a potent polyvalent tyrosine kinase inhibitor that not only prevents further angiogenesis but also provides a potent anti-vascular efficacy even resulting in regression of selected experimental tumor types (Laird et al. 2000).

In summary, the results of this study suggest that a successful intervention in advanced tumors and long-term disease control with anti-angiogenic compounds can be best achieved with a combination

therapy, targeting not only endothelial cells but also pericytes. Therefore, besides selectively targeting VEGF/VEGFR-2 as the pivotal pathway for angiogenesis, additional pathways involved in vascular stabilization and maturation should be included into the target profile.

The results obtained in Part II of the study support a fundamental role for EphB4 signaling in postnatal blood vessel remodeling and morphogenesis. This function is independent of the EphB4 receptor tyrosine kinase activity, and therefore not related to EphB4 forward signaling. The results of our study establish a novel concept in vascular biology by demonstrating that morphogenesis and organization of the postnatal vascular system are regulated by vascular guidance molecules. This is especially surprising in the context of tumor angiogenesis, which has been regarded as being unregulated and chaotic so far. Intervention with the fucntion of these molecules in future may provide novel opportunities for the development of therapeutic strategies aimed at a ´vascular reprograming´ of neoplastic and non-neoplastic tissues.

We have demonstrated that ephrinB2 and EphB4 are overexpressed in human and experimental malignant brain tumors. Therefore, we set out to study the function of endothelial EphB4 signaling in tumor angiogenesis first. The fact that EphB4 was expressed by both tumor cells and endothelial cells necessitated a vessel specific transgenic approach, in contrast to previous work where EphB4 signaling was unspecifically inhibited by expression of soluble EphB4 (Martiny-Baron et al. 2004) or its manipulation was restricted to tumor cells (Noren et al. 2004). Endothelial overexpression of EphB4wt did not affect initial angiogenesis, as indicated by a regular vascular initiation of the tumors, but markedly affected vascular morphogenesis, as indicated by a switch from angiogenic sprouting to circumferential vessel growth. Furthermore, overexpression of EphB4wt reduced the leakiness of tumor blood vessels. The fact that overexpression of EphB4dn phenocopied the EphB4wt effect, suggests that, EphB4 reverse signaling through ephrinB2 represents the predominant signaling pathway in this context

Eph receptor family molecules are characterized by versatile functions in a wide range of morphogenetic processes (Adams 2002). Knockout mice lacking ephrinB2 showed severe defects in the vasculature (Adams et al. 1999): although primitive blood vessels were formed, the remodeling of the early vascular network into an organized network of small capillaries and larger vessels failed. A similar phenotype was described for EphB4-deficient mice (Gerety et al. 1999): embryos carrying mutations of either the ligand or receptor showed a normal initial formation of the vasculature, but revealed severe defects in the later process of vascular remodeling and network formation.

Similar to these observations in the developing embryo, alteration of EphB4 signaling in tumor endothelial cells did not affect initial tumor vessel formation, but markedly affected subsequent morphogensis and remodeling of the tumor vascular system. However, it was also of interest to note that the phenomenon of reduced vascular leakiness was reminiscent of the phenotype of Ang-1 overexpressing mice (Thurston et al. 1999) suggesting a molecular link between ephrinB2/EphB4 and the vessel stabilizing Ang-1/Tie2 system. In line with this hypothesis, both e-EphB4wt tumors and e-EphB4dn tumors showed an increased Ang-1 expression and a reduced Ang-2 expression, leading to an inversed Ang-1/Ang-2 ratio.

Our studies have revealed that activation of EphB4 reverse signaling via ephrinB2 results in a fundamental switch in the vascularization program of the tumor. Under physiological conditions, vascularization and remodeling of the initial vascular system is primarily dependent on angiogenic sprouting. In contrast, activation of EphB4 reverse signaling results in a defective angiogenic sprout formation and vessel interconnection due to an inability of individual endothelial cells to leave the context of the main vascular tree. Instead, tumor endothelial cells proliferate within the vessel wall leading to circumferential growth of the initial vascular tree. In addition to their inability to leave the vessel wall, endothelial cells are characterized by an increased proliferative activity which is in line with the previous observation that activation of EphB4 reverse signaling may exert a mitogenic activity (Masood et al.

2005), (Zhang et al. 2001). Interestingly, a similar effect on blood vessel diameters was observed in transgenic mice, overexpressing ephrinB2 specifically in endothelial cells under the control of the Tie2 promoter (Oike et al. 2002).

The results of our study not only reveal a novel function for EphB4 during tumor vascularization, but also establish a novel concept in tumor biology by demonstrating that morphogenesis and organization of the postnatal vascular system are regulated by guidance molecules. So far, the vascular phenotype and the angioarchitecture of an individual tumor have been regarded as something re-active, i.e. the indirect result of an unregulated and chaotic tumor growth behavior. In contrast to this current notion, the present study now suggests that morphogenesis and organization of the tumor vascular system follow a pro-active, molecular program with EphB4 representing one mediator that regulates vessel branching and interconnection. If this holds true, vascularization of a tumor would be driven by two distinct molecular vascularization programs acting hand-in-hand, the vascular initiation program (driven by VEGF and other endothelial cell mitogens) and the vascular patterning program (driven by EphB4 and putatively other vascular guidance molecules). Although interference with the latter program may not directly translate into a successful anti-tumor strategy, a better understanding of this program and the underlying mechanisms may provide novel opportunities for the development of therapeutic strategies aimed at a ´vascular reprograming´ of tumors.

REFERENCES

Adams RH: Vascular patterning by Eph receptor tyrosine kinases and ephrins. *Semin Cell Dev Biol* 13: 55-60, 2002.

Adams RH, Diella F, Hennig S, Helmbacher F, Deutsch U, Klein R: The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* 104: 57-69, 2001.

Adams RH, Wilkinson GA, Weiss C, et al: Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. *Genes Dev* 13: 295-306, 1999.

Bange J, Prechtl D, Cheburkin Y, et al: Cancer progression and tumor cell motility are associated with the FGFR4 Arg(388) allele. *Cancer Res* 62: 840-847, 2002.

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E: Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 103: 159-165, 1999.

Benjamin LE, Hemo I, Keshet E: A plasticity window for blood vessel remodelling is defined by pericyte coverage of the preformed endothelial network and is regulated by PDGF-B and VEGF. *Development* 125: 1591-1598, 1998.

Benjamin LE, Keshet E: Conditional switching of vascular endothelial growth factor (VEGF) expression in tumors: induction of endothelial cell shedding and regression of hemangioblastoma-like vessels by VEGF withdrawal. *Proc Natl Acad Sci USA* 94: 8761-8766, 1997.

Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D: Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest* 111: 1287-1295, 2003.

Brem S, Tsanaclis AM, Gately S, Gross JL, Herblin WF: Immunolocalization of basic fibroblast growth factor to the microvasculature of human brain tumors. *Cancer* 70: 2673-2680, 1992.

Daniel TO, Stein E, Cerretti DP, St John PL, Robert B, Abrahamson DR: ELK and LERK-2 in developing kidney and microvascular endothelial assembly. *Kidney Int Suppl* 57: S73-81, 1996.

Easty DJ, Hill SP, Hsu MY, et al: Up-regulation of ephrin-A1 during melanoma progression. *Int J Cancer* 84: 494-501, 1999.

Erber R, Eichelsbacher U, Powajbo V, et al: EphB4 controls blood vascular morphogenesis during postnatal angiogenesis. *Embo J* 25: 628-641, 2006.

Erber R, Thurnher A, Katsen AD, et al: Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. *Faseb J* 18: 338-340, 2004.

Farhadi MR, Capelle HH, Erber R, Ullrich A, Vajkoczy P: Combined inhibition of vascular endothelial growth factor and platelet-derived growth factor signaling: effects on the angiogenesis, microcirculation, and growth of orthotopic malignant gliomas. *J Neurosurg* 102: 363-370, 2005.

Flanagan JG, Vanderhaeghen P: The ephrins and Eph receptors in neural development. *Annu Rev Neurosci* 21: 309-345, 1998.

Gale NW, Yancopoulos GD: Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev* 13: 1055-1066, 1999.

Gerber HP, McMurtrey A, Kowalski J, et al: Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation. *J Biol Chem* 273: 30336-30343, 1998.

Gerety SS, Wang HU, Chen ZF, Anderson DJ: Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. *Mol Cell* 4: 403-414, 1999.

Gordon MS: Vascular endothelial growth factor as a target for antiangiogenic therapy. *J Clin Oncol* 18: 45S-46S, 2000.

Guha A: Platelet-derived growth factor: a general review with emphasis on astrocytomas. *Pediatr Neurosurg* 17: 14-20, 1991.

Hawighorst T, Skobe M, Streit M, et al: Activation of the tie2 receptor by angiopoietin-1 enhances tumor vessel maturation and impairs squamous cell carcinoma growth. *Am J Pathol* 160: 1381-1392, 2002.

Helbling PM, Saulnier DM, Brandli AW: The receptor tyrosine kinase EphB4 and ephrin-B ligands restrict angiogenic growth of embryonic veins in Xenopus laevis. *Development* 127: 269-278, 2000.

Hellstrom M, Gerhardt H, Kalen M, et al: Lack of pericytes leads to endothelial hyperplasia and abnormal vascular morphogenesis. *J Cell Biol* 153: 543-553, 2001.

Herbst RS, Lee AT, Tran HT, Abbruzzese JL: Clinical studies of angiogenesis inhibitors: the University of Texas MD Anderson Center Trial of Human Endostatin. *Curr Oncol Rep* 3: 131-140, 2001.

Hermansson M, Nister M, Betsholtz C, Heldin CH, Westermark B, Funa K: Endothelial cell hyperplasia in human glioblastoma: coexpression of mRNA for platelet-derived growth factor (PDGF) B chain and PDGF receptor suggests autocrine growth stimulation. *Proc Natl Acad Sci USA* 85: 7748-7752, 1988.

Holder N, Klein R: Eph receptors and ephrins: effectors of morphogenesis. *Development* 126: 2033-2044, 1999.

Kerbel RS, Yu J, Tran J, et al: Possible mechanisms of acquired resistance to anti-angiogenic drugs: implications for the use of combination therapy approaches. *Cancer Metastasis Rev* 20: 79-86, 2001.

Kerger H, Groth G, Kalenka A, Vajkoczy P, Tsai AG, Intaglietta M: pO(2) measurements by phosphorescence quenching: characteristics and applications of an automated system. *Microvasc Res* 65: 32-38, 2003.

Kim KJ, Li B, Winer J, et al: Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature* 362: 841-844, 1993.

Laird AD, Vajkoczy P, Shawver LK, et al: SU6668 is a potent antiangiogenic and antitumor agent that induces regression of established tumors. *Cancer Res* 60: 4152-4160, 2000.

Martiny-Baron G, Korff T, Schaffner F, et al: Inhibition of Tumor Growth and Angiogenesis by Soluble EphB4. *Neoplasia* 6: 248-257, 2004.

Masood R, Xia G, Smith DL, et al: Ephrin B2 expression in Kaposi sarcoma is induced by human herpesvirus type 8: phenotype switch from venous to arterial endothelium. *Blood* 105: 1310-1318, 2005.

McBride JL, Ruiz JC: Ephrin-A1 is expressed at sites of vascular development in the mouse. *Mech Dev* 77: 201-204, 1998.

Mignatti P, Mazzieri R, Rifkin DB: Expression of the urokinase receptor in vascular endothelial cells is stimulated by basic fibroblast growth factor. *J Cell Biol* 113: 1193-1201, 1991.

Millauer B, Shawver LK, Plate KH, Risau W, Ullrich A: Glioblastoma growth inhibited in vivo by a dominant-negative Flk-1 mutant. *Nature* 367: 576-579, 1994.

Montesano R, Vassalli JD, Baird A, Guillemin R, Orci L: Basic fibroblast growth factor induces angiogenesis in vitro. *Proc Natl Acad Sci USA* 83: 7297-7301, 1986.

Morrison RS, Yamaguchi F, Bruner JM, Tang M, McKeehan W, Berger MS: Fibroblast growth factor receptor gene expression and immunoreactivity are elevated in human glioblastoma multiforme. *Cancer Res* 54: 2794-2799, 1994.

Moscatelli D, Presta M, Rifkin DB: Purification of a factor from human placenta that stimulates capillary endothelial cell protease production, DNA synthesis, and migration. *Proc Natl Acad Sci USA* 83: 2091-2095, 1986.

Murphy PR, Sato Y, Sato R, Friesen HG: Regulation of multiple basic fibroblast growth factor messenger ribonucleic acid transcripts by protein kinase C activators. *Mol Endocrinol* 2: 1196-1201, 1988.

Nelson NJ: Angiogenesis research is on fast forward. *J Natl Cancer Inst* 91: 820-822, 1999.

Noren NK, Lu M, Freeman AL, Koolpe M, Pasquale EB: Interplay between EphB4 on tumor cells and vascular ephrin-B2 regulates tumor growth. *Proc Natl Acad Sci USA* 101: 5583-5588, 2004.

Ogawa K, Pasqualini R, Lindberg RA, Kain R, Freeman AL, Pasquale EB: The ephrin-A1 ligand and its receptor, EphA2, are expressed during tumor neovascularization. *Oncogene* 19: 6043-6052, 2000.

Oike Y, Ito Y, Hamada K, et al: Regulation of vasculogenesis and angiogenesis by EphB/ephrin-B2 signaling between endothelial cells and surrounding mesenchymal cells. *Blood* 100: 1326-1333, 2002.

Pandey A, Shao H, Marks RM, Polverini PJ, Dixit VM: Role of B61, the ligand for the Eck receptor tyrosine kinase, in TNF-alpha-induced angiogenesis. *Science* 268: 567-569, 1995.

Plate KH: Gene therapy of malignant glioma via inhibition of tumor angiogenesis. *Cancer Metastasis Rev* 15: 237-240, 1996.

Plate KH, Breier G, Farrell CL, Risau W: Platelet-derived growth factor receptor-beta is induced during tumor development and upregulated during tumor progression in endothelial cells in human gliomas. *Lab Invest* 67: 529-534, 1992

Plate KH, Breier G, Millauer B, Ullrich A, Risau W: Up-regulation of vascular endothelial growth factor and its cognate receptors in a rat glioma model of tumor angiogenesis. *Cancer Res* 53: 5822-5827, 1993.

Samoto K, Ikezaki K, Ono M, et al: Expression of vascular endothelial growth factor and its possible relation with neovascularization in human brain tumors. *Cancer Res* 55: 1189-1193, 1995.

Sato TN, Tozawa Y, Deutsch U, et al: Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376: 70-74, 1995.

Shin D, Garcia-Cardena G, Hayashi S, et al: Expression of ephrinB2 identifies a stable genetic difference between arterial and venous vascular smooth muscle as well as endothelial cells, and marks subsets of microvessels at sites of adult neovascularization. *Dev Biol* 230: 139-150, 2001.

Stan AC, Nemati MN, Pietsch T, Walter GF, Dietz H: In vivo inhibition of angiogenesis and growth of the human U-87 malignant glial tumor by treatment with an antibody against basic fibroblast growth factor. *J Neurosurg* 82: 1044-1052, 1995.

Stefanik DF, Rizkalla LR, Soi A, Goldblatt SA, Rizkalla WM: Acidic and basic fibroblast growth factors are present in glioblastoma multiforme. *Cancer Res* 51: 5760-5765, 1991.

Stein E, Lane AA, Cerretti DP, et al: Eph receptors discriminate specific ligand oligomers to determine alternative signaling complexes, attachment, and assembly responses. *Genes Dev* 12: 667-678, 1998.

Stratmann A, Acker T, Burger AM, Amann K, Risau W, Plate KH: Differential inhibition of tumor angiogenesis by tie2 and vascular endothelial growth factor receptor-2 dominant-negative receptor mutants. *Int J Cancer* 91: 273-282, 2001.

Strawn LM, Mann E, Elliger SS, et al: Inhibition of glioma cell growth by a truncated platelet-derived growth factor-beta receptor. *J Biol Chem* 269: 21215-21222, 1994.

Strawn LM, McMahon G, App H, et al: Flk-1 as a target for tumor growth inhibition. *Cancer Res* 56: 3540-3545, 1996.

Stupp R, Mason WP, van den Bent MJ, et al: Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *N Engl J Med* 352: 987-996, 2005.

Tang XX, Brodeur GM, Campling BG, Ikegaki N: Coexpression of transcripts encoding EPHB receptor protein tyrosine kinases and their ephrin-B ligands in human small cell lung carcinoma. *Clin Cancer Res* 5: 455-460, 1999.

Thomas KA: Fibroblast growth factors. *Faseb J* 1: 434-440., 1987.

Thurston G, Suri C, Smith K, et al: Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* 286: 2511-2514, 1999.

Tsai JC, Goldman CK, Gillespie GY: Vascular endothelial growth factor in human glioma cell lines: induced secretion by EGF, PDGF-BB, and bFGF. *J Neurosurg* 82: 864-873, 1995.

Vajkoczy P, Lehr HA, Hubner C, Arfors KE, Menger MD: Prevention of pancreatic islet xenograft rejection by dietary vitamin E. *Am J Pathol* 150: 1487-1495, 1997.

Vajkoczy P, Schilling L, Ullrich A, Schmiedek P, Menger MD: Characterization of angiogenesis and microcirculation of high-grade glioma: an intravital multifluorescence microscopic approach in the athymic nude mouse. *J Cereb Blood Flow Metab* 18: 510-520, 1998.

Vajkoczy P, Menger MD: Vascular microenvironment in gliomas. *J Neurooncol* 50: 99-108, 2000.

Vajkoczy P, Blum S, Lamparter M, et al: Multistep nature of microvascular recruitment of ex vivo-expanded embryonic endothelial progenitor cells during tumor angiogenesis. *J Exp Med* 197: 1755-1765, 2003.

Vajkoczy P, Menger MD, Vollmar B, et al: Inhibition of tumor growth, angiogenesis, and microcirculation by the novel Flk-1 inhibitor SU5416 as assessed by intravital multi-fluorescence videomicroscopy. *Neoplasia* 1: 31-41, 1999a.

Vajkoczy P, Goldbrunner R, Farhadi M, et al: Glioma cell migration is associated with glioma-induced angiogenesis in vivo. *Int J Dev Neurosci* 17: 557-563, 1999b.

Vajkoczy P, Vollmar B, Wolf B, Menger MD: Effects of cyclosporine A on the process of vascularization of freely transplanted islets of Langerhans. *J Mol Med* 77: 111-114, 1999c.

Vajkoczy P, Thurnher A, Hirth KP, et al: Measuring VEGF-Flk-1 activity and consequences of VEGF-Flk-1 targeting in vivo using intravital microscopy: clinical applications. *Oncologist* 5 Suppl 1: 16-19, 2000.

Vajkoczy P, Menger MD, Goldbrunner R, et al: Targeting angiogenesis inhibits tumor infiltration and expression of the pro-invasive protein SPARC. *Int J Cancer* 87: 261-268, 2000a.

Vajkoczy P, Laschinger M, Engelhardt B: Alpha4-integrin-VCAM-1 binding mediates G protein-independent capture of encephalitogenic T cell blasts to CNS white matter microvessels. *J Clin Invest* 108: 557-565, 2001

Vajkoczy P, Farhadi M, Gaumann A, et al: Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. *J Clin Invest* 109: 777-785, 2002.

Wang HU, Chen ZF, Anderson DJ: Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. *Cell* 93: 741-753, 1998.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J: Vascular-specific growth factors and blood vessel formation. *Nature* 407: 242-248, 2000.

Zagzag D, Miller DC, Sato Y, Rifkin DB, Burstein DE: Immunohistochemical localization of basic fibroblast growth factor in astrocytomas. *Cancer Res* 50: 7393-7398, 1990.

Zhang XQ, Takakura N, Oike Y, et al: Stromal cells expressing ephrin-B2 promote the growth and sprouting of ephrin-B2(+) endothelial cells. *Blood* 98: 1028-1037, 2001.

Angiopoietin/Tie signaling

Contribution of the Tie/Angiopoietin Receptor Ligand System to Growth, Morphology and Function of Blood Vessels in Adult Mice

Dr. Urban Deutsch

present address:
Theodor-Kocher-Institute
University of Bern
Freiestr. 1, CH-3012 Bern, Switzerland

formerly:
Max-Planck-Institute for Physiological and Clinical Research, Bad Nauheim (until 6/2001)
Max-Planck-Institute for Molecular Biomedicine, Münster (until 12/2003)

Member of the SPP 1069 from 2001 until 2006

SUMMARY

We have established an endothelial cell-specific tetracycline-inducible expression system in transgenic mice to analyse the role of Angiopoietins in the adult vascular system. To this end, we have employed Tie-2 regulatory sequences demonstrated to direct expression into virtually all endothelial cell of mouse embryos and during adulthood. Tet-activator mice were raised for multiple versions of transactivator proteins (TAs) with the classical tTA (TET-OFF) and the optimised rtTA2-M2 (TET-ON) variants being the most effective. These systems work well during embryonic development up to early postnatal phases and exhibit differential activity in different adult organs (tTA) or are inactive in adult tissues (rtTA2-M2). Different types of responder constructs have been used to raise several series of responder mouse lines, of which simple unidirectional constructs are most reliable. Co-injection of activator constructs with insulator flanked responder constructs reduced the complexity of the breeding scheme but show less continuous expression in endothelial cells and are thus not generally recommendable. Using Tie2-tTA mouse lines reliable expression of both reporter genes as well as a number of responder genes could be achieved. For example, tet-induced expression of E-selectin protein was demonstrated to occur at similar levels as on endothelium activated by inflammatory mediators. Induced responder gene expression in Tie2-tTA double transgenic mice is endothelial cell (EC) selective and can be completed suppressed be feeding Doxycycline (Dox). Conversely, responder gene expression in Tie2-rtTA2-M2 double transgenic mice is EC-specifically induced upon application of Dox and completely lacking, when Dox is absent. To investigate the role of Angiopoietins in adult mice, we established multiple inducible Ang-1 and Ang-2 transgenic mouse lines. Double transgenic inducible Ang-1 mice do not show any conspicuous phenotypic alterations in the absence of additional challenge. However, in double transgenic inducible Ang-2 mice of a particular line, vascular changes are readily apparent starting at about 3 – 4 months of age. These mice show increases in blood vessel size leading to red appearance of their ears. With increasing age, these animals develop reddish swollen faces. The spleens of double transgenic mice grow to 4 times the size of that of single transgenic mice, heart size is increased by about 60%. Generally, chronic expression of moderate levels of Ang-2 appears to cause myocardial hyperplasia, splenomegaly and hallmarks of inflammation in at least some organs.

INTRODUCTION

Gene targeting studies have shown that VEGF-A and its receptors Flk-1 and Flt-1 are essential for endothelial cell lineage specification, differentiation and blood vessel formation. Reduced oxygen levels present in tissues, or at early embryological stages, determine neovascularization via an increase in the production of VEGF, a factor that exerts lineage determining effects on angioblasts and mitogenic and chemotactic effects on differentiated endothelial cells and permeability changes in established blood vessels. The enormous complexity of blood vessel morphology reveals that regulation of endothelial cell growth is not sufficient to create the vasculature of adult vertebrates. Obviously, additional signals are required to produce the complex architecture of a mature vascular tree (Risau, 1997).

Other genes with embryonic lethal phenotypes, include the receptor tyrosine kinases Tie-1 and Tie-2 as well as the Tie-2 ligand Angiopoietin-1, molecular components of the TGFβ signalling pathways, members of the EphB-receptor / ephrinB-ligand family such as EphB4 and ephrinB2 and also select members of the Notch receptor / jagged and Delta-like ligand families (for review see (Carmeliet, 2000; Gale and Yancopoulos, 1999; Yancopoulos et al., 2000)). Many of these gene products have multiple differerent activities in development and adulthood, depending on the specific vascular bed and during pathological situations.

The endothelial cell specific receptor tyrosine kinases, Tie-1 and Tie-2 govern processes involved in endothelial cell sprouting, remodelling of primitive vascular networks and blood vessel maturation and integrity. Tie-2 and its angiopoietin ligands (Ang-1 and Ang-2), are involved in the regulation of angiogenic sprouting, vascular remodelling and recruitment of perivascular during embryonic development. Ang-1 and Ang-2 (as well as Ang-3) are factors, which exhibit agonistic and antagonistic actions upon Tie-2 receptor phosphorylation, respectively. Ang-1, -2 and –3, all bind to Tie-2. Ang-1 binding leads to activation of the Tie-2 kinase, whereas Ang-2 and Ang-3 do not induce tyrosine phosphorylation of Tie-2 and instead rather compete with Ang-1 binding and thereby downregulate Ang-1 induced phosphorylation (Maisonpierre et al., 1997). Structure/ function analyses revealed the multimeric structure of the Angiopoietins and their Tie-2 binding and activation behavior. The minimal functional units of Ang-1 and Ang-2 are dimers, with tetramers and hexamers being the predominant multimers (Davis et al., 2003). The C-terminal fibrinogen-like domains of Ang-1 and Ang-2 carry the information for their distinct activities. A perplexing feature of Ang-2 function however, is that in cell culture the antagonistic action is observed in endothelial cells but not Tie-2 transfected fibroblastic cell lines, in which Ang-2 readily induces Tie-2 phosphorylation. It is still unknown what accounts for this difference. Ang-1 is initially expressed mainly in the developing heart and is later found in most perivascular cells. In contrast, Ang-2 is expressed in larger vessels and in areas of ongoing tissue remodelling or regression.

The Ang-1 mutant phenotype resembles that of Tie-2 regarding defects in sprouting and vascular remodelling (Sato et al., 1995; Suri et al., 1996). Furthermore, endothelial overexpression of Ang-2 in transgenic embryos leads to defects similar to the Tie-2 knock-out phenotype likely because Ang-2 causes constitutive inhibition of Tie-2 activity (Maisonpierre et al., 1997). Ang-2 null mice were reported not to survive beyond about three weeks of age, when they are edematous and hemorrhagic and suffer from a chylus ascites, which is the results of a defective lymphatic vasculature (Gale et al., 2002). Furthermore, in Ang-2 null mice the hyaloid vasculature that normally regresses in a stereotpyed fashion, consistently persists. However, in Ang-2 null mice backcrossed to the C57Bl/6 inbred strain, early postnatal lethality is no longer observed. In gene targeted mice, in which the first exon of the Ang-2 gene is replaced by the Ang-1 coding sequence, the defect in lymphatic vessel development is rescued, while the hyaloid vasculature still fails to regress (Gale et al., 2002). These results have been interpreted as evidence for an agonistic role of Ang-2 in

lymphatic endothelium (as opposed to an antagonistic function in vascular endothelium). There is still no explanation for the molecular differences that could account for an agonistic function of Ang-2 on lymphatic endothelium.

Some aspects of the Ang-2 phenotype are reminiscent of that of Tie-1 deficient mice, which die between E13.5 and birth from the consequences of generalized edema formation (Sato et al., 1995). Furthermore, in an analysis determining the cell autonomous function of Tie-1 it became apparent that Tie-1 is required for angiogenic sprouting at later developmental stages (at E15.5) (Partanen et al., 1996). More importantly, embryos lacking both Tie-1 and Tie-2 exhibit a phenotype even more severe than observed in the Tie-2 deficiency. These data strongly suggest that Tie-1 and –2 functionally interact and are mutually dependent on their functions (Puri et al., 1999).

Angiopoietins also affect blood vessel morphology and function in the adult organism. In transgenic mice that have been engineered to express Ang-1 in the skin, mild hypervascularity as well as an increased resistance toward vascular permeability were observed (Thurston et al., 1999). A similar result was obtained by adenoviral gene transfer of Ang-1. In various settings of tumor progression, Ang-2 is an early marker for angiogenic endothelium in the tumor. Its expression is associated with the loss of α-SMA from the vessel wall presumed to lead to destabilisation as well as regression of these vessels in the absence of survival factors such as VEGF (Holash et al., 1999). Based on these sets of findings, it is now believed that Ang-1 and Ang-2 conversely regulate SMC investment and thus the later fate of mature blood vessels depending on the presence or absence of additional factors such as VEGF or PDGF-B.

Even though many of the molecular components involved in blood vessel formation have now been identified, their exact mechanisms of action are still not fully understood. Furthermore, it has become increasingly clear that many of the pathways, in which these molecules participate, functionally intersect and influence each other in multiple ways. However, the biggest obstacle to the understanding of the roles of those proteins is the fact that mutations in their genes invariably lead to lethal phenotpyes during early embryogenesis or before the animals reach adulthood. Therefore, in vivo analysis of their contribution during adulthood as well as in settings of pathological alterations of the circulatory system was precluded. This obstacle for genetic analysis in adult mice can only be overcome by the use of inducible gene expression systems.

The most frequently used strategy for inducible gene expression in transgenic animals utilizes the so-called tetracycline-inducible expression system initially developed by Bujard and colleagues. This system is based on a tetracycline-regulatable transcriptional activator that can bind to and initiate transcription from tet-operator containing promoters. In the original (TET-OFF) version of the system, DNA-binding and thus activity of this artificial transcription factor is abolished by addition of tetracyline-analogues (Gossen and Bujard, 1992). A reverse system was also developed based on a mutated tet-repressor that exhibits a reverse tetracycline-modulated DNA-binding phenotype. In this TET-ON system, Doxycyline treatment induces DNA-binding and thereby gene expression. Most successful applications have utilized the TET-OFF system. The relatively low frequency of cases, in which the TET-ON system utilizing the rtTA gene was successfully used, is most likely due to inadequate expression levels of rtTA protein. Apparently, this problem can be at least partially overcome by adjusting the rtTA open reading frame sequence to human codon bias, by removing cryptic splice sites within the ORF and by replacing the VP16 transactivation domain by artificial activation domains comprized of peptide repeats. The most recently created version of the rtTA cassette (rtTA2S-M2) incorporates all these modifications (Urlinger et al., 2000).

Several practical advantages favor the use of a TET-ON system over that of a TET-OFF system. Most importantly, for inducible expression of a potentially toxic or harmful gene product, the TET-OFF system requires permanent feeding of transgenic mice with the Tetracycline analogue

Doxycycline (Dox) to maintain the transgene in the uninduced state. Prolonged feeding of Dox leads to accumulation of the antibiotic in tissues and requires long periods of Dox withdrawal for gene induction. Instead, the TET-ON system allows not only for rapid induction after Dox treat-ment, but also for local induction by the topical application of Doxycycline at the required organ or tissue site.

METHODS

The technical expertise employed during this study focuses on transgenic mouse technology, standard molecular biology and phenotypic analyses by histological and immunohistochemical techniques.

Constructs for the production of TET-responder and activator mice were produced by standard DNA cloning techniques and their identity and integrity verified by restriction analysis and sequencing. Transgenic mice were produced by pronuclear injection of different types of constructs after removal of the plasmid vector backbone or linearization. Injections were performed into fertilized oocytes from C57BL/6 // C3H hybrid females at the MPI in Bad Nauheim. Founder animals were identified by PCR genotyping and transgenic lines were raised after successful breeding. Functionality of activator mice was tested by breeding to reporter mouse strains.

Responder lines for inducible expression of Angiopoietin-1 and –2 were bred to Tie-2 activator mice to produce double transgenic offspring, which were analysed for obvious phenotypic alterations. Phenotypic analysis is by macroscopic inspection, histological evaluation, antibody staining for both vascular and lymphatic endothelial and hematopoietic cell markers and ultrasctructural analysis via transmission electron microscopy (in collaboration with Hartwig Wolburg, Tübingen).

RESULTS

In an early phase of this project, the prominent goal was to establish an endothelial cell-specific tetracycline-inducible system using transcriptional control elements of the Tie-2 gene that we had previously characterized in detail (Schlaeger et al., 1997). A 2 kB upstream fragment from the mouse Tie-2 gene in combination with a 10 kB fragment from the large first intron of the Tie-2 gene is sufficient to drive LacZ reporter gene expression into virtually all endothelial cell of the mouse embryo and adult mouse organs. Based on these regulatory sequences, we consecutively created transgene constructs and transgenic founder mice carrying several types of tet-activators (Fig. 1 A). For reasons of practicabilty, we started with the reverse tetracycline inducible transcriptional activator (rtTA). These lines were functionally tested by breeding with an established LacZ reporter mouse line, called nZL2 (provided by Hermann Bujard; Fig. 1 B: Luc-TRE-LacZ). None of the 12 lines established showed detectable reporter activity, when bred to the nZL2 line. Next, we produced 5 additional lines with a construct for an improved version of rtTA (called rtTA-FFF), in which the potentially toxic VP16 transactivating domain (TAD) was replaced by a triple repeat of an octapeptide with strong transactivating potential. One out of 5 lines raised show very weak endothelial expression (data not shown). Furthermore, we hypothezised that the nZL2 reporter line might not be suitable for induced expression in endothelial cells. To exclude this possibility, we raised independent reporter mice using a TRE-LacZ construct, which contained the minimal promoter of the endothelial cell-specifically expressed Tie-2 gene. Meanwhile, it had become apparent from several published studies that the original tetracycline inducible transcriptional activator (tTA) was functional in the context of different types of regulatory sequences, while rtTA was rarely found to work in a transgenic setting. Therefore, we decided to raise lines with the tTA open reading frame inserted between the Tie-2 elements. From 6 lines raised we could demonstrate strong induced endothelial expression with three independent lines in E11.5 day mouse embryos, when bred to nZL2 or one of the newly raised reporter lines (# 2717) containing the Tie-2 minimal promoter.Finally, we decided to raise yet

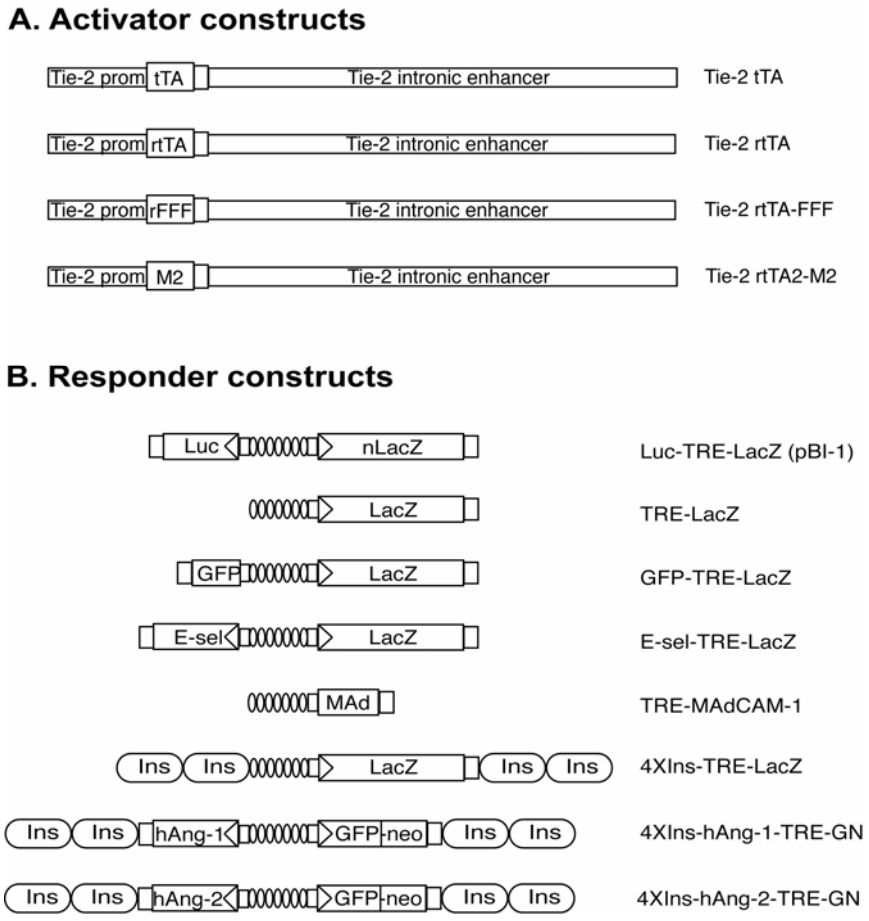


Fig. 1: TET-System Constructs

A. Activator Constructs:
2 kB Tie2 promoter upstream of activator open reading frames, followed by SV40pA addition signal, flanked by 10 kB Tie2 intronic enhancer fragment.
tTA: tet-inducible transciptional activator; rtTA: reverse tTA; rtTA–FFF: VP16 TAD replaced by repeat of activating peptide; rtTA2–M2: improved rtTA.

B. Responder Constructs:
TRE: TET-response element; Luc: luciferase; LacZ: β-galactosidase ORF; n: nuclear; GFP: enhanced green fluoresecent protein;
E-sel: E-selectin; MAd: MAdCAM-1;
Ins: chicken β-globin insulator; hAng-1: human Angiopoietin-1 ORF;
hAng–2: human Angiopoietin-2 ORF;

an additional series of activator mouse lines using a further improved version of rtTA, called rtTA2-M2, in which codon usage was adjusted to human codon bias, cryptic splice sites has been removed and repeat sequences altered to avoid loss by recombination. From 12 transgenic mouse lines raised using the Tie2-rtTA2-M2 construct, 3 showed endothelial cell-specific expression in mouse embryos upon application of Doxycycline. To date, we have not been able to demonstrate inducible expression in adult double transgenic Tie2-rtTA2-M2 mice.

We further characterized the functional Tie2-tTA mouse lines for inducible expression in the endothelia of adult organs. Using various responder mouse lines (nZL2 or E-sel-TRE-LacZ #2899) it became apparent that LacZ activity was not equally complete in different vascular beds. While a large proportion of vessels in brain and tongue show reporter activity, expression in splenic and intestinal vessels was sparse.

Table 2: Tet-Responder Mice

Responder Constructs	F_0	No. of lines ind. *in vitro*	Lines est.	ID. No. of lines maintained	Endothelial inducibility	
					Embryonic	Adult
nZL2 * (bidirectional nLacZ + Luciferase)	-	n.a.	-	3564	yes	yes
GFPtetO$_7$LacZ ◊	-	n.a.	-	n.a.	n.d.	n.d.
TRE BH LacZ (LacZ indicator w./ Tie-2 min. prom.)	17	n.d.	8	2717	yes	yes
TRE-MAdCAM (mMAdCAM-1)	5	n.d.	4	17910 19814	n.d.	yes yes
4XIns hAng-1-TRE-GFPneo	19	n.d.	15		n.d.	n.d.
4XIns hAng-2-TRE-GFPneo	17	n.d.	8	16772	n.d. (viable)	n.d. (altered phenotype)
4XIns TRE LacZ (Insulator flanked LacZ indicator)	4	n.d.	3	-	yes (patchy)	n.d.

* provided by Hermann Bujard (Heidelberg, Germany).
◊ provided by Rolf Sprengel (Heidelberg, Germany).

One drawback of TET-inducible systems is the need for the time-consuming and expensive breeding of two transgenic mouse strains to create mice that carry both an activator and a responder construct in their genome. Co-injection of both constructs into the same fertilized oocytes is generally precluded whenever the regulatory sequences used to drive expression of the TET-activator protein into a specific cell lineage contain an enhancer-like element (such as those of the Tie-2 gene). To circumvent this problem, we created inducible TRE-containing constructs, in which the expression cassette, LacZ, was flanked by so-called insulator sequences from the chicken β-globin gene, which had been shown to insulate expression cassettes from the influence of neighbouring chromatin elements such as enhancers. From 6 lines raised following co-injection of the Tie2-tTA and an insulator flanked inducible LacZ (4XIns TRE-LacZ) constructs, two expressed endothelial cell-specifically in E10.5 day transgenic embryos (Fig. 2). Compared to double transgenic embryos raised from breeding of two separate lines, expression was somewhat more patchy.

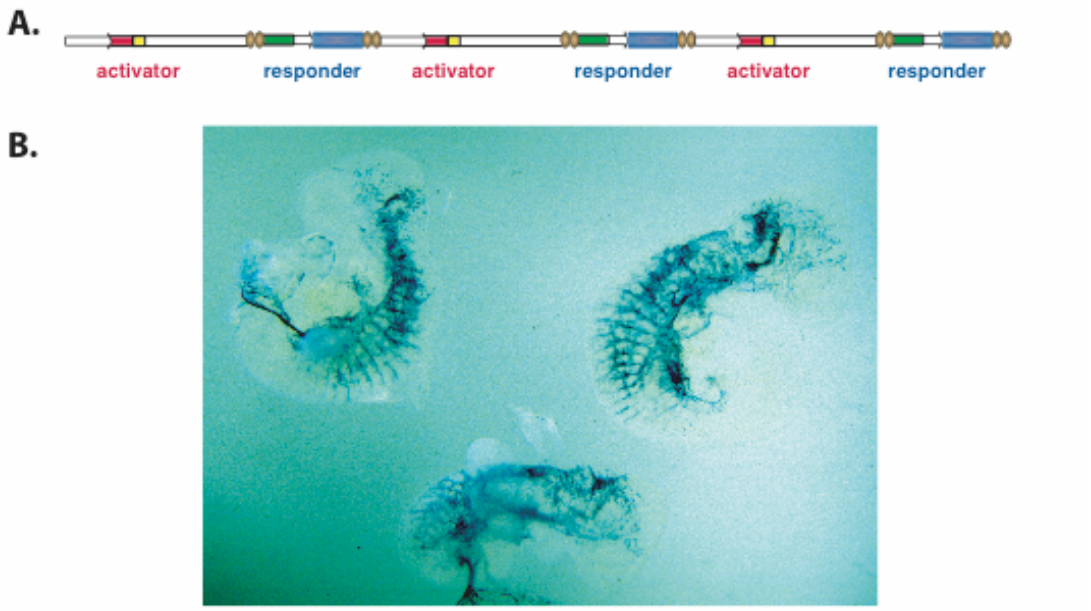


Fig. 2: Whole mount analysis of E10.5 embryos transgenic for co-injected Tie-2 tTA and insulator flanked TRE-LacZ constructs. Endothelial expression in co-injected embryos is more patchy than in double transgenic Tie2 tTA//TRE-LacZ embryos derived from mating of activator and responder lines.

Based on this outcome, we decided to not follow this approach as a general strategy. Surprisingly, in one of these "co-injected" lines (#9469), almost all cardiac vessels showed strong induced expression in adult mice, which should allow a much stronger endothelial-specific expression in the heart than observed in any of the other tTA lines investigated.

Transgenic mouse lines for inducible expression of Angiopoietins

For the production of transgenic mouse lines for inducible expression of Angiopoietins we created constructs that were designed for two purposes: to raise transgenic mouse lines and to produce stably transfected breast carcinoma cell lines that should allow both inducible expression as well as rapid and specific selection of regulatable cell clones. In a collaboration with Karl Plate, Frankfurt, these tumour cell lines were successfully used to investigate the role of Angiopoietins on angiogenesis, tumour growth and tumour vessel morphology (Knedla et al., manuscript in revision).

Based on the efficacy of the construct in the tumour cell experiments, we decided to use the same constructs (Fig. 1) for pronuclear injection to raise transgenic founder mice. The constructs allow for production of two transcripts from a bi-directional expression cassette, a GFP-neo fusion protein coding sequence on one side and a human Angiopoietin-1 or –2 coding sequence on the other side. The human Ang-1 and Ang-2 sequences were used to allow to distinguish their expression from the endogenous mouse gene products. 19 founder mice were produced that were transgenic for the inducible Ang-1 construct. From these founders a total of 15 transgenic lines were established. Injection of the inducible Ang-2 constructs yielded 17 founder mice of which only 8 lines could be established. We attribute this low number of lines to potentially detrimental effects of dysregulated Ang-2 expression in transgenic mice, in which expression might be integration site dependent.

Based on the finding that direct expression of Ang-2 under the control of those Tie-2 regulatory elements that we used for the successful expression of the tet-inducible transactivators tTA and rtTA2-M2 causes embryonic lethality, we assumed that mice double transgenic for Tie2-tTA and a

functional inducible Ang-2 transgene should not be viable. However, all 8 remaining transgenic Ang-2 inducible lines yielded double transgenic offspring at the expected Mendelian frequency. Thus, expression of Ang-2 could not be as strong as in the Tie2 promoter/enhancer-driven Ang-2 transgenics published previously.

We now analysed all 8 inducible Ang-2 lines as Tie2-tTA double transgenic offspring without application of Doxycycline (for suppression Dox was usually provided either in the drinking water or as addition to the food at 100 mg/kg). In one out of the 8 lines we observed the following phenotype: Starting at about 3 months of age, mice double transgenic for the inducible Ang-2 transgene (line #16772) and the Tie2-tTA transgene (#7770) develop red appearing ears with increased lumen size of the prominent blood vessels that then progress to hemorrhaging. Later, these mice also show red swollen snouts. Furthermore, their scrotum is significantly enlarged, apparently due to edema formation. When sacrificed, spleens are found to be up to 5 fold increased compared to single transgenic littermates. In addition, the hearts of double transgenic animals is increased to up to two-fold. In contrast, the kidneys of such mice are slightly smaller and have a somewhat ruffled appearing surface. Finally, double transgenic mice exhibit increased mortality, which has become more pronounced a later generations, when backcrossed to the inbred strain C57BL/6.

Preliminary histological analysis of spleens of double and single transgenic littermates indicates that the increase in spleen size is due to an increase in erythropoesis. In collaboration with Hartwig Wolburg, we have analyzed multiple organs for changes in endothelial junctional integrity and apoptotic or necrotic cell death, without detecting obvious alterations (data not shown).

DISCUSSION

We have set up both TET-OFF and TET-ON systems for inducible expression of responder genes in the endothelia of transgenic mouse embryos using Tie2 transcriptional control elements. While various Tie2 promoter/enhancer driven TET-ON transactivators failed to show useful expression in adult organs, the original TET-OFF version tTA is effective in adult organs. The reasons for this discrepancy are not clear but are also born out by the majority of published TET-systems being of the TET-OFF type with rarely a TET-ON transactivator mouse working efficiently.

Combination of Tie2-tTA transgenes with various responder mouse lines led to incomplete expression of inducible genes in the blood vessels of different tissues. Generally, expression was in almost all vessels of the tongues, in at most 50% of brain vessels and in even fewer vessels of the spleen and the intestine. There was also considerable variability in the completeness of vascular expression in different responder mouse lines when bred to the same Tie2-tTA transactivator line #7770. However, one type of construct used for inducible of murine MAdCAM-1 (part of a collaboration with Britta Engelhardt, Bern) allowed to raised a particularly high proportion of inducible responder lines with robust expression in almost all vessels of many organs (brain, spinal cord, retina, kidney, lung and others). As a example, brain sections of one of these lines are shown in Fig. 3. From the sum of these expression data of multiple activator and responder lines carrying several different inducible uni-directional or bi-directional constructs it emerges that not only the activator mouse lines are instrumental in driving cell type-specific expression but the responder constructs also strongly impact on the level and therefore very likely completeness of cell-specific expression. To achieve the best possible result in the context of this project, a combination of an endothelial cell-specific Tie2-tTA activator line with a simple uni-directional responder line (containing a bovine growth hormone polyadenylation sequence seems to be the best choice.

TET-OFF versions of transactivator mouse lines are fraught with a particular generic disadvantage: to suppress unwanted gene expression from the responder construct, double

transgenic mice have to be kept under a Doxycycline regimen at all times. Dox is accumulated in mouse tissues over time and induction requires the elimination of the antibiotic from the body of the mouse before expression of the responder gene initiates. It is therefore instrumental to titrate the least amount of Dox for reliable suppression of expression and to maintain the mice under Dox treatment for the least possible amount of time. Induction of transgene expression is gradual and will be not complete during the first week of Dox withdrawal in adult mice.

It is gratifying to find that suppression of expression by Doxycycline is complete and leakiness was never observed in the selected activator/responder combinations. Similarly, the cell type restriction of induced expression to endothelial reflected the pattern observed previously in Tie2-LacZ transgenic mice. This system is therefore suitable for the induced expression of cre-recombinase to allow for lineage tracing studies (collaboration with Ernesto Bockamp, Mainz; manuscript in revision) as well as inducible gene deletion using floxed gene targeted alleles.

Transgenic mice carrying both inducible Ang-1 and Tie2-tTA activator constructs are phenotypically normal. At present, we have not characterized the expression of the remaining 15 lines. One of the 8 remaining inducible Ang-2 transgenic lines showed interesting phenotypic alterations when also carrying a Tie2-tTA activator transgene. These mice showed alterations not

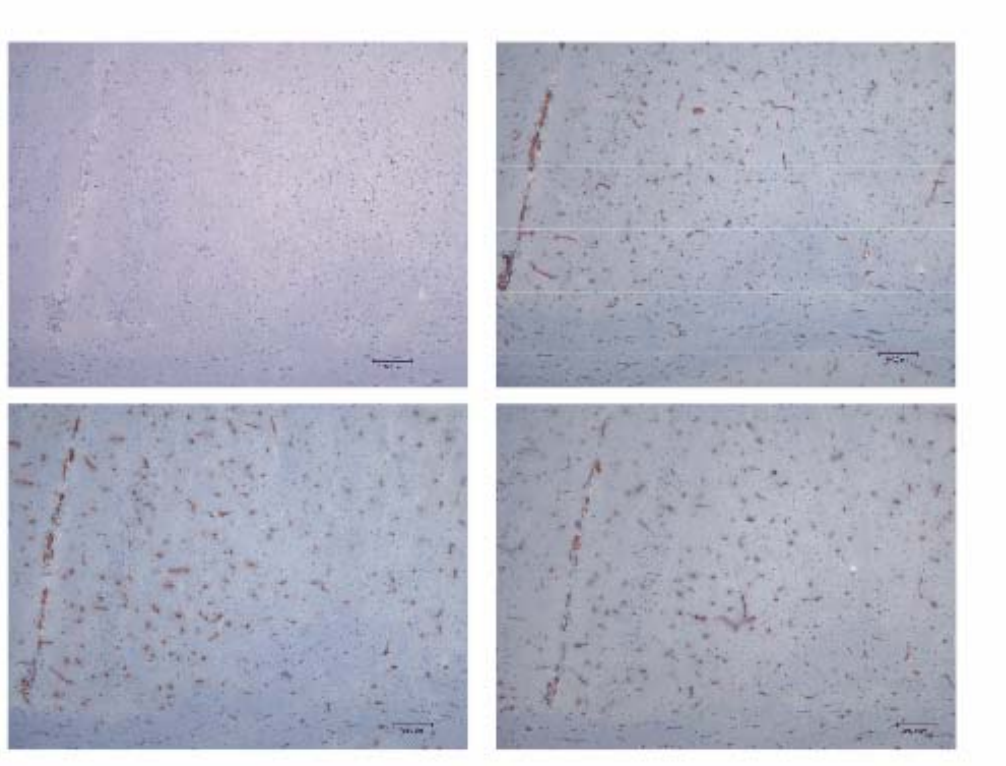


Fig. 3: Immunohistochemical analysis of TRE-MAdCAM-1//Tie2-tTA double transgenic mice (17910//7770). Stainings of serial brain sections of a double transgenic mouse maintained in the absence of Doxycycline. Upper left panel: Isotype control staining. Upper right panel: MEC13.3 staining for the pan-endothelial PECAM-1. Lower left panel: Staining for induced expression of MAdCAM-1 using the mAb MECA89. Lower right panel: Staining for induced expression of MAdCAM-1 using the mAb MECA367. Note that MAdCAM-1 is induced in virtually all PECAM-1 positive vessels. (MAdCAM-1 is known to be not expressed in CNS parenchymal blood vessels).

only in vessel architecture in the ear but also signs of compromized blood vessel integrity as was expected from earlier studies in for example tumour models. Preliminary observations also hint at a pro-inflammatory role of chronic expression of Ang-2 as would have to be suggested based on the observation that Ang-2 is required for neutrophil transmigration in different models of inflammation.

At present, it is unclear how induced expression of Ang-2 in the vasculature might cause an increase in spleen size. It should be noted that the Tie2 gene is active in a subpopulation of hematopoietic precursor cells and the Tie2 promoter/enhancer elements might drive tTA expression into this lineage as well to allow for expansion of particular hematopoietic lineages causing splenomegaly. Likewise, induced expression in bone marrow endothelial cells could have similar consequences.

The changes to an inflammatory vascular phenotype have to be investigated further. Preliminary analysis in the affected hearts and kidneys do not reveal obvious changes in the amount of CD45-positive hematopoietic cells present in these organs (data not shown). Histological analysis of the spleens however of double transgenic Tie2-tTA//TRE-Ang-2 mice indicates disruption of the normal architecture of the red pulp that needs to be analysed in more detail.

REFERENCES

Palmer A, Zimmer M, Erdmann KS, Porthin A, Heumann R, Deutsch U, Klein R: EphrinB phosphorylation and reverse signalling: regulation by Src kinases and PTP-BL phosphatase. *Molecular Cell* 9: 725–737, 2002.

Carmeliet, P: Gene targeting and gene transfer to unravel the molecular basis of the formation and disorders of blood vessels. *Verh K Acad Geneeskd Belg* 62: 31-68, 2000.

Davis S, Papadopoulos N, Aldrich TH, Maisonpierre PC, Huang T, Kovac L, Xu A, Leidich R, Radziejewska E, Rafique A, Goldberg J, Jain V, Bailey K, Karow M, Fandl J, Samuelsson SJ, Ioffe E, Rudge JS, Daly TJ, Radziejewski C, Yancopoulos GD: Angiopoietins have distinct modular domains essential for receptor binding, dimerization and superclustering. *Nat Struct Biol* 10: 38-44, 2003.

Fiedler U, Krissl T, Koidl S, Weiss C, Koblizek T, Deutsch U, Martiny-Baron G, Marme D, Augustin HG: Angiopoietin-1 and angiopoietin-2 share the same binding domains in the Tie-2 receptor involving the first IgG-like loop and the EGF-like repeats. *J Biol Chem* 278: 1721-7, 2003.

Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD: Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Dev Cell* 3: 411-423, 2002.

Gale NW, Yancopoulos GD. Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev* 13: 1055-1066, 1999.

Gossen M, Bujard H: Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci USA* 89: 5547-5551, 1992.

Hammes HP, Lin J, Wagner P, Feng Y, vom Hagen F, Krzizok T, Renner O, Breier G, Brownlee M, Deutsch U: Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. *Diabetes* 53: 1104-1110, 2004.

Hammes HP, Lin J, Renner O, Shani M, Lundqvist A, Betsholtz C, Brownlee M, Deutsch U: Pericytes and the pathogenesis of diabetic retinopathy. *Diabetes* 51: 3107-12, 2002.

Hoffmann J, Feng Y, vom Hagen F, Hillenbrand A, Lin J, Erber R, Vajkoczy P, Gourzoulidou E, Waldmann H, Giannis A, Wolburg H, Shani M, Jaeger V, Weich HA, Preissner KT, Hoffmann S, Deutsch U, Hammes HP: Endothelial survival factors and spatial completion, but not pericyte coverage of retinal capillaries determine vessel plasticity. *FASEB J* 19: 2035-6, 2005.

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ: Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science* 284: 1994-1998, 1999.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277: 55-60, 1997.

May T, Mueller PP, Weich H, Froese N, Deutsch U, Wirth D, Kroger A, Hauser H: Establishment of murine cell lines by constitutive and conditional immortalization. *J Biotech* 120: 99-110, 2005.

Partanen J, Puri MC, Schwartz L, Fischer KD, Bernstein A, Rossant J: Cell autonomous functions of the receptor tyrosine kinase TIE in a late phase of angiogenic capillary growth and endothelial cell survival during murine development. *Development* 122: 3013-3021, 1996.

Puri MC, Partanen J, Rossant J, Bernstein A: Interaction of the TEK and TIE receptor tyrosine kinases during cardiovascular development. *Development* 126: 4569-4580, 1999.

Risau W: Mechanisms of angiogenesis. *Nature* 386: 671-674, 1997.

Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y: Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376: 70-74, 1995.

Schlaeger TM, Bartunkova S, Lawitts JA, Teichmann G, Risau W, Deutsch U, Sato TN: Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc Natl Acad Sci USA* 94: 3058-3063, 1997.

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD: Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87: 1171-1180, 1996.

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM: Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science 28:* 2511-2514, 1999.

vom Hagen F, Feng Y, Hillenbrand A, Hoffmann S, Shani M, Deutsch U, Hammes HP: Early loss of arteriolar smooth muscle cells: more than just a pericyte loss in diabetic retinopathy. *Exp Clin Endocrinol Diabetes* 113: 573-576, 2005.

Urlinger S, Baron U, Thellmann M, Hasan MT, Bujard H, Hillen W: Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci USA* 97: 7963-7968, 2000.

Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, Holash J: Vascular-specific growth factors and blood vessel formation. *Nature* 407: 242-248, 2000.

Regulation of Endothelial Cell-specific Angiopoietin-2 Expression and Function

Dr. Ulrike Fiedler
Dept. of Vascular Biology and Angiogenesis Research
Tumor Biology Center Freiburg
Breisacher Str. 117, 79106 Freiburg, Germany

Prof. Dr. Dieter Marmé
Institute of Molecular Oncology, Tumor Biology Center Freiburg

Dr. Georg Martiny-Baron, Novartis Inc., Basel, Switzerland (formerly Tumor Biology Center Freiburg)

Member of the SPP 1069 from 2002 until 2005 (U.F.)
Member of the SPP 1069 from 2000 until 2005 (D.M)
Member of the SPP 1069 from January 2000 until June 2000 (G. M.-B.)

SUMMARY

Angiopoietin-2 (Ang-2) is a non-signal transducing ligand of the endothelial receptor tyrosine kinase Tie2. Ang-2 is produced by endothelial cells and acts as an autocrine regulator mediating destabilization of the vasculature by inhibiting angiopoietin-1 induced Tie2 signaling. During the three funding periods of the SPP1069 we studied the mechanisms regulating endothelial cell specific Ang-2 expression and functions. We identified unique positive and negative regulatory mechanisms of endothelial cell Ang-2 expression by analyzing the Ang-2 promoter. We could show that Ang-1 and Ang-2 bind to the same sites in Tie2 with similar affinity, suggesting that an innate endothelial cell specific mechanism controls agonistic and antagonistic angiopoietin/Tie signaling. Furthermore, we identified Ang-2 as a Weibel-Palade body stored molecule which is rapidly released from endothelial cells upon stimulation, suggesting that Ang-2 controls vascular homeostasis. In line with this hypothesis we could show that Ang-2 mediates inflammatory responses by sensitizing the endothelium towards cytokine stimulation. In conclusion, our data support the model that Ang-2 is the dynamically-regulated antagonistic player of the angiopoietin / Tie-system and thus a key regulator of vascular homeostasis and responsiveness.

INTRODUCTION

Angiogenesis, the formation of blood vessels from pre-existing vessels, is controlled by a hierarchically structured signaling cascade of endothelial cell specifically expressed receptor tyrosine kinases. These receptor / ligand systems control different steps in angiogenesis, e.g. the VEGF / VEGF receptor system controls formation of the first vascular plexus, the angiopoietin / Tie system regulates maturation and remodeling of the vascular system and the Ephrin / Eph system controls vascular remodeling and vascular identity (Gale and Yancopoulos, 1999; Carmeliet, 2000).

The VEGF/VEGFR system was identified first and it has taken 15 years from identification to clinical translation. The angiopoietin/Tie system (Ang/Tie) was identified in 1996/97 as the second vascular-specific rate-limiting receptor tyrosine kinase signaling system (Suri et al., 1996; Davis et al., 1996, Maisonpierre et al., 1997). It is believed to have important translational therapeutic prospect and a number of pharmaceutical companies support screening programs aimed at identifying small molecular weight Tie-2 inhibitors. Yet, even after 8 years of angiopoietin research,

the Ang/Tie system is molecularly poorly understood and therapeutically far from being solidly validated.

Two Tie-receptors have been identified so far, Tie1 and Tie2. Both molecules are preferentially expressed by endothelial cells and exhibit a split tyrosine kinase domain. Tie1 and Tie2 show strong homology and were originally isolated as orphan receptors. They are composed of three EGF homology repeats flanked by two Ig-like loops. The second Ig-like loop is followed by a fibronectin (FN) Type III domain hooked on the transmembrane domain. The Tie-receptors contain an intracellular tyrosine kinase domain (Sato, et al., 1993). No specific ligand for Tie1 could be identified until now. There is evidence that Ang-1 is capable to bind to membrane anchored Tie1 at high concentrations, but not to the soluble Tie1 ectodomain (Saharinen et al., 2005). However, the role of Ang-1 mediated or ligand dependent Tie1 signaling is not well understood until today.

Four ligands have been isolated for Tie2, Ang-1, Ang-2, Ang-3 and Ang-4. Among them Ang-1 and Ang-2 are the best characterised ones. Ang-3 is the mouse ortholog of human Ang-4. Both are species specific Tie2 agonists, however their functions *in vivo* are still enigmatic (Valenzuela, et al. 1999, Kim et al., 2004). Furthermore several angiopoietin-related proteins have been identified, which do not bind to the Tie-receptors, but have functions in regulating angiogenesis (Kim, et al. 2000). Angiopoietin-1 (Ang-1) has been identified as the agonistic ligand of Tie-2 (Davis et al., 1996; Suri et al., 1996). Ang-1 is produced by many cell types and acts in a paracrine manner on endothelial cells. Ang-1 has been described as a transcriptionally-regulated molecule in some tumors (Statemann et al., 1998; Sugimachi et al., 2003) and during inflammation (Brown et al., 2004). Yet, its transcriptional regulation is rather moderate compared to other angiogenesis-regulating molecules such as VEGF and Ang-2. Ang-1 regulates endothelial cell survival and blood vessel maturation (Suri et al., 1996). It exerts a vessel sealing effect (Thurston et al, 2000), acts anti-inflammatory (Gamble et al., 2000; Jeon et al., 2003; Ramsauer et al., 2002), protects against cardiac allograft arteriosclerosis (Nykanen et al., 2003) and radiation-induced endothelial cell damage (Cho et al., 2004). In the adult, low level constitutive Tie-2 activation may be required to maintain the mature quiescent phenotype of the resting vascular endothelium (Wong et al., 1997).

Angiopoietin-2 (Ang-2) has been identified as functional antagonist of Ang-1. It binds to Tie-2 without inducing rapid signal transduction in Tie-2-expressing endothelial cells (Maisionpoierre et al., 1997). The opposing effects of Ang-1 and Ang-2 support a model of constitutive Ang-1/Tie-2 interactions controlling vascular homeostasis as default pathway (Wong et al., 1997) and Ang-2 acting as dynamically regulated antagonizing cytokine (Hanahan and Folkman, 1996; Cameliet, 2000). Surprisingly, the loss of the Ang-2 gene and function is compatible with life as evidenced by the observation that Ang-2-deficient mice are born apparently normal (Gale et al., 2002). The functionally unaffected blood vascular system of Ang-2-deficient mice has only minor abnormalities (e.g., perturbed vessel regression phenotype of the eye's hyaloid blood vessels). Yet, depending on the genetic background of the mice, a significant fraction of newborn mice develops a lethal chylous ascites within the first 14 days as a consequence of a mechanistically hitherto unexplained lymphatic phenotype (Gale et al., 2002). In contrast to the mild phenotype of Ang-2-deficient mice, mice transgenically overexpressing Ang-2 have an embryonic lethal phenotype that essentially phenocopies the Ang-1 and the Tie-2 null phenotypes (Suri et al., 1996; Davis et al., 1996; Maisonpierre et al, 1997). The similarity of the Ang-1 loss-of-function phenotype with the Ang-2 gain-of-function phenotype strongly supports the antagonistic concept of Ang-1 and Ang-2. Yet, the embryonic lethal phenotype of systemically Ang-2 overexpressing mice also demonstrates that Ang-2 is a potentially dangerous molecule whose dosage and spatiotemporal appearance must be tightly regulated. The Ang-2 dosage concept is also supported by the observation that local overexpression of Ang-2 in the heart is compatible with life (Visconti et al., 2002), whereas strong overexpression of

Ang-2 with systemic dissemination in a large organ such as the skin leads to an embryonic lethal phenotype similar to the systemic overexpression of Ang-2 (Maisionpierre et al., 1997). Similarly, an activating Tie-2 mutation causes venous malformations that are composed of dilated endothelial channels covered by a variable amount of smooth muscle cells demonstrating that a precise balance of Tie-2 signals is critical (Vikkula et al., 1996).

Taken together all findings lead to the model that Ang-2 is the dynamic player in the angiopoietin / Tie system. Therefore we focused in our project on mechanisms regulating Ang-2 expression and its spatiotemporal activities during the entire funding period. Specifically, (i) we mapped the Ang-1 and Ang-2 binding sites in Tie2 to study whether differential receptor binding elicits specific vascular responses (ii) we analysed mechanisms regulating Ang-2 expression and bio-availability by studying transcriptional regulation of the Ang-2 gene and Ang-2 protein distribution in endothelial cells and (iii) we analysed Ang-2 activities on resting and activated endothelium *in vitro* and *in vivo*.

METHODS

Until today the availability of suitable reagents to study the angiopoietin / Tie-system are limited. Both, recombinant proteins and antibodies were not available when the project was started. Consequently, we invested time and resources to develop appropriate molecular tools and reagents which are listed below:

Recombinant ligands

We have generated baculovirus-based expression and purification systems for full length multimeric Ang-1 and dimeric Ang-2. We have succeeded in purifying recombinant full length bioactive Ang-1 and Ang-2 that we have also made available to a number of collaborating laboratories. Recently, we have also made these unique reagents commercially available to the scientific community (www.reliatech.de).

Receptor constructs

We have generated a number of Tie-1 and Tie-2 receptor mutants and successfully expressed these in different cell populations. We have and are continuing to employ these constructs in ligand binding and functional signaling experiments. Additionally, we have generated and expressed a number of truncated recombinant extracellular soluble Tie-2 constructs. Recombinant proteins have been used for ligand binding experiments and immuncytochemical analyses.

Antibodies

We invested some time and money to generate monoclonal antibodies discriminating between Ang-1 and Ang-2 by various approaches. We performed DNA-, protein- and peptid-vaccination strategies in collaboration with Andreas Lingnau in the Tumor Biology Center Freiburg and antibody generating companies. Moreover, we performed a single-chain antibody library screening in collaboration with Prof. Dr. Dario Neri form the ETH Zürich, Switzerland. None of the approaches has been successful. Finally RnD Systems developed an Ang-2 antibody that was suitable for immuncytochemical studies and we joined forces with Regeneron Pharmaceuticals and received antibodies specifically recognizing human Ang-2 for immunhistochemical and western blot analyses.

Viral reagents

We have generated retroviral and adenoviral vectors for myc-tagged Ang-1 and Ang-2. These have proven useful and versatile tools for the expression of Ang-1 and Ang-2 *in vitro* and *in vivo*.

siRNA transfections

We have tested multiple approaches to generate Ang-2 deficient endothelial cells. One approach was the isolation and immortalization of endothelial cells from Ang-2 deficient mice. In the second approach we tested 10 different Geneblocs designed by Atugen (Munich, Germany) for the down regulation of Ang-2 in endothelial cells. None of the tested Geneblocs was functional. Finally, we switched to the siRNA technology and identified two siRNAs from Ambion that specifically down-regulate human Ang-2 in endothelial cells.

Ang-2 promoter constructs

We have cloned 6713 bp of the human Ang-2 promoter from a human genomic library containing 4427 bp upstream of the transcriptional start site, 476 bp untranslated region, the first exon encompassing 288 bp (96aa) and 1522 bp of the first intron. We fused various parts of the promoter fragment to a luciferase reporter and tested promoter activity in endothelial cells and non-endothelial cells.

Transgenic mice

We generated transgenic mice carrying various Ang-2 promoter-LacZ constructs to study activation in physiological and pathological angiogenesis. Moreover we were able to implement a Material Transfer Agreement with Regeneron Pharmaceuticals in January 2003 that has given us access to Ang-2-deficient mice. Ang-2-deficient mice have been characterized as having a postnatal lethal phenotype. Close to 98% of normally born mice die within 14 days of birth (Gale et al., 2002). We have received the mice in a 129 genetic background for the isolation of Ang-2-deficient endothelial cells. Surprisingly, when backcrossing these mice in C57/BL6 mice, we were able to generate viable adult mice indicating that the penetrance of the postnatal lethal Ang-2 phenotype is dependent on the genetic background of the mice (Fiedler et al., 2006). Based on this observation we have established an extensive breeding colony that has allowed us to pursue LacZ-reporter expression analyses and functional experiments in homozygous Ang-2-deficient mice.

RESULTS

1. Mapping of the Ang-1 and Ang-2 binding sites in Tie-2

<u>Quantitative ELISA- and immunoprecipitation-based mapping of the angiopoietin-binding sites within the Tie-2 receptor</u>

The mechanisms underlying the differential agonistic/antagonistic functions of Ang-1 and Ang-2 towards Tie-2 have hitherto not been unraveled. We speculated that differential receptor binding of the ligands may be responsible for the antagonistic functions of Ang-1 and Ang-2 and consequently set up experiments aimed at identifying the ligand binding sites to the Tie-2 receptor.

Angiopoietin binding was studied in a competition ELISA-based assay in which binding of immobilized Ang-1 and Ang-2 to the biotinylated full length extracellular Tie-2 domain (sTie-2[1-730]-Fc) is inhibited by increasing concentrations of the different amino terminal truncation mutants of

soluble Tie-2-Fc. A concentration of 10 pmol competing soluble Tie-2-Fc was capable to inhibit binding of biotinylated sTie-2-Fc to Ang-1 and Ang-2. Analysis of the different truncation mutants of the extracellular domain of Tie-2 (Fig.1) in this assay revealed that sTie-2[1-440]-Fc and sTie-2[1-360]-Fc are similarly capable to inhibit binding of Ang-1 and Ang-2 to Tie-2, indicating that the angiopoietin-binding sites in Tie2 are located within the first Ig-like loop and the EGF-like repeats. The shortest construct consisting just of the amino terminal Ig-like domain of Tie-2 (sTie-2[1-199]-Fc) did not bind Ang-1 and Ang-2, indicating that the first Ig-like domain is not sufficient to effectively bind Ang-1 and Ang-2.

Based on the results of the ELISA based angiopoietin binding experiments, we further studied binding of Ang-1 and Ang-2 to the extracellular domain of Tie-2 in angiopoietin pull down assays. These experiments were performed by incubating full length extracellular Tie-2-Fc as well as the different truncation mutants of Tie-2-Fc with myc-tagged Ang-1 and myc-tagged Ang-2 which was followed by Protein-A-Sepharose precipitation and anti-myc Western blot analysis. Corresponding to and confirming the ELISA based binding experiments, full length extracellular sTie-2-Fc as well as sTie-2[1-440]-Fc and sTie-2[1-360]-Fc were capable to bind and pull down Ang-1 and Ang-2. In contrast, the shortest sTie-2-Fc fusion protein consisting just of the amino terminal Tie-2 Ig-like domain is not capable to precipitate Ang-1 and Ang-2. Taken together, the results of the ELISA-based competition experiments and the pull down experiments indicate: (i) the first Ig-like loop together with the EGF-like repeats of Tie-2 are sufficient for strong Ang-1 and Ang-2 binding, (ii) the second Ig-like loop of Tie-2 confers some specificity for Ang-1 over Ang-2 binding, and (iii) the first Ig-like loop of Tie-2 is not sufficient to bind the angiopoietins.

To further corroborate these finding, we generated additional truncated sTie-2-Fc mutants which lacked varying amino terminal parts of the Tie-2 receptor (Fig. 1): (i) sTie-2[211-360]-Fc consisting of the EGF-like repeats, (ii) sTie-2[211-730]-Fc being composed of the entire extracellular domain of Tie-2 just lacking the first Ig-like domain; and (iii) sTie-2[341-730]-Fc consisting of the C terminal extracellular domains of Tie-2 lacking the first Ig-like loop as well as the EGF-like repeats. The first set of experiments had indicated that the EGF-like repeats might be involved in angiopoietin binding suggesting that sTie-2[211-360]-Fc and sTie-2[211-730]-Fc might be capable to bind Ang-1 and Ang-2. Surprisingly, neither of the additional Tie-2 truncation mutants, sTie-2[211-360]-Fc, sTie-2[211-730]-Fc, and sTie-2[341-730]-Fc, was able to bind Ang-1 or Ang-2 in precipitation experiments as well as in the ELISA based competition assays.

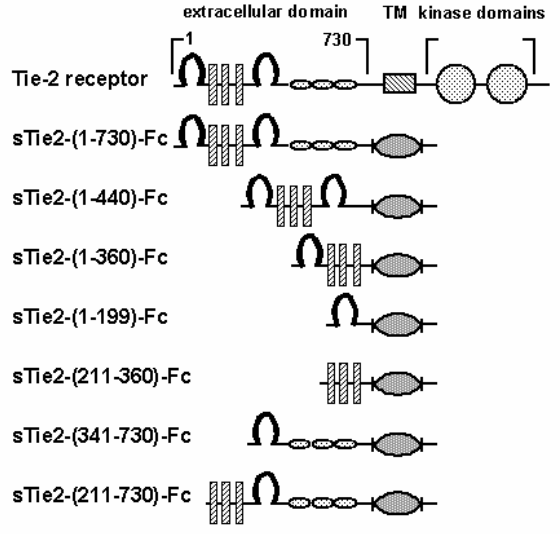


Fig. 1: Domain structure of the Tie-2 receptor and schematic diagram of the soluble Tie-2-Fc truncation mutants. The extracellular domain of Tie-2 consists of an amino terminal Ig-like domain followed by EGF-like repeats, a second Ig-like domain, and fibronectin (FN) type III domains. Fc fusion proteins were generated by replacing the transmembrane and kinase domain of Tie-2 by human Fc (constant part of IgG1). Different portions of the extracellular domain of Tie-2 were fused to Fc (the numbers denote the amino acids of the extracellular Tie-2 domain of each of the constructs).

Collectively, the data show that neither the first Ig-like domain alone nor the EGF-like repeats are sufficient to bind Ang-1 and Ang-2, but that both domains together (sTie-2[1-360]-Fc) are necessary and sufficient to bind Ang-1 and Ang-2 (Fiedler et al., 2003).

Ang-1 and Ang-2 binding properties of Tie-2 isolated from endothelioma cells established from wildtype mouse embryos and embryos expressing mutated Tie-2 protein

Mice with a targeted insertion of a neomycin expression cassette in exon 2 of the Tie-2 gene die on embryonic day 10.5 as a consequence of severe vascular defects. We have received endothelioma cell lines from these mutant embryos and their corresponding heterozygous and wildtype littermates from Dr. Urban Deutsch (University of Bern, SPP1069 member) to further study the mechanisms of Tie-2 signaling. As expected, cells from wildtype mice express full length Tie-2 receptor (166 kDa, Fig. 2A). Surprisingly, endothelioma cells from homozygous Tie-2 mutant embryos expressed a truncated Tie-2 receptor with an apparent molecular weight of 133 kDa. Correspondingly, both full length Tie-2 as well as the truncated Tie-2 molecule was detectable in endothelial cells isolated from heterozygous embryos (Fig. 2A). Based on these observations, we cloned the cDNA coding for the truncated murine Tie-2 receptor in mutant endothelioma cells (mTie-2 -/-). Sequence analysis revealed that the mutant mTie-2 receptor is produced as a transmembrane receptor with a complete intracellular domain, containing a signal sequence but lacking the first 104 amino acids of the extracellular domain corresponding to parts of the first Ig-like loop. Based on these findings, we propose that the entire exon 2 including the neomycin expression cassette is removed in the Tie-2 mutant mice by aberrant splicing from the primary Tie-2 transcript produced from the targeted allele

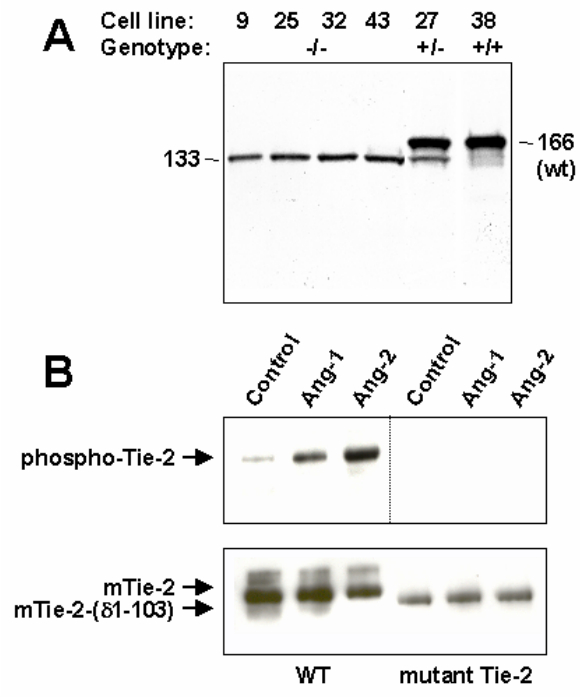


Fig. 2: Detection of mutant Tie-2 protein in endotheliomas derived from mouse embryos with a targeted mutation of Tie-2. *A*, Polyoma middle T immortalized endothelioma cells were generated from mouse embryos homozygous for a targeted insertion into exon 2 of the Tie-2 gene [cell lines 9, 25, 32, 43]; heterozygously Tie-2 targeted mouse embryos [cell line 27], and wildtype mouse embryos [cell line 38]. Cell lysates were separated by 7.5% SDS PAGE, blotted, and probed with an anti-Tie-2 antibody. Wildtype endothelioma cells express full length 166 kDa Tie-2. In contrast, all homozygous Tie-2 mutant endothelioma cells express a smaller Tie-2 band with an apparent molecular weight around 133 kDa. Heterozygous mutant endothelioma cells express the wildtype 166 kDa band as well as the mutant 133 kDa band. *B*, CHO cells were transfected with either a plasmid coding for full length mTie-2 (lanes 1-3) or a plasmid that codes for the mutant murine Tie-2[delta aa 1-103] (lanes 4-6). Cells were starved for 12 h and stimulated with 5 µg/ml myc-Ang-1 or myc-Ang-2 for 15 min. Cell lysates immunoprecipitated with an anti-Tie-2 antibody, separated by SDS PAGE, blotted, and probed with an anti-P-tyrosine antibody (upper panel) and reprobed with an anti-Tie-2 antibody. Both, Ang-1 and Ang-2 are capable to induce Tie-2 phosphorylation. In contrast, the mutant Tie-2 lacking aa 1-103 is not capable to become phosphorylated upon Ang-1 or Ang-2 stimulation.

leading to an in frame fusion of exons 1 and 3. In order to study the signal transduction properties of full length mTie-2 receptor and the mutant mTie-2 receptor (mTie-2[delta aa1-103]), we stably overexpressed both molecules in CHO cells and stimulated the cells with Ang-1 and Ang-2. Stimulation of full length Tie-2 expressing CHO cells results in rapid autophosphorylation upon Ang-1 as well as Ang-2 addition (Fig. 2B). In contrast, mTie-2[delta aa1-103] does not become phosphorylated upon Ang-1 or Ang-2 stimulation (Fig. 2B). Corresponding experiment in wildtype and mTie-2[delta aa1-103] expressing endothelioma cells showed that Ang-1 is capable to induce Tie-2 phosphorylation in WT endothelioma but not in mutant endothelioma cells.

The experiment with WT and mutant Tie-2 suggested that the mutant mTie-2[delta aa1-103] receptor lacking parts of the first Ig-like domain is either not able to bind Ang-1 and Ang-2 or that mutant Tie-2 can bind the angiopoietins but can not undergo the conformational change that leads to autophosphorylation. To address these alternative possibilities, we studied the binding of myc-Ang-1 and myc-Ang-2 by CHO cells expressing either full length Tie-2 or the mutant mTie-2[delta aa1-103] and traced binding by cytochemical detection using anti-myc antibodies. These experiments showed that full length Tie-2 expressing CHO cells bind Ang-1 as well Ang-2 whereas mTie-2[delta aa1-103] expressing cells are not capable to bind Ang-1 and Ang-2 (data not shown). Collectively, these experiments show that the first 104 amino acids of the first Ig-like domain of the Tie-2 receptor are critically required for binding of Ang-1 and Ang-2 to the Tie-2 receptor *in vivo* (Fiedler et al., 2003).

2. Regulation of endothelial angiopoietin-2 expression

Ang-2 mRNA expression

There is numerous circumstantial evidence to suggest that Ang-1 is constitutively produced by many cell types and that Ang-2 is almost exclusively produced by endothelial cells (Stratmann et al., 1998; Mandriota et al., 1998; Holash et al., 1999; Hackett et al., 2000; Huang et al., 2002). This would imply that Ang-1 acts in a paracrine manner, whereas Ang-2 acts in an autocrine manner. We have confirmed and extended these observations by extensive RT-PCR analysis on a broad panel of endothelial cell and non-endothelial cell populations. These experiments revealed that all analyzed cultured human endothelial cell populations express detectable Ang-2 (umbilical vein endothelial cells, HUVEC; aortic endothelial cells, HAoEC; saphenous vein endothelial cells, HSaVEC; umbilical artery endothelial cells, HUAEC; dermal microvascular endothelial cells, HDMVEC). In contrast, cultured smooth muscle cells (HUASMC), esophageal epithelial cells (KOP), striated muscle cells (KMU), primary fibroblasts, and monocytic cells (U937) do not express Ang-2. Similarly, of 10 analyzed arbitrarily selected tumor cell lines, only 1 cell line (MDBK) expressed detectable levels of Ang-2 mRNA. These findings were supported by detailed northern blot and RT-PCR analyses of human matched pairs of colon carcinoma biopsies. We observed overexpression of Ang-2 mRNA in 76 % of the tested matched pairs. Interestingly, both Ang-2 and VEGF mRNA were upregulated in 68 % of the tumor samples, indicating that Ang-2 and VEGF overexpression are coupled. Further *in situ* hybridisation experiments and more importantly histological analysis showed that Ang-2 is exclusively expressed by the endothelial compartment of the tumor (Fig. 3). In summary the data show that Ang-2 is almost exclusively expressed by endothelial cells *in vivo* and t*in vitro* (Hegen et al., 2004).

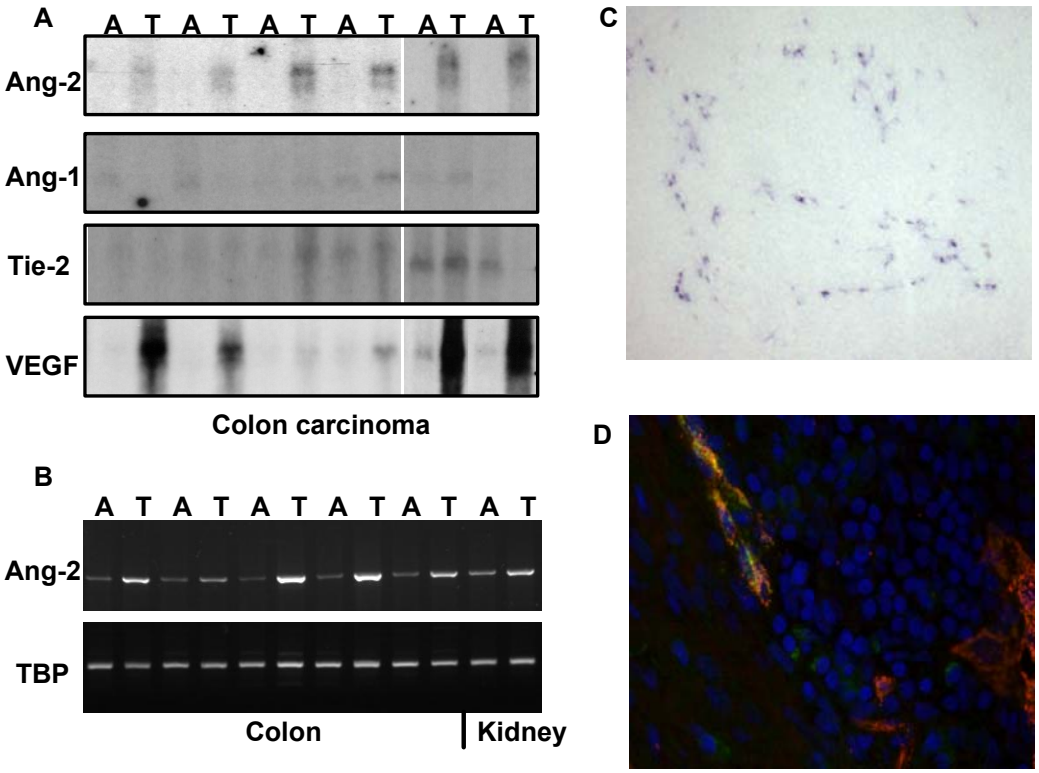


Fig. 3: Ang-2 expression in tumors. Analysis of Ang-2 expression in matched pairs of human colon carcinomas and adjacent normal tissue. Northern blot analysis of Ang-2, Ang-1, VEGF and Tie2 expression (A) and RT-PCR analysis (B). *In situ* hybridisation for Ang-2 mRNA expression in subcutaneous Lewis-Lung tumor (C) and immunhistochemical staining for vWF (red) and Ang-2 (green) on a human colon carcinoma biopsy. Both Ang-2 and VEGF mRNA are upregulated in the tumor tissues. Prominent Ang-2 expression is detectable in the tumor endothelium.

Regulation of the human Ang-2 promoter

To study regulation of Ang-2 expression in endothelial cells, we decided to analyze the properties of the Ang-2 promoter. To this end we screened a human λ-phage genomic library, using a probe encoding the first 291 bp of the Ang-2 coding region. We have isolated a 6713 bp fragment containing: 4427 bp upstream of the transcriptional start site, 476 bp untranslated region, the first exon encompassing 288 bp (98aa) and 1522 bp of the first intron. Sequence analysis revealed that: (i) the human Ang-2 promoter is a TATA-less promoter and contains no consensus initiator sequence; (ii) a CpG island is overlapping with the transcriptional start site, which suggested regulation by imprinting and (iii) the following transcription factors are potential regulators of promoter activity: Ets-1, Elf-1, GATA-1, Sp1, AP1, Smad-3, Smad-4, retinoic acid receptors as well as Arnt (the heterodimerisation partner of HIF-1alpha).

We fused several promoter fragments with varying length to a luciferase reporter gene and analysed the promoter activities in bovine aortic endothelial cells, two tumor cell lines (R30C breast carcinoma cells and A375 melanoma cells), NIH3T3 fibroblasts and in HEK 293 embryonic kidney cells. The Ang-2 promoter constructs were only active in bovine aortic endothelial cells, indicating that the Ang-2 promoter is an endothelial cell specific promoter (Fig. 4). Further analysis of the various length promoter constructs revealed that a negative regulatory element is located between bp –4427 to –2004. Deletion of this fragment results in a three fold higher expression of the luciferase reporter in endothelial cells. Fusion of this fragment to the strong SV40 promoter resulted

in repression of the promoter activity in endothelial and non-endothelial cells, indicating that the fragment contains a pleiotropic repressor. Further deletion of upstream sequences showed that the promoter fragment from bp –109 to + 476 is sufficient to confer endothelial cell specific promoter activation. Furthermore, activity of promoter fragments lacking the negative regulatory elements could be stimulated by VEGF and bFGF. In order to analyse mechanisms regulating endothelial cell specific Ang-2 expression, we studied: (i) whether the Ang-2 gene is an imprinted gene and (ii) which transcription factors mediate endothelial cell specific expression. We observed that Ang-2 is not an imprinted gene, while Tie2 expression is regulated by imprinting. In order to identify transcription factors mediating endothelial cell specific Ang-2 promoter activity, we tested the contribution of Ets-like transcription factors on the Ang-2 expression.

Ets-1 is expressed in endothelial precursors during vasculogenesis and in endothelial cells during angiogenesis (Sato, et al. 2001). Furthermore, it has been shown that Ets-1 is conferring endothelial cell specific expression of promoters of genes involved in angiogenesis: uPA, Tie2, Tie1, VEGFR1 and VEGFR-2 (Nerlov et al., 1991; Dube et al., 1999; Iljin et al., 1999; Wakiya et al., 1996; Kappel, et al., 2000). Elf-1 is another member of the Ets transcription factor family, binding to Ets-binding sites. It has been shown that Elf-1 is a positive regulator of Tie-1 and Tie-2 expression (Dube et al., 2001). We found multiple Ets- and Elf-binding sites within the isolated Ang-2 promoter fragment. Therefore, we cotransfected the Ang-2 reporter constructs with expression plasmids for the transcription factors Ets-1, dominant negative Ets-1 and Elf-1 into bovine aortic endothelial cells and A375 melanoma cells (plasmids were a gift from Dr. M.H. Sieweke and Dr. D. Skalnik). We found that Ets-1 and Elf-1 stimulated Ang-2 promoter activity 4 to 6 fold in endothelial cells and that dominant negative Ets-1 is

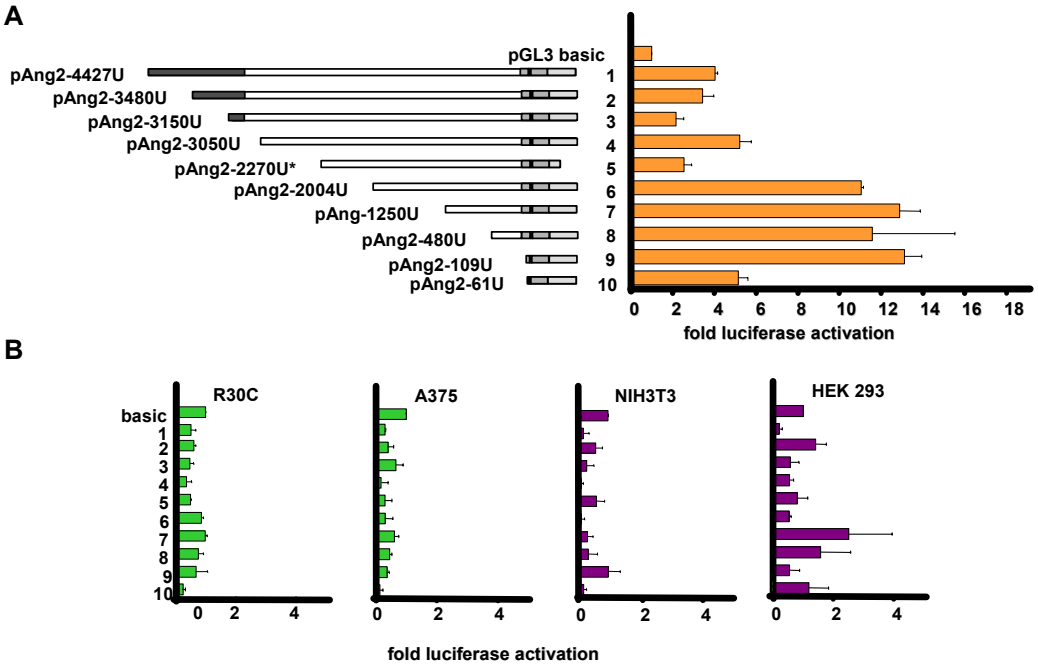


Fig. 4: Functional analysis of the human Ang-2 promoter *in vitro*. Truncation mutants of the human Ang-2 promoter were fused to the luciferase gene. The number marking each construct indicates the base pairs to the transcriptional start site/the number of bp of the UTR. (A) Results of transient transfections in BAEC; (B) results of transfection into R30C mammary carcinoma cells; A375 melanoma cells, NIH3T3 mouse fibroblast cells and HEK293 human embryonic kidney cells. Promoter activity is calculated as fold activation compared to promoter-less control vector pGL3-basic.

repressing Ang-2 promoter activity. Moreover, Ets-1 and Elf-1 also stimulate Ang-2 mRNA expression in bovine aortic endothelial cells. Interestingly, Ets-1 failed to induce Ang-2 promoter expression in A375 melanoma cells, while Elf-1 could stimulate Ang-2 promoter activity in these cells. This indicates that expression of Elf-1 is sufficient for upregulation of Ang-2 expression, whereas Ets-1 requires an endothelial cell specific modification to achieve its activity. Indeed, Ets-1 becomes phosphorylated by ERK2, a component of the MAP-kinase pathway. Upon this modification, Ets-1 achieves its transactivating activity (Seidel and Graves, 2002).

Regulation of Ang-2 expression in diabetes

To study the regulation of Ang-2 expression in diabetic retinopathy we collaborated with Prof. Dr. Hans-Peter Hammes (University of Mannheim, SPP1069 member). We were able to show that Ang-2 mRNA expression and promoter activity were stimulated by high glucose levels, indicating that Ang-2 is upregulated in diabetic retinopathy. This is in line with the hypothesis that Ang-2 overexpression is causing pericyte drop out in diabetic retinae and causes vessel regression (Hammes et al., 2004). Moreover, we were able to inhibit high glucose mediated Ang-2 promoter activation and mRNA expression using inhibitors of the glucose metabolism, scavengers of reactive oxygen species and a PARP-inhibitor (probucol, CCCP, TTFA, MnTBAP, benfothiamine, PJ34). In conclusion, this indicates that the development of diabetic retinopathy might be inhibited by blocking Ang-2 overexpression.

Activation of Ang-2 expression *in vivo*

In vivo Ang-2 mRNA-expression is only observed at sites of vascular remodelling and not in large quiescent vessels (Stratmann et al., 1998). This suggests that the microenvironment, e.g. the contact of endothelial cells and mural cells is regulating Ang-2 expression. To analyse the regulation of the Ang-2 promoter by mural cells *in vivo*, we have generated transgenic mice expressing human Ang-2 promoter-LacZ-constructs in collaboration with Prof. Dr. Georg Breier (University of Dresden, SPP1069 member). We have generated transgenic mice expressing an Ang-2 promoter construct containing negative regulatory elements (pAng-2-3051/+476) and two constructs lacking the negative regulatory elements (pAng-2-2004/+476; pAng-2-109/+476). In addition we generated the same constructs including sequences of the first intron, in case an intronic enhancer is mediating promoter activity *in vivo*, as it has been reported for the Tie2 and Flk-promoter (Schlaeger et al., 1997; Kappel et al., 1999). LacZ expression was analysed during development and tumor angiogenesis. Unfortunately, none of the constructs showed LacZ-expression. To ensure that we were screening the right tissues at the correct time points, we compared LacZ-expression in transgenic Ang-2-promoter mice with LacZ-expression in heterozygous and homozygous Ang-2-lacZ mice. These mice are a gift from Regeneron and have a LacZ-insertion in the Ang-2 gene locus leading to disruption of Ang-2 expression (Gale et al., 2002). In these mice LacZ-expression was detectable in large vessels, like in the dorsal aorta, during embryonic development. In adult mice, LacZ-expression was almost absent and only inducible in a subset of tumor blood vessels. Taken together we infere that the analyzed Ang-2 promoter fragments miss sequences for *in vivo* activation.

3. Storage of angiopoietin-2 in endothelial cell Weibel-Palade bodies

The finding that Ang-2 is mainly expressed in endothelial cells *in vitro* and *in vivo* supports the concept that Ang-2 acts as an autocrine regulator of endothelial cell functions. We consequently decided to study endothelial cells Ang-2 production and presentation in more detail. Tracing of angiopoietin protein expression by staining cultured endothelial cells identified Ang-2 as distributed

into granules. Based on these findings, we examined Ang-1 and Ang-2 production in endothelial cell and smooth muscle cell populations that were retrovirally transduced to overexpress full length myc-tagged Ang-1 and Ang-2. Myc staining of Ang-2 in HUVE cells identified the same granular pattern as was observed in non-transfected cells.

Storage of Ang-2 in endothelial cells

The characteristic granular pattern of transfected and endogenous Ang-2 in endothelial cells prompted us to speculate that endothelial cell Ang-2 is stored in Weibel-Palade bodies, the primary endothelial storage granule of the procoagulant von Willebrand Factor (vWF). Double staining of von Willebrand Factor and endogenous Ang-2 in HUVE cells revealed the co-localization of Ang-2 and vWF and identified Ang-2 as a Weibel-Palade body stored molecule (Fig. 5). Similarly, human umbilical artery endothelial cells (HUAEC), human aortic endothelial cells (HAoEC), and human saphenous vein endothelial cells (HSaVEC) were all found to store Ang-2 in Weibel-Palade bodies demonstrating that Ang-2 storage in Weibel-Palade bodies is not restricted to cultured HUVE cells but rather occurring in all Ang-2-producing endothelial cell populations. In contrast, α-granules, the vWF storage granule of platelets, do not contain stored Ang-2. Extending and confirming these immunofluorescence co-localization experiments, endothelial cells vWF and myc-Ang-2 were also found to be co-localized by double labeling immunogold cytochemistry. Von Willebrand Factor (vWF) is the primary constituent of Weibel-Palade bodies and expression of vWF in certain non-endothelial cells has been shown to induce the formation of Weibel-Palade bodies (Hannah et al., 2002). Correspondingly, vWF is required for the trafficking of Ang-2 into Weibel-Palade bodies as evidenced by the observation that Ang-2 is expressed in a uniform cytoplasmic pattern in endothelial cells in the absence of vWF (Fiedler et al., 2004).

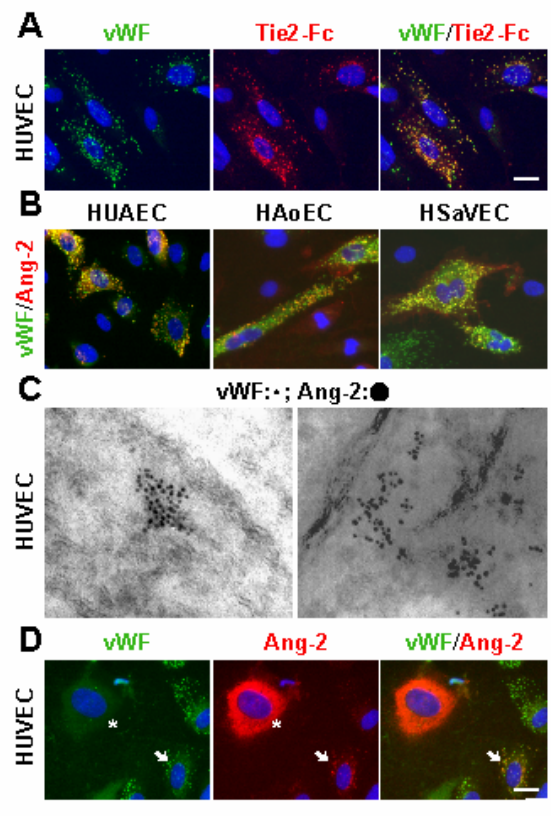


Fig. 5: Endothelial cell Ang-2 is localized in Weibel-Palade bodies (WBP). (A) Co-localization of endogenous Ang-2 (detected by Tie-2-Fc) and von Willebrand Factor (vWF) in HUVE cells. **(B)** Co-localization of Ang-2 and vWF in HUAEC, HAoEC, and HSaVEC. **(C)** Ultrastructural double immunoelectron microscopy of Ang-2 (large 10 nm particles) and vWF (small 6 nm particles) demonstrating co-localization of Ang-2 and vWF. The poor Weibel-Palade body morphology is due to the fixation procedure which imposes limitations on the postembedding immuno-cytochemistry. **(D)** vWF is required for Ang-2 storage in Weibel-Palade bodies (arrow). Cells overexpressing Ang-2 but not vWF express Ang-2 in a uniform cyto-plasmic pattern (asterisks). Scale bar in A, B, D: 20 µm; original magnification in C: 45.000x.

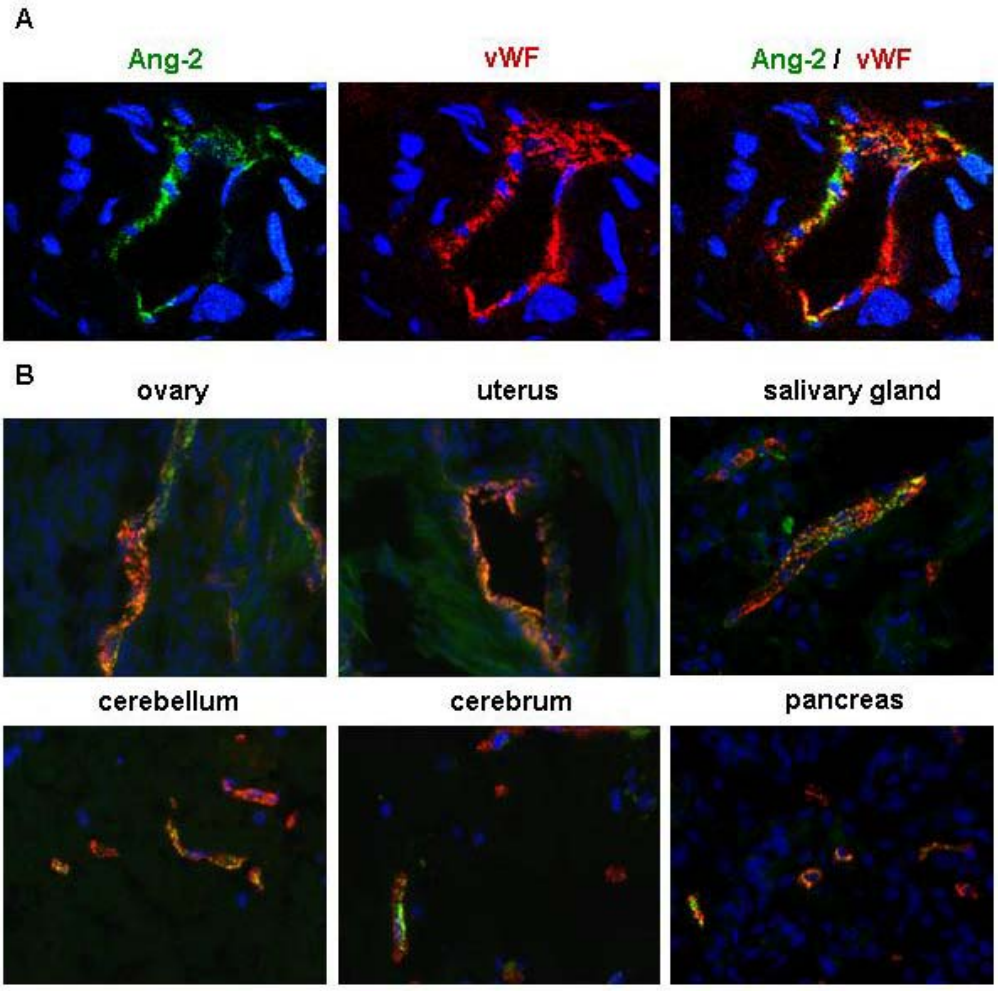


Fig. 6: Ang-2 expression and storage in human tissues. Human tissues were stained for vWF (red) and Ang-2 (green). High resolution confocal microscopy on human colon tissue reveals Ang-2 expression and storage in Weibel-Palade bodies *in vivo* (**A**). Furthermore, Ang-2 expression and storage was detected in all analyzed tissues (**B**). Yet, there is regional heterogeneity. Granular Ang-2 is detectable in most endothelial cells of the female reproductive system. Prominent expression is also detectable in the brain. Ang-2 is found in a subset of endothelial cells in most other tissues. Some tissues (e.g., skeletal muscle, cardiac muscle) contain hardly any detectable Weibel-Palade body-stored Ang-2.

Ang-2 release from Weibel-Palade bodies

The detection of Ang-2 in Weibel-Palade bodies suggested that Ang-2 is a stored molecule that may become rapidly available upon release. We consequently stimulated myc-Ang-2 overexpressing HUVE cells with secretagogues of vWF release and traced the disappearance of the intracellularly stored Ang-2 pool and the accumulation in the supernatant of stimulated cells. Ang-2 becomes detectable in the supernatant of endothelial cells stimulated for 5min with the PKC-activating phorbol ester PMA, accumulating to maximum concentrations in the supernatant within 20 min. Tracing of intracellular vWF and Ang-2 upon PMA stimulation revealed that vWF and Ang-2 export follows the same temporal kinetic. Weak vWF/Ang-2 co-localization is still detectable after 20 min and becomes essentially undetectable after 30 min of stimulation.

PMA proved to be the strongest stimulator of endothelial cell Ang-2 release. Similarly, the Ca^{2+} ionophore ionomycin and the intracellular Ca^{2+} store discharging ionophore thapsigargin are able to induce the release of Ang-2 into the supernatant of stimulated HUVE cells. In fact, the Ca^{2+} chanel

blocker nifedipine inhibits PMA-induced Ang-2 release indicating that calcium flow is the primary regulator of Ang-2 release as was previously demonstrated for von Willebrand Factor.

Stimulation of HUVE cells with histamine, TNFα, Ang-1, VEGF, FGF-2, TGFß, $CoCl_2$ (hypoxia mimicry) or high glucose does not induce Ang-2 release. Yet, stimulation of human dermal microvascular endothelial cells (HDMVEC) with either histamine or thrombin or HUVEC with thrombin induces the rapid release of Ang-2 associated with a characteristic perinuclear accumulation of Ang-2 prior to release of intracellular storage granules (Fiedler et al., 2004).

Ang-2 storage *in vivo*

The finding that endothelial cells store Ang-2 in and release it from Weibel-Palade bodies *in vitro* raised the question, whether Ang-2 is also stored in Weibel-Palade bodies *in vivo*. To this end we performed Ang-2 / vWF double stainings on human tissue and tumor samples. We verified Ang-2 storage in Weibel-Palade bodies *in vivo* (Fig. 6). Yet, there was regional heterogeneity. Stored Ang-2 was detectable in most endothelial cells of the female reproductive system (uterus, ovary, and cervix). Substantial expression was also detectable in the brain. Ang-2 was found in a subset of endothelial cells in most other tissues. Some tissues (e.g., skeletal muscle, cardiac muscle) contained hardly any detectable Weibel-Palade body-stored Ang-2 (Fiedler et al., 2006). Interestingly, although we expected that Ang-2 is constitutively released from tumor vessels, we observed prominent Ang-2 storage in some tumor vessels. Further analyses will show: (i) whether Ang-2 is stored in quiescent or activated vessels, (ii) whether Ang-2 is stored in vessels overexpressing Ang-2 mRNA, (iii) whether Ang-2 is exclusively secreted by trafficking through Weibel-Palade bodies *in vivo* or (iv) whether there is a Weibel-Palade body independent route of Ang-2 secretion. In addition, we found Ang-2 storage in lymphatic endothelial cells *in vitro* and in lymphatic vessels *in vivo*. The role of Ang-2 storage in lymphatic endothelium and its role in lymphangiogensis is one of the main topics of our current research.

4. Functional consequences of Ang-2 storage and release *in vitro* and *in vivo*

Ang-2 mediates destabilisation of the endothelium *in vitro*

Angiopoietin-2 expression and storage in endothelial cells suggested that Ang-2 acts autocrine on the endothelium and directed our research to analyses of autocrine Ang-2 functions *in vitro* and *in vivo*. To this end we joined forces with Prof. Dr. Hellmut G. Augustins Group (Tumor Biology Center Freiburg, SPP1069 member). First we analysed Ang-2 functions on the endothelium *in vitro* using a three-dimensional co-culture spheroid system. The three-dimensional co-culture system of endothelial cells and smooth muscle cells mimics the physiological assembly of the blood vessel with a quiescent monolayer of endothelial cells covering a core of smooth muscle cells. Stimulation of the co-culture spheroid with Ang-1 and VEGF had no obvious effect on the stability and the quiescence of the co-culture spheroid. However, stimulation of the co-culture spheroid with Ang-2 perturbed integrity of the endothelial cells monolayer, suggesting that Ang-2 is interfering with vessel wall integrity and quiescence. Further analyses showed that Ang-2 mediated disintegration of the co-culture spheroid occurred very fast, within 30 minutes and could be rescued by Ang-1, VEGF and sTie2-Fc. In order to study the effect of Ang-2 release from Weibel Palade bodies on the stability of the co-culture spheroid, we stimulated Ang-2 release by PMA treatment of the co-culture spheroid. PMA stimulation resulted in perturbation of the endothelial cell monolayer of the co-culture spheroid and could be rescued by co-stimulation with Ang-1 and VEGF. Surprisingly, soluble Tie2-Fc did not

rescue Ang-2 mediated co-culture spheroid perturbation, indicating that secreted Ang-2 is inaccessible for sTie2-Fc in this setting. To verify that released Ang-2 is mediating co-culture spheroid perturbation, we performed the same experiment with Ang-2 deficient endothelial cells (specific knockdown of Ang-2 in endothelial cells using siRNA). PMA treatment of co-culture spheroids formed by Ang-2 deficient endothelial cells and smooth muscle cells did trigger endothelial cell monolayer disintegration. This indicates that PMA mediated Ang-2 release destabilises the endothelium (Scharpfenecker et al. 2005).

Ang-2 functions in the inflammatory response

The finding that release of endogenous Ang-2 is interfering with the endotheliums integrity led to the hypothesis that Ang-2 mediates fast endothelial functions, e.g. inflammatory responses. To test this hypothesis, we performed inflammation experiments in Ang-2 deficient mice in collaboration with Hellmut Augustin (Tumor Biology Center Freiburg, SPP1069 member). Ang-2 deficient mice have been described to die within the first 14 days after birth due to massive chylous ascites. Breeding the mice in a C57/BL6 background resulted in a loss of the lethal phenotype and the generation of viable adult Ang-2 deficient mice. We used these mice in three different inflammation experiments. First we performed thioglycollate-mediated peritonitis experiments in Ang-2 deficient and wildtype mice. In wildtype mice thioglycollate induced massive neutrophil influx into the peritoneal cavity but not in Ang-2 deficient mice. This phenotype of the Ang-2 deficient mice could be rescued by administration of recombinant Ang-2, suggesting that the non-responsiveness to inflammatory stimuli is a direct consequence of Ang-2 deficiency. In a second approach we stimulated an inflammatory response in the peritoneal cavity of Ang-2 deficient mice and wildtype mice by injection of Staphylococcus aureus. Again Ang-2 deficient mice could not elicit a rapid inflammatory response. To study which step in the inflammatory cascade is impaired in Ang-2 deficient mice, we joined forces with Dr. Peter Vajkoczy (University of Mannheim, SPP1069 member) and preformed intravital microscopical studies. To this end we used a dorsal skin fold chamber model and analysed the different steps of inflammation after TNFα injection. We observed that Ang-2 deficiency leads to reduced firm adhesion of recruited leukocytes. In turn, the relative fraction of rolling leukocytes increased in Ang-2 deficient mice, indicating that the P-selectin mediated initiation of inflammation is not impaired, but leukocyte adhesion. This suggested that Ang-2 deficiency prevents TNFα-induced adhesion molecule expression. To test this hypothesis we performed *in vitro* monocyte adhesion experiment on confluent HUVEC monolayers stimulated with varying amounts of TNFα in the absence and presence of Ang-2. Ang-2 mediated monocyte adhesion to the HUVEC monolayer stimulated with low subsaturating concentrations of TNFα. Ang-2 by itself did not affect monocyte adhesion. This suggested that Ang-2 is sensitizing the endothelium towards TNFα stimulation. In order to investigate the molecular mechanism by which Ang-2 is facilitating TNFα induced monocyte adhesion, we analysed adhesion molecule expression using subsaturating concentrations of TNFα in the absence and presence of Ang-2. Ang-2 facilitates TNFα induced ICAM-1 and VCAM-1 expression of HUVEC. Moreover ICAM-1 expression was stimulated in wildtype cells but not in the Ang-2-deficient endothelial (specific down regulation of Ang-2 by siRNA) cells upon TNFα stimulation, indicating that Ang-2 facilitates TNFα-induced monocyte adhesion (Fiedler et al., 2006).

DISCUSSION

The angiopoietin / Tie system was studied intensively by many researchers during the last three funding periods (six years). Most scientists focussed on the role of the receptor tyrosine kinase Tie2 and its agonistic ligand Ang-1. Ang-1 is expressed by mural cells and some tumor cells. Its expression is only slightly regulated. Ang-1 mediated Tie2 signaling regulates endothelial cells

survival and blood vessel maturation (Suri et al., 1996). Ang-1 acts anti-permeable, anti-inflammatory, protects against infection and allograft arteriosclerosis (Thurston et al., 2000; Gamble et al., 2000; Witzenbichler et al., 2005; Nykanen et al., 2003). Ang-1 is the Tie2 agonist and mediates Tie2 phosphorylation. It is commonly appreciated that low-level Tie-2 phosphorylation is required to maintain the mature quiescent phenotype of the resting vascular endothelium in the adult (Wong et al., 1997; Kruse and Fiedler et al, unpublished). Moreover, Ang-1 induces blood and lymph angiogenesis and enlargement of blood vessels upon overexpression (Thurston et al., 1999; Tammela et al., 2005). In summary, a tight balance of Tie-2 signaling appears to be required for maintenance of the vascular bed. Genetic experiments revealed that Ang-2 is the natural antagonist of Ang-1 / Tie2 signaling by binding to the receptor without induction of signal transduction (Maisionpierre et al., 1997). The opposing effect of Ang-1 and Ang-2 supported a model of constitutive Ang-1/Tie2 signaling controlling vascular homeostasis as a default pathway and Ang-2 acting as a dynamic antagonistic cytokine. Based on this hypothesis we focussed our research on Ang-2 regulation and functions. Ang-2 expression is tightly controlled. Expression analysis of our and other groups showed strong upregulation of Ang-2 mRNA expression in human and murine tumors (Statmann et al., 1998, Holash et al., 2000). In situ hybridisation experiments showed that Ang-2 expression is restricted to the vascular compartment of the tumor. Interestingly, Ang-2 mRNA expression is only observed in a few tumor vessels, which might be tumor-neovessels and is almost absent in all other vessels in murine tissues (Koidl and Fiedler, unpublished). Analyses of LacZ-expression in mice with a LacZ-insertion into the Ang-2 locus (Ang-2 deficient mice, gift from Regeneron) verified our finding. We found strong LacZ expression in tumor vessels and very weak to no expression in the resting murine vasculature (Hegen and Fiedler, unpublished). These results are perfectly in line with the findings that Ang-2 overexpression is not compatible with life, indicating that dosage and spatio-temporal Ang-2 appearance needs to be tightly controlled. To shed further light into the regulation of Ang-2 expression we have cloned and analysed the human Ang-2 promoter. We have isolated a genomic DNA fragment of 6713 bp encompassing 4427 bp upstream of the transcriptional start site, 476 bp untranslated region, the first exon until bp 288 and 1522 bp of the first intron. Furthermore, we identified a CpG-island surrounding the transcriptional start site, suggesting regulation of promoter activity by imprinting. Detailed analysis revealed no regulation of Ang-2 promoter activity by imprinting, but identified the Tie2-gene as an imprinted gene. Deletion analysis of the promoter fragment identified two strong negative regulatory elements, one within the upstream region of the Ang-2 promoter encompassing bp -4427 to -2227 and one within the first intron. Deletion of these fragments resulted in a strong activation of promoter activity, identifying a promoter fragment encompassing bp – 109 to +476 as being sufficient to mediate endothelial cell specific promoter activity. Our analysis showed that the Ang-2 promoter is an endothelial cell specific promoter, showing no activity in all tested non-endothelial cells. Moreover we identified Ets-1 as endothelial cell specific regulator of Ang-2 promoter activity and Elf-1 as being capable to induce Ang-2 promoter activity in non-endothelial cells too. Since Ets-1 is not specifically expressed in endothelial cells this suggested that other transcription factors or signals act together with Ets-1 to mediate endothelial cell specific Ang-2 expression. Analyses of signals regulating Ang-2 promoter activity in endothelial cells showed that VEGF and FGF are potent inducers of Ang-2 promoter activity, implying that these factors might drive Ang-2 expression in tumor vessels. Indeed we found a strong correlation of Ang-2 and VEGF overexpression in human tumor samples (Hegen et al., 2004). Interestingly, we identified high glucose levels as potent inducers of Ang-2 promoter activity and mRNA expression (Kruse and Fiedler, unpublished). Upregulation was completely abrogated if cells were co-stimulated with drugs that interfere with glucose metabolism and reactive oxygen species formation. This finding puts perspective in targeting Ang-2 expression in patients with diabetic retinopathy.

The expression of Ang-2 in endothelial cells suggested that Ang-2 may act in an autocrine manner on endothelial cells to control the quiescent and activated state of the endothelium. This notion was further supported by the identification of Ang-2 as a Weibel-Palade body stored protein in endothelial cells (Fiedler et al., 2004). Immunocytochemical analyses identified Ang-2 to be stored in granules in all tested endothelial cell; e.g. aortic, arterial, venous, microvascular and lymphatic endothelial cells. Light and electron microscopy double staining with von Willebrand factor, revealed that Ang-2 is a Weibel-Palade body stored molecule, like P-selectin, CD63, Endothelin-1, tPA, etc.. Ang-2 is rapidly released from Weibel-Palade bodies by challenging cells with secretagogues that increase intracellular calcium or cAMP levels, like thrombin, histamine and PMA. Release was induced within minutes and completed after 20 minutes. Thus, Ang-2 is rapidly available on the cell surface of endothelial cells and is regulating rapid vascular reactions. Moreover, we found that stored Ang-2 has a half-life of at least 18 hours and appears in Weibel-Palade bodies again 6 hours after release. Fortunately we received an antibody from our collaborators at Regeneron Pharmaceuticals (Tarrytown, New York, United States) that allowed us to stain Ang-2 in human tissue samples. We identified Ang-2 to be stored in Weibel-Palade bodies in a subset of endothelial cells in most tissues. Interestingly, there was regional heterogeneity. Stored Ang-2 was detectable in most endothelial cells of the female reproductive system (uterus, ovary, cervix), prominently expressed in brain and almost absent in muscle tissues (Fiedler et al., 2006). Moreover, we identified Ang-2 to be stored in Weibel-Palade bodies in lymphatic vessels. Surprisingly, prominent Ang-2 storage was observed in some tumor vessels (Koidl and Fiedler, unpublished). We expected Ang-2 to be constitutively released from tumor vessels due to its strong overexpression and the fact that only subsets of tumor vessels express vWF. In conclusion, these findings raise a number of interesting questions which need to be addressed in the future, e.g.: (i) Why is only a subset of endothelial cells storing detectable amounts of Ang-2 *in vivo*?, (ii) Is Ang-2 secreted only through Weibel-Palade bodies *in vivo*, or are there other trafficking pathways?, (iii) Do Ang-2 mRNA expression and Ang-2 protein storage correlate?, (iv) How stable is stored Ang-2 *in vivo*?, (v) What is the Ang-2 turn over?, (vi) What are the signals triggering Ang-2 release *in vivo*? and (vii) What is the role of stored Ang-2 *in vivo*?

Our finding that Ang-2 is stored in Weibel-Palade bodies *in vitro* and *in vivo* and becomes rapidly released, points to a function of Ang-2 in rapid vascular homeostatic reactions such as inflammation and coagulation. To this end we studied the role of Ang-2 in inflammation. We made use of the fact that Ang-2 deficient mice are viable in the C57/BL6 background. We compared the inflammatory response in wildtype and Ang-2 deficient mice in short-term peritonitis experiments and a dorsal skin-fold chamber experiment in which we induced inflammation by TNFα. Ang-2 deficient mice did not elicit an inflammatory response in both experimental setups. Moreover, we detected leukocyte rolling but not adhesion in Ang-2 deficient mice, suggesting that expression of adhesion molecules is impaired in response to TNFα stimulation. Cell culture experiments revealed that Ang-2 promotes adhesion of monocytes by sensitizing the endothelial cells towards TNFα and modulating TNFα-induced endothelial cell adhesion molecule expression. Taken together, our findings identify Ang-2 as an autocrine regulator of endothelial cell inflammatory responses. Ang-2 thereby acts as a switch of vascular responsiveness exerting a permissive role for the activities of proinflammatory cytokines (Fiedler et al., 2006).

In order to get deeper insights into the mechanisms by which Ang-2 and particularly stored Ang-2 is mediating vascular responsiveness, we performed cell culture experiments using a co-culture spheroid model that mimics the vessel wall (Korff et al., 2001). We observed that exogenous recombinant Ang-2 disturbs co-culture spheroid integrity by inducing detachment of endothelial cells. In addition release of stored Ang-2 from Weibel-Palade bodies has a similar effect suggesting that release of Ang-2 from Weibel-Palade bodies interferes with the quiescent state of the endothelium. Constitutive Ang-1 mediated Tie2 signaling is suggested to mediate quiescence of the vascular bed.

In line with this notion we found that Ang-1 rescues Ang-2 mediated perturbation of the endothelium. Moreover, an inhibitor of Tie2 signaling is also disturbing integrity of the co-culture spheroid. This indicates that Ang-2 interferes with Tie2 signaling and mediates activation of the endothelium (Scharpfenecker et al., 2005). The exact mechanism by which Ang-2 interferes with Tie2 signaling is not fully understood. Genetic experiments revealed that mice deficient for Tie2 and Ang-1 have a similar phenotype as mice embryonically overexpressing Ang-2, namely the loss of vessel maturation and remodelling leading to death of the embryo around day E10.5 – E12 (Davis et al., 1996; Suri et al., 1996, Maisionpierre et al., 1997). Correspondingly, experiment in endothelial cells show that Ang-1 binding to Tie2 leads to receptor tyrosine phosphorylation which can be blocked by Ang-2. Surprisingly, both Ang-1 and Ang-2 induce Tie2 phosphorylation upon expression of Tie2 in non-endothelial cells (Maisonpierre et al., 1997). This suggests that either Ang-1 and Ang-2 bind to different sites in Tie2 and transduce different signals or that an endothelial cell immanent regulatory mechanism is contributing to the antagonistic Ang-2 effect on Tie2 phosphorylation and signaling. In order to study whether Ang-1 and Ang-2 bind to the same binding sites in Tie2, we mapped the Ang-1 and Ang-2 binding sites in Tie2. We generated various Tie2-Fc fusion proteins containing different domains of Tie2 and observed that Ang-1 and Ang-2 bind to the same sites in Tie2 with similar affinity. Ang-1 and Ang-2 binding to Tie2 requires both the first Ig-like loop and the EGF-like repeats. This finding was further corroborated by the observation that a Tie2 version lacking the first 104 aa does not bind Ang-1 and Ang-2. Taken together, this indicates that differential receptor binding is not responsible for the different Ang-1 and Ang-2 functions (Fiedler et al., 2003). Thus, an endothelial cell innate mechanism controls agonistic Ang-1 and antagonistic Ang-2 functions. One attractive model would be that the second Tie-receptor Tie1 is controlling agonistic and antagonistic angiopoietin functions. There is increasing evidence that Tie1 and Tie2 act together: (i) Tie1 and Tie2 can form heterodimers (Marron et al., 2000) and (ii) Ang-1 binds both membrane bound Tie1 and Tie2 and induces tyrosine-phosphorylation (Saharinen et al., 2005). Moreover, we have observed that shedding of Tie1 by VEGF leads to the interaction of the membrane-anchored intracellular domain of Tie1 with Tie2 (Mössinger, Thomas and Fiedler, unpublished). Further experiments will show whether full-length Tie1 or the shedded version are controlling agonistic and antagonistic angiopoietin functions.

In conclusion our studies reveal that Ang-2 is a critical regulator of vascular homeostasis and responsiveness towards cytokines. First, Ang-2 is stored in endothelial cells and released upon activation. Ang-2 release perturbs vascular integrity and sensitizes the vasculature to cytokine responses. Secondly, Ang-2 becomes upregulated in activated endothelial cells *in vitro* and *in vivo*, which might induce a second wave of the angiogenic response upon stimulation. This suggests that Ang-2 is both a primer of the angiogenic cascade by disturbing the quiescent state of the endothelium and a driver of angiogenesis by promoting long term cytokine stimulated angiogenesis. In summary we propose the following model (Fig. 7): Constitutive Ang-1 expression is mediating constitutive Tie2 phosphorylation and controls the quiescent mature state of the endothelium. Stimulation of Weibel-Palade body release from the endothelium by histamine, thrombin, superoxide generation due to cellular stress, etc., results in release of Ang-2 and high local Ang-2 concentrations that perturb the quiescent state of the endothelium. The activated endothelium is now susceptible for cytokine stimulation, leading to inflammatory or angiogenic responses. In a second line of regulation Ang-2 becomes upregulated and induces vessel regression in the absence of an angiogenic cytokine or to angiogenesis in its presence. This model suggests that Ang-2 is involved in many rapid vascular homeostatic reactions that are linked to many diseases like, coagulation, inflammation, retinopathy, atherosclerosis and psoriasis. Altogether this provides a strong rational for the exploitation of anti-Ang-2 therapies beyond antiangiogenic cancer therapies.

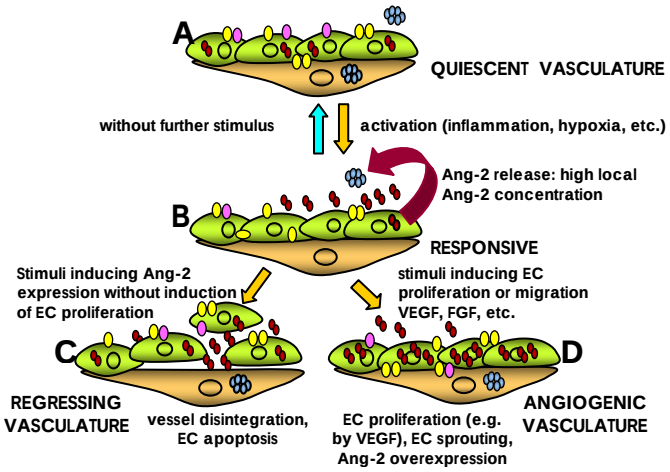


Fig. 7: Model of Angiopoietin/Tie interactions in the regulation of vascular quiescence (A), vascular responsiveness (B), vascular regression (C), and angiogenesis (D). **(A)**: The quiescent endothelial cell phenotype is maintained by constitutive oligomeric Ang-1 (blue) activation of Tie-2 (yellow). Tie-1 (purple) can heterodimerize with Tie-2 and may act as a modulator of Ang-1/Tie-2 signaling. Quiescent endothelial cells contain stored dimeric Ang-2 (red). **(B)**: Activation of endothelial cells leads to liberation of autocrine Ang-2 and subsequent transcriptional Ang-2 upregulation which interferes with constitutive Ang-1/Tie-2 signaling and yields endothelial cells responsive to the activities of other cytokines (e.g., inflammatory or angiogenic). **(C)**: Continued exposure of endothelial cells to Ang-2 in the absence of other cytokines leads to endothelial apoptosis and subsequent vessel regression. **(D)**: Conversely, continued Ang-2 primes endothelial cells towards angiogenic stimuli. Transcriptional upregulation of Ang-2 in endothelial cells is an early event of the angiogenic cascade.

REFERENCES

Carmeliet P: Mechanisms of angiogenesis and arteriogenesis: *Nat Med*. 4: 389-95, 2000.

Cho CH, Kammerer RA, Lee HJ, Yasunaga K, Kim KT, Choi HH, Kim W, Kim SH, Park SK, Lee GM, Koh GY: Designed angiopoietin-1 variant, COMP-Ang1, protects against radiation-induced endothelial cell apoptosis. *Proc Natl Acad Sci* 101: 5553-8, 2004.

Fiedler U, Krissl T, Koidl S, Weiss C, Koblizek T, Deutsch U, Martiny-Baron G, Marme D, Augustin HG: Angiopoietin-1 and angiopoietin-2 share the same binding domains in the Tie-2 receptor involving the first Ig-like loop and the epidermal growth factor-like repeats. *J Biol Chem* 278: 1721-7, 2003.

Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, Kriz W, Thurston G, Augustin HG: The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood* 103: 4150-6. 2004.

Fiedler U*, Reiss Y*, Scharpfenecker M*, Grunow V, Thurston G, Gale NW, Sobke A, Herrmann M, Preissner K, Vajkoczy P, Augustin HG: Angiopoietin-2 sensitizes endothelial cells to TNFα and has a crucial role in the induction of inflammation. *Nat Med* 12: 235-9, 2006.

Gale NW, Yancopoulos GD: Growth factors acting via endothelial cell-specific receptor tyrosine kinases: VEGFs, angiopoietins, and ephrins in vascular development. *Genes Dev* 13: 1055-66, 1999.

Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D, Suri C, Campochiaro PA, Wiegand SJ, Yancopoulos GD: Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Dev Cell* 3: 411-23, 2002.

Gamble JR, Drew J, Trezise L, Underwood A, Parsons M, Kasminkas L, Rudge J, Yancopoulos G, Vadas MA: Angiopoietin-1 is an antipermeability and anti-inflammatory agent In vitro and targets cell junctions. *Circ Res* 87: 603-7, 2000.

Hammes HP, Lin J, Wagner P, Feng Y, Vom Hagen F, Krzizok T, Renner O, Breier G, Brownlee M, Deutsch U: Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. *Diabetes* 53: 1104-10, 2004.

Hanahan D, Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86: 353-64, 1996.

Hannah MJ, Williams R, Kaur J, Hewlett LJ, Cutler DF: Biogenesis of Weibel-Palade bodies. *Cell Dev Biol* 13: 313-24, 2002.

Hegen A, Koidl S, Weindel K, Marmé D, Augustin HG, Fiedler U: Strong positive and negative regulation of endothelial cell specific Angiopoietin-2 expression. *Arterioscler Thromb Vasc Biol* 24: 1803-9, 2004.

Holash J, Maisonpierre PC, Compton D, Boland P, Alexander CR, Zagzag D, Yancopoulos GD, Wiegand SJ: Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science.* 284: 1994-8, 1999.

Jeon BH, Khanday F, Deshpande S, Haile A, Ozaki M, Irani K: Tie-ing the antiinflammatory effect of angiopoietin-1 to inhibition of NF-kappaB. *Circ Res* 92: 586-8, 2003.

Kappel A, Ronicke V, Damert A, Flamme I, Risau W, Breier G: Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice. *Blood* 93: 4284-92, 1999.

Kim I, Moon SO, Koh KN, Kim H, Uhm CS, Kwak HJ, Kim NG, Koh GY: Molecular cloning, expression, and characterization of angiopoietin-related protein angiopoietin-related protein induces endothelial cell sprouting. *J Biol Chem* 274: 26523-8, 1999.

Korff T, Kimmina S, Martiny-Baron G, Augustin HG: Blood vessel maturation in a 3-dimensional spheroidal coculture model: direct contact with smooth muscle cells regulates endothelial cell quiescence and abrogates VEGF responsiveness. *FASEB J* 15: 447-57, 2001.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277: 55-60, 1997.

Mandriota SJ, Pepper MS: Regulation of angiopoietin-2 mRNA levels in bovine microvascular endothelial cells by cytokines and hypoxia. *Circ Res* 83: 852-9, 1998.

Marron MB, Hughes DP, Edge MD, Forder CL, Brindle NP: Evidence for heterotypic interaction between the receptor tyrosine kinases TIE-1 and TIE-2. *J Biol Chem* 275: 39741-6, 2000.

Nykanen AI, Krebs R, Saaristo A, Turunen P, Alitalo K, Yla-Herttuala S, Koskinen PK, Lemstrom KB: Angiopoietin-1 protects against the development of cardiac allograft arteriosclerosis. *Circulation* 107: 1308-14, 2003.

Ramsauer M, D'Amore PA: Getting Tie(2)d up in angiogenesis. *J Clin Invest* 110: 1615-7, 2002.

Saharinen P, Kerkela K, Ekman N, Marron M, Brindle N, Lee GM, Augustin H, Koh GY, Alitalo K: Multiple angiopoietin recombinant proteins activate the Tie1 receptor tyrosine kinase and promote its interaction with Tie2. *J Cell Biol* 169: 239-43, 2005.

Sato TN, Qin Y, Kozak CA, Audus KL: Tie-1 and tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc Natl Acad Sci USA* 90: 9355-8, 1993.

Scharpfenecker M, Fiedler U, Reiss Y, Augustin HG: The Tie-2 ligand Angiopoietin-2 destabilizes quiescent endothelium trough an internal intractine loop mechanism: *J Cell Sci* 118: 771-80, 2005.

Schlaeger TM, Bartunkova S, Lawitts JA, Teichmann G, Risau W, Deutsch U, Sato TN: Uniform vascular-endothelial-cell-specific gene expression in both embryonic and adult transgenic mice. *Proc Natl Acad Sci USA* 94: 3058-63, 1997.

Stratmann A, Risau W, Plate KH: Cell type-specific expression of angiopoietin-1 and angiopoietin-2 suggests a role in glioblastoma angiogenesis. *Am J Pathol* 153: 1459-66, 1998.

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD: Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87: 1171-80, 1996.

Tammela T, Saaristo A, Lohela M, Morisada T, Tornberg J, Norrmen C, Oike Y, Pajusola K, Thurston G, Suda T, Yla-Herttuala S, Alitalo K: Angiopoietin-1 promotes lymphatic sprouting and hyperplasia. *Blood* 105: 4642-8, 2005.

Thurston G, Suri C, Smith K, McClain J, Sato TN, Yancopoulos GD, McDonald DM: Leakage-resistant blood vessels in mice transgenically overexpressing angiopoietin-1. *Science* 286: 2511-4, 1999.

Thurston G, Rudge JS, Ioffe E, Zhou H, Ross L, Croll SD, Glazer N, Holash J, McDonald DM, Yancopoulos GD: Ang-1 protects the adult vasculature against plasma leakage. *Nat Med* 6: 460-3, 2000.

Valenzuela DM, Griffiths JA, Rojas J, Aldrich TH, Jones PF, Zhou H, McClain J, Copeland NG, Gilbert DJ, Jenkins NA, Huang T, Papadopoulos N, Maisonpierre PC, Davis S, Yancopoulos GD: Angiopoietins 3 and 4: diverging gene counterparts in mice and humans. *Proc Natl Acad Sci USA* 96: 1904-9, 1999.

Wong AL, Haroon ZA, Werner S, Dewhirst MW, Greenberg CS, Peters KG: Tie2 expression and phosphorylation in angiogenic and quiescent adult tissues. *Circ Res* 81: 567-74, 1997.

Veikkola TM, et al: Role of Angiopoietin-2 in regulation of lymphatic and hematopoietic development. *Proc. XIIth Int. Vasc. Biol. Meeting*; p.71, 2002.

Vikkula M, Boon LM, Carraway KL 3rd, Calvert JT, Diamonti AJ, Goumnerov B, Pasyk KA, Marchuk DA, Warman ML, Cantley LC, Mulliken JB, Olsen BR: Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase Tie2. *Cell* 87: 1181-90, 1996.

Visconti RP, Richardson CD, Sato TN: Orchestration of angiogenesis and arteriovenous contribution by angiopoietins and vascular endothelial growth factor (VEGF). *Proc Natl Acad Sci USA* 99: 8219-24, 2002.

Angiopoietin-2 and its Modulation in Diabetic Retinopathy

Prof. Dr. Hans-Peter Hammes

5th Medical Clinic
University of Heidelberg
Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

Member from the SPP 1069 from 2002 until 2006

SUMMARY

Proliferative diabetic retinopathy is the leading cause of blindness in developed countries. The earliest morphological change in the diabetic retina is the loss of pericytes, followed by the formation of acellular capillaries. Several ligand-receptor systems have been suggested to be involved in this process. The Angiopoietin(Ang)-Tie2 system plays an important role in physiological angiogenesis, and may actively participate in the initiation of vascular pathological changes in diabetic retinopathy. While (Ang)-1 activates the receptor-tyrosin kinase Tie-2, Ang-2, a natural antagonist of Ang-1, interrupts the phosphorylation of Tie-2 induced by Ang-1. Upregulated Ang-2 prior to pericyte dropout has been demonstrated in the diabetic retinopathy model. For a better understanding and a possible basis for future interventions, we established and characterized a new transgenic mouse which overexpresses Ang-2 under control of the photoreceptor opsin promoter. This mouse exhibits promotion in developmental retinal angiogenesis and in the model of hypoxia-induced retinal neovascularization. Overexpression of Ang-2 in the retina induces pericyte loss in retinal capillaries, and results in serious vascular morphological change in experimental diabetic retinopathy. Consistent with these data we showed that injection of recombinant Ang-2 leads to pericyte loss in the adult rat eye, indicatingthat Ang-2 is essential for the initiation of pericyte dropout. Moreover, we used an Ang-2LacZ knock-in mouse line, which carries the reporter gene LacZ to assess the expression and regulation of Ang-2 under different conditions. This mouse, which is equivalent to a knock-out mouse, shows aberrant retinal vascular angiogenesis and remodeling. In postnatal angiogenesis/remodeling, Ang-2 is expressed in the endothelial cells, in particular in endothelial cells within sprouts, but not in the pericytes. A reduction of Ang-2 expression by half protects the diabetic retina from pericyte loss and partially from formation of acellular capillaries. In vitro, expression of Ang-2 is elevated in endothelial cells and Müller cells cultured in high glucose through activation of its promoter. Experiments using metabolic signal blockers and catalytic antioxidants consistently demonstrated an inhibition of Ang-2 transcription in vitro. In vivo, the reduction of Ang-2 expression and improvement of retinal vascular pathological change were realized by using these compounds in different animal models for early diabetic retinopathy.

INTRODUCTION

Diabetic retinopathy is one of the major microvascular complications in both type 1 and type 2 diabetes. Proliferative diabetic retinopathy is the leading cause of blindness among adults of working age (Thylefors et al., 1995)

Studies on retinal capillaries of both diabetic humans and diabetic animals revealed that the loss of pericytes is the first morphologic change in a diabetic retina. Endothelial cells subsequently disappear, leaving behind acellular capillaries, which are no longer perfused (Kohner and Henkind, 1970). Finally, capillary nonperfusion with hypoxia stimulates proliferative retinopathy, which is

characterized by the growth of new vessels from the retina into the vitreous and defined as retinal neovascularization in proliferative diabetic retinopathy.

The interaction between endothelial cells and pericytes has been reviewed elsewhere (Armulik et al., 2005). The coordinated cell-cell interaction of the vascular wall is characteristic of the mature, quiescent blood vessel network. Within this context, the recruitment of pericytes to the capillary wall during vascular development is an important step in the maturation of the vascular system and provides relative insensitivity of the vessel system to proliferative signals. Endothelial cells can initiate, but not complete angiogenesis. Mural cells such as pericytes are essential for the stabilization of the provisional capillary as they inhibit endothelial proliferation and migration, and stimulate matrix synthesis. Several other molecules have been implicated in the recruitment of mural cells/smooth muscle cells with protective effects against vessel rupture or regression. VEGF-VEGFR system and the Angiopoietin-Tie system belong to the modulators of endothelial-pericyte interactions.

It has been evident that several angiogenic pathways are involved in proliferative diabetic retinopathy (Aiello et al., 1994, , Patel et al., 2005, Watanabe et al., 2005). VEGF is implicated as an important factor in embryonic vasculogenesis and angiogenesis as well as in postnatal physiological and pathological angiogenesis. VEGF is hypoxia inducible and has endothelial-specific mitogenic activity, it is increased in the vitreous of patients with active neovascularizations in the eye, and VEGF-inhibition can reduce experimental proliferative retinopathy. It is identified as a primary mediator of intraocular angiogenesis and permeability in proliferative diabetic retinopathy (Leung et al., 1989). However, VEGF is not only important during the angio-responsive periods of retinopathy, but most likely already during early periods of increased endothelial damage by incipient/permanent hyperglycemia (Hammes et al., 1998). Experiments in diabetic rats show that VEGF and its receptors are upregulated by hyperglycemia (Hammes et al., 1998) and by AGE (Lu et al., 1998) suggesting that VEGF may serve as a survival factor for increased endothelial cell damage by hyperglycemia or its sequelae.

Recently, new factors have been identified that appear to modulate the angiogenic response by affecting both endothelial cells and pericytes. One member is the endothelial receptor tyrosine kinase Tie2 and its two ligands Ang-1 and Ang-2. According to gene-targeting experiments in vivo, Ang-1 signaling via Tie-2 is involved in endothelial cell survival, capillary sprouting and vascular remodeling (Davis et al., 1996, Sato et al., 1995, Suri et al., 1996). Importantly, Ang-1 has also been implicated in the stabilization of vessels by recruiting pericytes and smooth muscle cells to the vessel wall. Ang-2, a natural antagonist of Ang-1, can antagonize the effect of Ang-1–stimulated Tie-2 activation by inhibiting the autophosphorylation of Tie-2 (Maisonpierre et al., 1997). Several lines of evidence suggest that Ang-2 in combination with vascular endothelial growth factor (VEGF) leads to sprouting angiogenesis while Ang-2 in the absence of growth-promoting signals renders vessels susceptible to regression (Maisonpierre et al., 1997, Hanahan 1997, Holash et al., 1999, Jain et al., 2003, Carmeliet et al., 2003).

Ang 2 is normally only expressed in the female reproduction system in which repetitive physiologic angiogenesis occurs. This suggests that Ang 2 activity renders the vasculature sensitive to proliferative signals, in part by blocking Ang 1-induced recruitment of growth-inhibiting mesenchymal cells. Ang 2 is also upregulated in cancer, and in proliferative retinopathy. Oh et al. showed Ang 2 mRNA upregulation in response to hypoxia and to VEGF in cultured cells and in the hypoxia-induced retinal neovascularization model of the mouse. In response to hypoxia, Ang 2 showed a moderate increase in the ganglion cell layer and an intense signal in the inner nuclear layer close to vessels. At the time of peak neovascularization which (around p17 in this model), an intense signal in neovascular tufts was detected. Ang 2 was also found in all neovascular membranes which were examined in a study with subretinal neovascularization in age-related macular degeneration. Of note, there was largely congruent expression with VEGF, suggesting that both growth factors are also crucial in subretinal neovascularization. Using the reporter gene LacZ

under the control of the Ang 2 promotor, Hackett et al. have reported on the expression of Ang 2, in horizontal cells, in cells of unknown origin and cells adjacent to preretinal neovascularizations.

The biochemical mechanisms by which chronic hyperglycemia lead to vascular damage involves increased flux of glucose metabolites through the sorbitol, the hexosamine pathway, increased production of advanced glycation end products, and activation of the protein kinase C pathway by increased de-novo synthesis of diacyl-glycerol. Recently, these seemingly independent biochemical pathways have been linked by the findings that one single mechanisms, hyperglycemia-induced mitochondrial overproduction of reactive oxygen species, is the underlying cause, which, mediated through the enzyme poly-ADP-ribose polymerase blocks activities of the critical glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase (for a review, see Brownlee et al., 2005).

The underlying cause of initial pericyte dropout is unclear at present. Pericyte loss may be the consequence of hyperglycemia and its sequelae such as formation of reactive oxygen intermediates and AGEs. Blood glucose normalization by intensified insulin treatment prevents pericyte loss almost completely and antioxidant treatment also inhibits pericyte dropout significantly. The formation and accumulation of advanced glycation end products (AGE) was found in pericytes. As AGE may be toxic for pericytes, it was speculated that they contribute to the pericyte loss. The experimental studies using pharmacologic approaches to prevent early diabetic retinopathy show that the induction of cell death via NFkB pathway might be possible for pericyte dropout (Remon et al., 2002). The discrepancies between the occurrence of pericyte loss and the time course of accumulation of toxic products in these cells suggests that the initiation of pericyte dropout in diabetic retinopathy results from mechanisms different than the simple cellular accumulation of toxic intermediates. The link between pericyte dropout and the subsequent development of acellular capillaries which represent the driving force for ischemia-driven retinal vascular remodelling and neovascularization remains speculative. Accordingly the closed association between VEGF and Ang-2 in physiological as well as in pathophysiological angiogenesis, we speculated that Ang-2 may play an important role in the initiation of diabetic retinopathy.

Therefore, our studies aimed to dissect the effect of hyperglycemia on Ang-2 expression, and to study the relation between Ang-2 expression and pericyte loss in the diabetic retina. Furthermore, we established a transgenic mouse line by inducing murine retina-specific constitutive overexpression of Ang-2. This mouse line and another mouse line with genetical Ang-2 inactivation were used to study in a specific focus on vasoregressive (diabetic) and vasoproliferative (retinopathy of prematurity, ROP) model systems. We also studied Ang-2 expression using in-vitro cell systems. In these systems, the modulation of Ang-2 by a number of pharmaceutic substances, such as metabolic signal blockers and catalytic reactive oxygen scavengers was investigated.

METHODS

Within the period of the SPP 1069 program we established or modified several methods in our lab to investigate the role of angiopoietin-2 in the physiological and pathophysiological angiogenesis. Generally, we use animal models for in vivo and cell culture system for in vitro and retinal explantation for ex vivo. The animal models have been described in detail elsewhere (Hammes, et al., 1991, Hammes, et al., 1996).

Diabetic mouse model

In brief, eight week old mice were made diabetic by peritoneal injection of 150 mg/kg body weight streptozotocin (STZ). For the diabetic rat model, we used six week-old Wistar rats which were rendered diabetic by intravenous STZ injection of 65 mg/kg body weight. Diabetes was monitored by

measurements of body weight (weekly), glucose levels (weekly to monthly), and glycated hemoglobin is measured at the end of study. The animals were sacrificed at different time intervals.

Retinal digestion preparation

Retinas are obtained at the end of the studies after enucleation of the eyes and immediately fixed in 4% buffered formalin. Retinal vascular preparations were performed using a pepsin-trypsin digestion technique as previously described (Hammes et al., 1991). Briefly, a combined 5% pepsin and 2.5% trypsin digestion was used to isolate the retinal vasculature. Subsequently, the samples were stained with periodic acid Schiff base (PAS). The total number of pericytes was counted in 10 randomly selected fields of the retina using an image analyzing system. Retinal digest preparations were used to disclose quantitative changes in pericyte coverage of retinal capillaries (pericyte recruitment), density of the vascular network (vascular remodeling) and the formation of acellular capillaries (capillary regression; i.e., loss of pericytes and endothelial cells).

Analysis of physiological retinal vascular angiogenesis

The retinal vasculature in the mouse develops after birth. Therefore, it is a suitable tissue to study the effect of different factors as modulated by transgenic techniques or pharmaceutical interventions on angiogenesis and remodeling. Developmental angiogenesis in the transgenic mice was studied in the retinae from animals at postnatal (p) day p5, p10, p15 and p26 stained with the vascular marker lectin *Bandeiraea Simplicifolia*. Retinal sprouting angiogenesis, outgrowth of the retinal vessels, retinal capillary density, diameters of retinal arterioles, venules and capillaries were quantified by image analysis.

ROP model - the mouse model of Retinopathy Of Prematurity

This mouse model was used to study proliferative retinopathy in vivo. Briefly, as illustrated in Fig. 1, mice at postnatal day 7 were exposed to 75% oxygen for 5 days with their nursing mother, and return to room air at day 12. At day 17, eyes were enucleated under deep anesthesia and fixed in 4% buffered formalin, and vertical paraffin sections (6 µm) are prepared. The numbers of nuclei in newly formed vessels at the vitreous side of the inner limiting membrane (ILM) are counted on the sections stained with Periodic Acid-Schiff (PAS) (Fig. 1A, 1B).

Analysis of pathological retinal vascular angiogenesis

The retinas were stained with lectin *Bandeiraea Simplicifolia* to visualize vessels. As summarised in figure 1D-1E, the vasoregression under hyperoxia and vessel regrowth under hypoxia were studied by measuring the avascular zone evoked in ROP model.

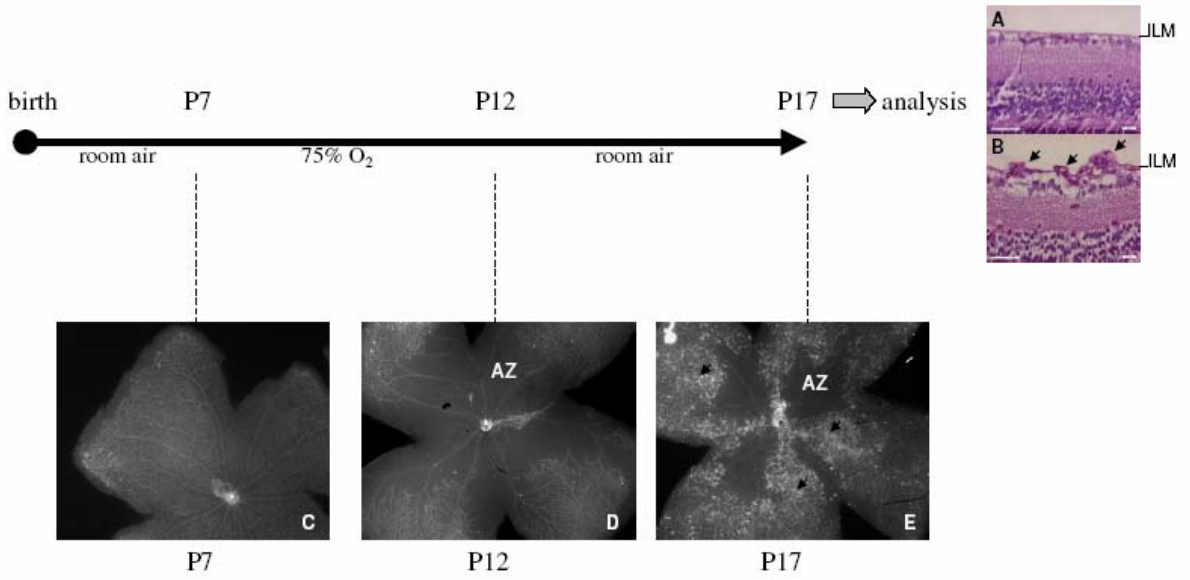


Fig. 1: Illustration of ROP model. B shows the retinal neovascularizations (arrows) in the ROP model which do not occur in the normal retina (A). C, D and E are the whole mount retinas at indicated time pointsstained with lectin to visualize the normal developed retinal vessels at P7 (C), regressed central retinal capillaries at P12 in ROP model (AZ in D), remained AZ and neovascularization (arrows in E) at P17 in ROP model.

Whole mount retina immunofluorescence

Whole-mount retina preparations are used from animals during retinal development, from ROP model or diabetic model for immunofluorescence staining with different cell specific markers to study the interaction of certain retinal cells and the retinal vascular cells to identify their contribution to the vascular morphological change under pathological conditions.

RESULTS

Angiopoietin-2 causes pericyte dropout in diabetic retinopathy, and even in the normal retina

We determined the time course of pericyte loss in the diabetic rat model over a period of 3 months of diabetes using quantitative retinal morphometry. It was found that pericyte loss started around two months of experimental diabetes (Fig. 2A-C). We then assessed by Western blot the expression change of Ang-2 in relation to pericyte loss, and found that Ang-2 was 37-fold upregulated before pericyte loss became evident in the diabetic retina (i.e., at three weeks of diabetes), and remained 2.5 fold upregulated after 3 months (Fig. 2D). Intravitreal injection of recombinant Ang-2 caused a dose-dependent pericyte loss in young non-diabetic rats (Fig. 2E). Mice with a heterozygous deletion of Ang-2 by knock in of a LacZ reporter construct (provided by Regeneron Pharmaceuticals, Tarrytown, NJ) were protected from diabetic pericyte loss, and partially protected from the formation of acellular capillaries (Fig. 2F, 2G). Together, these data indicated that the Ang-Tie system has a critical role in diabetic pericyte loss (Hammes et al., 2004).

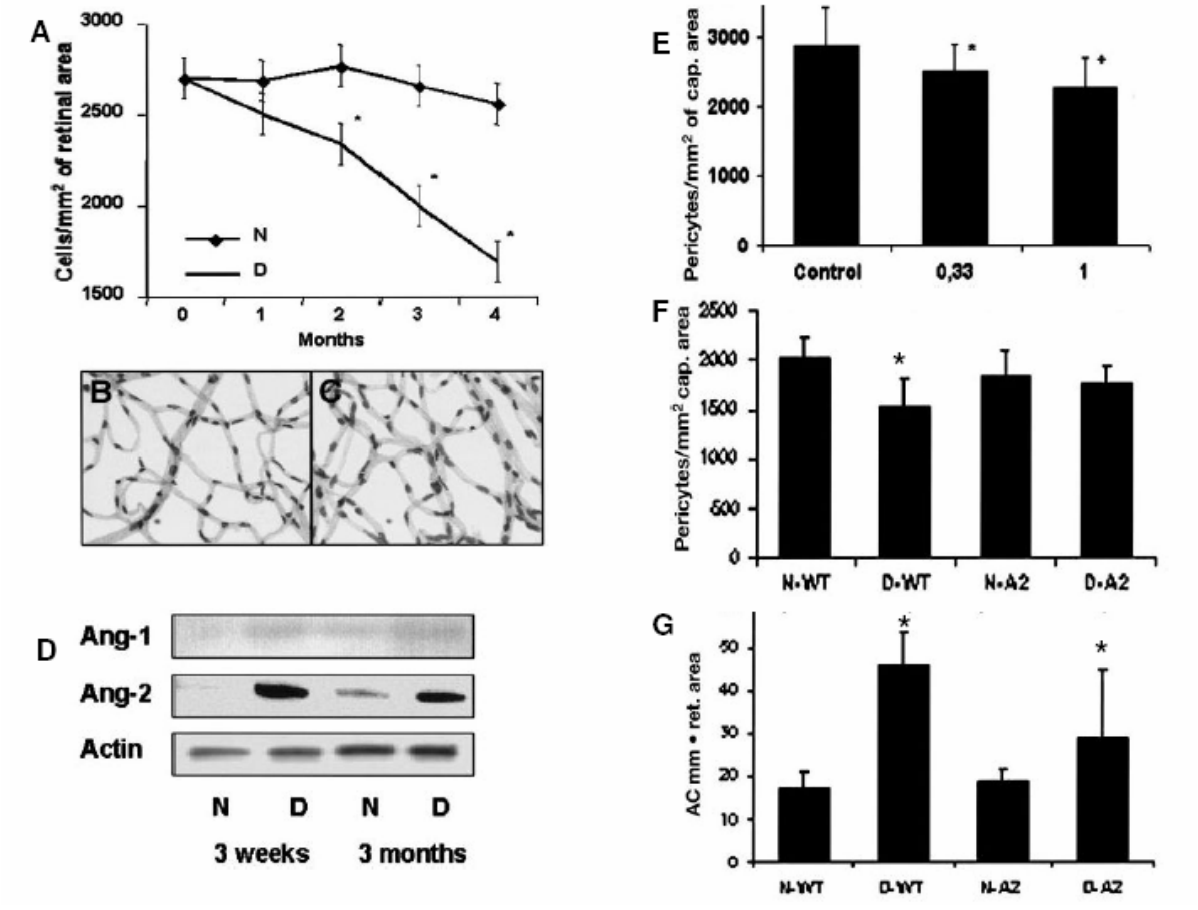


Fig. 2: Correlation of Ang-2 and pericyte loss. A. Time course of pericyte loss in diabetic rats (D) compared with controls (N) from quantitative retinal morphometry. B and C. Representative examples of retinal digest preparations stained with PAS in nondiabetic (*B*) and diabetic (*C*) rats after 3 months of diabetes. Pericytes were identified by their shape, staining intensity with PAS and relative position in the capillary. D. Representative demonstration of retinal expression of Ang-1 and Ang-2 in non-diabetic and diabetic rat at 3 weeks and 3 months after diabetes

induction (Western blot). E. Pericyte loss after intravitreal injection of 0.33 µg and 1µg recombinant Ang-2 in non-diabetic rats. F shows pericyte number and G acellular capillaries in non-diabetic (N) and diabetic (D) retinas from wild type (WT) and heterozygous Ang-2 deficient (A2) mice.

Overexpression of Ang-2 induces enhanced physiological angiogenesis and results in reduced capillary coverage with pericytes

To further study the effect of increased Ang-2 in the retina, we established a mouse line with constitutive overexpression of human Ang-2 in the photoreceptor layer (mOpsinhAng2 mouse). Ang-2 overexpression was confirmed by PCR and immunohistochemistry. Analysis of developmental sprouting in these mice revealed a significant transient acceleration in the formation of the deep capillary network in transgenic mice. Measurements of the growing edge in retinal whole mount preparations stained with lectin to visualize the vasculature revealed that no difference in growth speed was discernible in the superficial capillary network. This is consistent with the fact that Ang-2 produced by the photoreceptor layer predominantly acts on the formation of the deep capillary layer.

For analysis of overexpressed Ang-2 on the retinal vascular morphological change, we assessed the pericyte coverage on the retinal vasculature on retinal digest preparations of mOpsinhAng2 retinas. We found that adult mOpsinhAng2 mice had a 17% pericyte deficit, which equals a diabetic retina after 3 months' disease duration.

Overexpression of Ang-2 enhances pathological angiogenesis in hypoxia-induced retinal neovascularization

In order to determine the effect of Ang-2 overexpression to retinal neovascularization, we quantified preretinal neovascularizations in the ROP model. The transgenic mOpsinhAng2 mice showed a 51% increased number of neovascular nuclei on the vitreal side of the inner limiting membrane in comparison to their wild type littermates ($p<0.01$). Increased retinal angiogenesis in the deep capillary layer in the mOpsinhAng2 mice was associated with reduced pericyte coverage ($P<0.05$) and increased sprouting angiogenesis ($P<0.01$).

Overexpression of Ang-2 results in severe retinal vascular morphological changes in the diabetic mouse model

Furthermore, we studied the mOpsinhAng2 model during permanent hyperglycemia. After 6 months of diabetes, a significant increase of acellular capillaries and an increase in pericyte dropout was observed in the transgenic mice in comparison to wild type littermates. Analysis of apoptotic cells on retinal digest preparation revealed significantly increased numbers of vascular cells undergoing apoptosis in mopsinhAng2 retinas, of both, endothelial cell and pericyte identity.

Ang-2-deficiency results in aberrant development of the retinal vasculature

We further used a modified retinal digestion preparation method to study the effect of Ang-2-deficiency on the retinal vessels. We studied 2-months old Ang-2 LacZ knock-in mice (Ang2LacZ), which were kindly provided by Regeneron Pharmaceuticals. After backcrossing homozygous Ang-2 deficient mice on a C 57 Bl-6 background, animals substantially increased in survival time as noted previously (Fiedler et al., 2006). These animals exhibited delayed retinal vascular development and limited patterning of retinal vessels. The retinal venular outgrowth was reduced by approximately 50%, and capillaries in proximity to the venular site of the network were incompletely remodeled

compared to heterozygous and wild type mice. The major defect, however, was observed in retinal arterioles. Their growth towards the retinal periphery was largely impaired compared with the venules, and showed prominent tortuousity, and numerous aneurysms. A chaotic capillary network was also observed on the arteriolar side of the capillary network. Quantitative analysis of retinal vascular morphometry demonstrated that homozygous Ang-2-deficient mice had significantly reduced arteriolar and venular numbers compared with wild type and heterozygous mice. The arterioles had larger diameters than wild type arterioles, and the diameters of capillaries next to venules and arterioles were larger than in wild types or heterozygous Ang-2 knockout mice. The areas covered by capillaries (called capillary density) around venules were substantially reduced in the homozygous Ang-2 deficient mice, compared with wild type, and heterozygous mice.

Subsequently, we determined the Ang-2 expression in the retinal vasculature using the reporter gene LacZ. After LacZ staining, the retinal samples were digested with trypsin. There were two domains of retinal vessels to be identified and isolated: the numerous preretinal vessels and few intraretinal vessels. We observed a strong Ang-2 expression at the edges of preretinal vessels. In the areas in which arterioles were forming, strong LacZ staining was also detected. In established capillary network areas, LacZ staining was much weaker than in areas with abnormal arterioles. Notably, LacZ positive cells were found in aneurysms and in the endothelial cells on the sprouts, but not in the pericytes of the sprouts or in capillaries.

Ang-2 expression is upregulated by high glucose in-vitro, and is inhibited by scavengers of reactive oxygen species and metabolic signal blockers

We used endothelial cells exposed to high glucose to study glucose-dependent regulation of the Ang-2 promoter. Bovine aortic endothelial cells (BAEC) were grown in medium containing 5 mM glucose and 25 mM for stimulation. Using different Ang-2 promoter fragments transfected into BAEC, it was demonstrated that several of the fragments respond to high glucose stimulation, whereas a short fragment spanning –61/+476 bp did not. Time course of Ang-2 mRNA regulation under high glucose was analyzed in BAEC by RT-PCR. High glucose upregulated Ang-2 mRNA expression after 6 hours of stimulation. Promoter activity of Ang-2 was determined by detecting Luciferase activity in the Ang-2 promoter construct transfected into BAEC. The cells were stimulated with high glucose and treated with antioxidants, inhibitors of complex II of the mitochondrial respiratory chain, SOD mimetics, and metabolic signal blockers. Stimulation of promoter activity by high glucose was reversed by the treatment with these inhibitors.

Ang-2 mRNA expression was also analyzed in the presence and absence of the PARP inhibitor PJ34 (Du et al., 2003). The PARP inhibitor decreased Ang-2 mRNA levels in BAECs grown under high glucose conditions. Similar experiments in Müller cells revealed that benfotiamine had no effect on Ang-2 expression while PJ 34 significantly reduced Ang-2. These experiments were corroborated using diabetic Ang-2 LacZ reporter mouse. Treatment with PARP inhibitor PJ34 over 6 weeks reduced the expression of LacZ significantly. This is consistent with our immunohistochemical data obtained from rats treated for 6-9 months with benfotiamine, and with data from mice, treated with the PARP inhibitor PJ 34 for 6 months.

Novel experiments using the catalytic reactive oxygen scavenger R-(+)-a-lipoic acid showed that treatment over 30 weeks can significantly reduce pericyte dropout, and the formation of acellular capillaries (Fig. 3A, 3B) (Lin et al., 2006). R-(+)-a-lipoic acid reduced oxidative stress in the diabetic retina, the expression of RAGE and methylglyoxal type AGE which are known as the most important intracellular AGE which form during hyperglycemia. Notably, Ang-2 expression was completely

normalized by this type of treatment, suggesting that mechanisms resulting in oxidative stress and AGE formation are involved in transcriptional changes of Ang-2 (Fig. 3C).

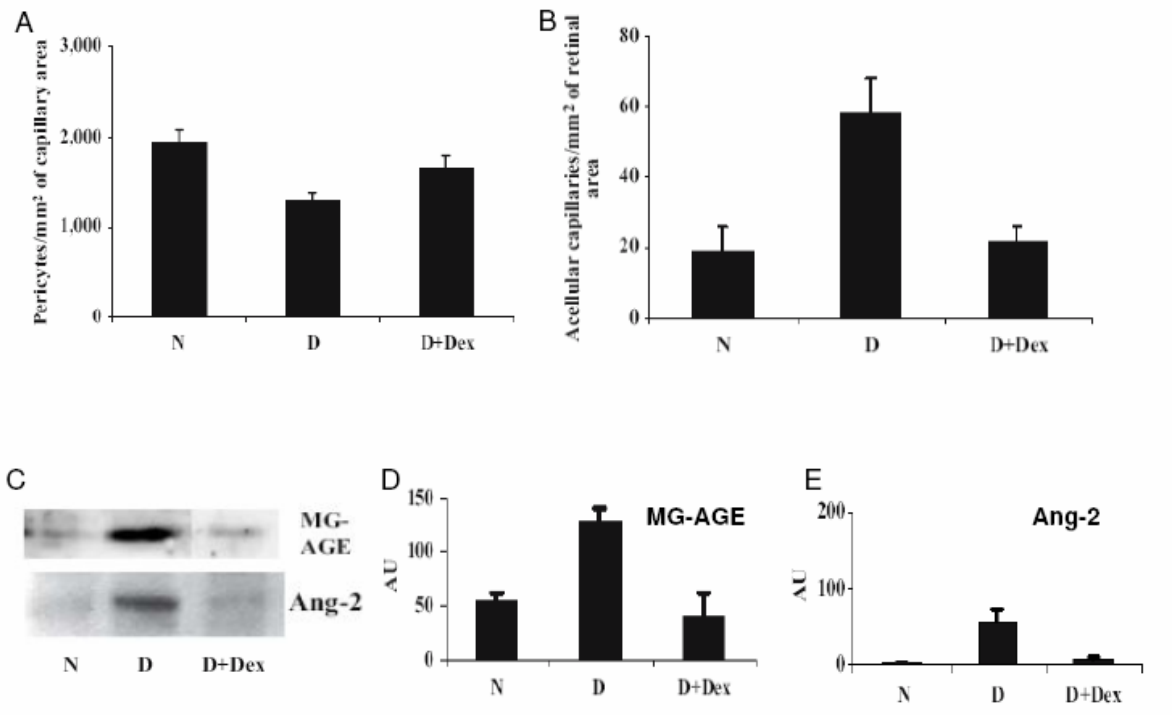


Fig. 3: Effect of catalytic reactive oxygen scavenger on the expression of Ang-2 in diabetic rats. A and B show the number of pericyte (A) and acellular capillary (B) from quantitative retinal morphometry. C. Influence of catalytic reactive oxygen scavenger R-(+)-a-lipoic (Dex) on the expression of metylglyoxal-AGE and Ang-2 in the 30 weeks diabetic retinas. N: non-diabetic; D: diabetic; D+Dex: diabetic mice treated with R-(+)-a-lipoic.

Important evidence for this concept has recently been obtained. As demonstrated before by in situ hybridization, apart from endothelial cells, Müller cells are a source for Ang-2 in the retina. Müller cells were taken to demonstrate the molecular mechanism how glycolysis and Ang-2 transcription are linked. Increased glycolytic flux induced by incubating Müller cells in vitro in high glucose over several days caused methylglyoxal modification of the corepressor mSin3A which results in an increased recruitment of O-Glc-Nac transferase to an mSin3A-Sp3 complex. As a consequence, SP3 is modified by O-linked N-acetylglucosamine, reducing the binding affinity of the repressor complex to a glucose-responsive GC-box in the Ang-2 promoter with subsequent transcriptional activation of Ang-2 (Yao et al., 2006).

DISCUSSION

Accumulated data indicate that the Ang-Tie system is involved in vascular morphogenesis, repair, and sprouting angiogenesis, both under physiological and pathological conditions. Ang-2 is regulated by a variety of conditions. Together with recent data implicating Ang-2 in the inflammatory response of the endothelium, we have identified the Ang-Tie system as crucial in diabetic pericyte loss, and endothelial (capillary) survival/repair in the diabetic, and the hypoxic retina.

Localization of Ang-2 in the retina

Under physiological conditions, the expression of Ang-2 in the mature retina is very low, compared with other growth-related ligands. There is low abundance of Ang-2 in horizontal cells, and possibly in endothelial cells. In vitro, Ang-2 is expressed in retinal endothelial cells, pericytes, horizontal cells and Müller cells (Chi et al., 2003; Fiedler et al., 2004). Upon stimulation such as hypoxia, Ang-2 is upregulated in endothelial cells of vascular sprouts, in cells of the ganglion cell layer, and in the inner nuclear layer.

Stimulation of Ang-2 in the retina – a role for glucose

Prolonged retinal stress such as chronic hyperglycemia induces a permanent overexpression of Ang-2 in specific cells such as Müller glia. Glucose dependent regulation of Ang-2 has also been demonstrated in endothelial cells. Upregulation of Ang-2 in both Müller cells and endothelial cells most likely shares similar mechanisms, although some variations in glucose-responsive promoter elements of both cell types may exist (Yao et al., 2006; Lin and Hammes et al. unpublished). The observation that methylglyoxal modification of the corepressor mSin3a alters SP3 binding to the Ang-2 GC box and subsequently leads to de-repression of Ang-2 transcription in Müller glia may explain the therapeutic effects of some of the compounds recently evaluated in the preclinical retinopathy model. For example, a catalytic antioxidant with self-regenerating properties through glycolytic metabolites inhibited the formation of acellular capillaries (Lin et al., 2006). Hyperglycemic upregulation of Ang-2 was associated with increased retinal methyl-glyoxal-type AGE accumulation, providing strong in-vivo evidence for the above hypothesis, i.e. that inhibition of ROS-production with subsequent reduction in (MG-type) AGE formation normalizes retinal (glia) Ang-2 expression. Similar results are obtained, when a metabolic signal blocker, i.e. the PARP-inhibitor PJ34 is administered to mice expressing an Ang-2 dependent reporter. PARP inhibition reduces the ADP-ribose mediated inhibitory effect on critical glycolytic enzymes thus alleviating upstream accumulation of reactive metabolites (Du et al., 2003). As a resultant, Ang-2 expression is completely normalized.

Ang-2 and pericyte loss

The concept proposed by Hanahan suggested that Ang-2 inhibits Ang-1 mediated signaling resulting in an antiproliferative and stabilized phenotype of the endothelium. Therefore, it was hypothesized that Ang-2, by opposing this effect, may contribute to angiogenesis and vasoregression partly by modulating pericyte function. However, our data revealed that very early during the course of experimental diabetes, Ang-2 is increased before pericyte loss initiates. It must be noted that in this model, neither intraretinal, nor preretinal neovascularization ensues. Furthermore, by injecting recombinant Ang-2 into the vitreous of non-diabetic rats, pericytes can be actively eliminated. Hyperglycemia-induced pericyte loss is prevented when Ang-2 expression is halved. This set of data suggests that Ang-2 is critical in the loss of pericytes in the early diabetic retina without primary link to angiogenesis. As indicated above, both, metabolic signal blockers, and catalytic antioxidants normalize Ang-2 expression, and pericyte dropout. However, it remains unclear whether endothelial or glial Ang-2 expression determines diabetic pericyte loss. Unpublished in vitro data suggest that probucol which a strong vitamine E analogue with chain breaking antioxidant properties does inhibit endothelial Ang-2 expression and pericyte dropout (Hammes, Fiedler, Feng et al., unpublished observations) while the lipid-soluble analog benfotiamine which alleviates experimental diabetic retinopathy through activation of transketolase inhibits endothelial Ang-2 without affecting pericyte dropout (Hammes et al., 2003). A possible correlate is that benfothiamine does not normalize Ang-2

expression from Müller cells exposed to high glucose, while in endothelial cells benfothiamine does normalize Ang-2 expression. We did not find a difference in transketolase activity in both cells (Lin et al., unpublished observations) so that the reason for this differential modulation of Ang-2 expression remains unclear. Other classical antioxidants which are not recycled under hyperglycemic conditions, and work stoichiometrically such as the butylpropylphenol nicanartine, are unable to correct capillary damage despite the partial inhibition of pericyte loss. This set of data further points to a crucial role of retinal glia in the control of pericyte maintenance and survival function for the capillary endothelium.

The cellular antagonism of Ang-2 and Ang-1 on the cognate receptor tyrosin kinase Tie-2 suggests a beneficial effect of the pharmacological application of Ang-1 in early diabetic retinopathy. Indeed, in vivo experiments have demonstrated a beneficial effect of Ang-1 in disturbed vessel permeability, which is another early sign of hyperglycemia-induced retinal damage. The effects on pericytes or on structural damage such as acellular capillaries was not studied (Joussen et al., 2002)

Ang-2 function in the retina – new models for the understanding of diabetic retinopathy

Photoreceptor-based overexpression of Ang-2 reduces the pericyte-recruiting signal to the deep capillary layers. The concomitant transient growth promotion of the deep layer finds its experimental counterpart in recent data from Oshima et al (2005). In mice with conditional overexpression of Ang-2, a transient increase in capillary formation was demonstrated. Of note, as in our model, no increased vasoregression was noted, most likely because of the rich availability of VEGF in the retina, as compared with other survival factors (Feng Y, Schreiter K, and Hammes HP, unpublished).

Moreover, Oshima et al found that early Ang-2 induction resulted in more new vessels, while delayed overexpression of Ang-2 (relative to the hypoxic signal) was accompanied by more vessel regression in the inner retina. These data comply with our findings since we observed more new vessels both within the retina, and in the preretinal location.

Retinal overexpression of Ang-2 induced decreased pericyte coverage similar to 3 month experimental diabetic retinopathy. These results suggest that the balance of Ang-2 to Ang-1 is involved in the determination of pericyte recruitment, at least in the deep capillary layers. A similar transient effect of vascularization dynamics in the developing retina was reported using an inducible Ang-2 expressing mouse model (Oshima et al., 2005). Therefore, we speculate that both autocrine signaling of Ang-2 in the endothelial cells and probably the paracrine Ang-2 from surrounding cells may induce the enhanced vascular angiogenesis in the developing retina.

The comparison of mice with retinal Ang-2 overexpression revealed a level of retinal damage which is similar to the phenotype of diabetic mice with several months of hyperglycemia. Setting the extent of pericyte dropout and acellular capillaries of 6 months diabetic wild type mice as reference, the relative amount of pericyte loss (71%) surpassed the level of endothelial damage (as expressed by acellular capillaries, 29%) by far. This suggests a somewhat higher susceptibility of pericytes to surrounding Ang-2 level than the endothelial cells which are more sensible to the hyperglycemia-induced damage.

In summary, our data suggest that Ang-2 is involved in diabetic pericyte loss, and that some of the effects exerted by pharmacological treatment of diabetic retinopathy are causally linked to the modulation of Ang-2 expression. Since the transcriptional activation of Ang-2 is caused by changes of intracellular AGEs, old and novel modulators may be evaluated for their effect on Ang-2 expression and the prevention of vessel damage.

REFERENCES

Aiello LP, Avery RL, Arrigg PG, et al. Vascular endothelial growth factor in ocular fluid of patients with diabetic retinopathy and other retinal disorders. N Engl J Med 331:1480-7, 1994

Armulik A, Abramsson A, Betsholtz C: Endothelial/pericyte interactions. *Circ Res* 97: 512-23, 2005.

Brownlee M: The pathobiology of diabetic complications: a unifying mechanism. *Diabetes* 54: 1615-25, 2005.

Carmeliet P: Angiogenesis in health and disease. *Nat Med* 9: 653-660, 2003.

Davis S, Aldrich TH, Jones PF, Acheson A, Compton DL, Jain V, Ryan TE, Bruno J, Radziejewski C, Maisonpierre PC, Yancopoulos GD: Isolation of angiopoietin-1, a ligand for the Tie2 receptor, by secretion-trap expression cloning. *Cell* 87: 1161-1169, 1996.

Du X, Matsumura T, Edelstein D, Rossetti L, Zsengeller Z, Szabo C, Brownlee M: Inhibition of GAPDH activity by poly(ADP-ribose) polymerase activates three major pathways of hyperglycemic damage in endothelial cells. *J Clin Invest* 112: 1049-1057, 2003.

Erber R, Eichelsbacher U, Powajbo V, Korn T, Djonov V, Hammes HP, Lin J, Grobholz R, Ullrich A, Vajkoczy A: EphB4 controls blood vascular morphogenesis during postnatal angiogenesis. EMBO J. 25(3):628-41, 2006

Erber R, Thurnher A, Katsen AD, Groth G, Kerger H, Hammes HP, Menger MD, Ullrich A, Vajkoczy P. Combined inhibition of VEGF and PDGF signaling enforces tumor vessel regression by interfering with pericyte-mediated endothelial cell survival mechanisms. FASEB J. 18(2):338-40, 2004

Fiedler U, Scharpfenecker M, Koidl S, Hegen A, Grunow V, Schmidt JM, Kriz W, Thurston G, Augustin HG. The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. Blood 103: 4150-6, 2004

Fiedler U, Reiss Y, Scharpfenecker M, Grunow V, Koidl S, Thurston G, Gale NW, Witzenrath M, Rosseau S, Suttorp N, Sobke A, Herrmann M, Preissner KT, Vajkoczy P, Augustin HG. Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. Nat Med 12:235-9, 2006

Hanahan D: Signaling vascular morphogenesis and maintenance. *Science* 277: 48-50, 1997.

Holash J, Wiegand SJ, Yancopoulos GD: New model of tumor angiogenesis: dynamic balance between vessel regression and growth mediated by angiopoietins and VEGF. *Oncogene* 18: 5356-5362, 1999.

Hammes HP, Martin S, Federlin K, Geisen K, Brownlee M: Aminoguanidine treatment inhibits the development of experimental diabetic retinopathy. *Proc Natl Acad Sci USA* 88: 11555-11558, 1991.

Hammes HP, Brownlee M, Jonczyk A, Sutter A, Preissner KTP: Subcutaneous injection of a cyclic peptide antagonist of vitronectin receptor-type integrins inhibits retinal neovascularization. *Nat Med* 2: 529-533, 1996.

Hammes HP, Du X, Edelstein D, Taguchi T, Matsumara T, Ju Q, Lin J, Bierhaus A, Nawroth P, Hannak D, Neumaier M, Bergfeld R, Giardino I, and Brownlee M: Benfotiamine blocks three major pathways of hyperglycemic damage and prevents experimental diabetic retinopathy. Nat Med 9, 294-299, 2003

Hammes HP, Lin J, Wagner P, Feng Y, vom Hagen F, Krzizok, T, Renner O, Breier G, Brownlee M, and Deutsch U: Diabetes-induced Angiopoietin-2 causes pericyte dropout in the normal retina: evidence for involvement in diabetic retinopathy. *Diabetes* 53: 1104-1110, 2004.

Hoffmann J*, Feng Y*, vom Hagen F, Hillenbrand A, Lin J, Erber R, Vajkoczy P, Gourzoulidou E, Waldmann H, Giannis, A, Wolburg H, Shani M, Jaeger V, Weich HA, Preissner KT, Hoffmann S, Deutsch U, Hammes HP: Endothelial survival factors and spatial completion, but not pericyte coverage of retinal capillaries determine vessel plasticity. FASEB J 19: 2035-6, 2005

Jain R: Molecular regulation of vessel maturation. *Nat Med* 9: 685-693, 2003.

Joussen AM, Poulaki V, Tsujikawa A, Qin W, Qaum T, Xu Q, Moromizato Y, Bursell SE, Wiegand SJ, Rudge J, Ioffe E, Yancopoulos GD, Adamis AP. Suppression of diabetic retinopathy with angiopoietin-1. Am J Pathol. 160(5):1683-93, 2002

Kohner EM, Henkind P: Correlation of fluorescein angiogram and retinal digest in diabetic retinopathy. *Am J Ophthalmol* 69: 403-414,1970.

Leung DW, Cachianes G, Kuang WJ, Goeddel DV, Ferrara N. Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306-9, 1989

Lin J, Bierhaus A, Bugert P, Dietrich N, Feng Y, Vom Hagen F, Nawroth P, Brownlee M, Hammes HP: Effect of R-(+)-alpha-lipoic acid on experimental diabetic retinopathy. Diabetologia. Mar 7, 2006

Lu M, Kuroki M, Amano S, Tolentino M, Keough K, Kim I, Bucala R, Adamis AP. Advanced glycation end products increase retinal vascular endothelial growth factor expression. *J Clin Invest*. 101:1219-24, 1998

Luhmann UFO, Lin J, Acar N, Lammel S, Feil S, Grimm C, Seeliger MW, Hammes HP, Berger W: Role of the Norrie Disease Pseudoglioma Gene in Sprouting Angiogenesis during Development of the Retinal Vasculature. Invest Ophthalmol Vis Sci 46:3372-82, 2005

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277: 55-60, 1997

Oh H, Takagi H, Suzuma K, Otani A, Matsumura M, Honda Y. , Hypoxia and vascular endothelial growth factor selectively up-regulate angiopoietin-2 in bovine microvascular endothelial cells. J Biol Chem. 28;274(22):15732-9, 1999

Okamoto T, Yamagishi S, Inagaki Y, Amano S, Koga K, Abe R, Takeuchi M, Ohno S, Yoshimura A, Makita Z. Angiogenesis induced by advanced glycation end products and its prevention by cerivastatin. FASEB J. 16(14):1928-30, 2002

Oshima Y, Oshima S, Nambu H, Kachi S, Takahashi K, Umeda N, Shen J, Dong A, Apte RS, Duh E, Hackett SF, Okoye G, Ishibashi K, Handa J, Melia M, Wiegand S, Yancopoulos G, Zack DJ, Campochiaro PA: Different effects of angiopoietin-2 in different vascular beds: new vessels are most sensitive. *FASEB J* 19: 963-5, 2005.

Patel JI, Hykin PG, Gregor ZJ, Boulton M, Cree IA. Angiopoietin concentrations in diabetic retinopathy. Br J Ophthalmol. 89(4):480-3, 2005

Raab S, Beck H, Gaumann A, Yuce A, Gerber HP, Plate K, Hammes HP, Ferrara N, Breier G. Impaired brain angiogenesis and neuronal apoptosis induced by conditional homozygous inactivation of vascular endothelial growth factor. Thromb Haemost. 91(3):595-605, 2004

Romeo G, Liu WH, Asnaghi V, Kern TS, Lorenzi M. Activation of nuclear factor-kappaB induced by diabetes and high glucose regulates a proapoptotic program in retinal pericytes. Diabetes 51:2241–2248, 2002

Thylefors B, Negrel AD, Pararajasegaram R, Dadzie KY: Global data on blindness. *Bull World Health Organ* 73: 115-121, 1995.

Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y: Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376: 70-74, 1995.

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD: Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87: 1171-1180, 1996.

vom Hagen F, Feng Y, Hillenbrand A, Hoffmann S, Shani M, Deutsch U, Hammes HP: Early loss of arteriolar smooth muscle cells: more than just a pericyte loss in diabetic retinopathy. Exp Clin Endo Diab 113: 573-6, 2005

Watanabe D, Suzuma K, Matsui S, Kurimoto M, Kiryu J, Kita M, Suzuma I, Ohashi H, Ojima T, Murakami T, Kobayashi T, Masuda S, Nagao M, Yoshimura N, Takagi H. Erythropoietin as a retinal angiogenic factor in proliferative diabetic retinopathy. N Engl J Med. 25;353(8):782-92, 2005

Yao D, Taguchi T, Matsumura T, Pestell R, Edelstein D; Giardino I, Suske G, Ahmed N, Thornalley PJ, Sarthy VP, Hammes HP, Brownlee M: Methylglyoxal modification of mSin3A links altered glycolysis to angiopoietin-2 transcription: A novel mechanism for regulating gene expression. Cell 124: 275-86, 2006.

Hypoxia

The Role of Vascular Endothelial Growth Factor and of Endothelial Transcriptional Regulators in Embryonic Angiogenesis

Prof. Dr. Georg Breier

Dept. of Pathology
Faculty of Medicine
Technical University of Dresden
Fetscherstr. 74, 01307 Dresden, Germany

Member of the SPP 1069 from 1999 until 2006

Co-PI: Prof. Dr. Matthias Clauss, Indianapolis, USA (formerly Max Planck Institute, Bad Nauheim)
Co-PI: PD Dr. Ingo Flamme, Bayer Inc., Wuppertal (formerly Max Planck Institute, Bad Nauheim)

SUMMARY

The development of new blood vessels during ontogeny is regulated by vascular endothelial cell specific growth factors and their signaling receptors which are expressed on the surface of endothelial cells. It is well established that the signaling system consisting of vascular endothelial growth factor (VEGF) and its receptors plays a key role in angiogenesis and vasculogenesis. However, when this project was initiated, many questions related to VEGF biology, signaling, and regulation of VEGF receptor expression were still unclear. We have thus studied (i) the function of VEGF in mouse brain development by conditional gene targeting, (ii) VEGF-induced signaling events in angiogenesis and vascular permeability, and (iii) the control of VEGF receptor-2 *(flk1)* gene expression by endothelial transcriptional regulators. In the first part of the project, we showed, by conditional gene targeting, that VEGF, via local secretion by neural progenitors, induces brain angiogenesis and guides the growth of capillaries toward the ventricular zone. VEGF thus acts as a key regulator of brain angiogenesis and provides instructive cues for the correct spatial organization of the vasculature. These findings established the critical importance of neuroectoderm-derived VEGF in the morphogenesis of the brain. In the second part of the project, we identified VEGF-receptor specific signaling events which regulate angiogenesis and vascular permeability. In particular, we identified the p38 MAP-kinase (MAPK) as a novel antiangiogenic signaling molecule. A p38alpha isoform mediated this activity through erk-1/2 MAPK inactivation. In addition, we provided evidence that the transcription factor egr-1 is a candidate mediator of VEGF effects downstream of erk-1/2, and found its inhibitor Nab2 to be antiangiogenic. Finally, we identified p38 MAPK and as an essential signaling molecule in VEGF-induced vascular permeability. Based on it opposing activities in terms of angiogenesis and vascular permeability we suggest that the p38 MAPK is a molecular switch between VEGF-mediated angiogenesis and vascular permeability. In the third part of the project, we identified several transcription factors - including ets-1 and HIF-2α – which are expressed in the endothelial lineage and regulate the expression of the murine *flk1* gene. To investigate the potential endothelial cell intrinisic function of hypoxia signaling in vivo, HIF activity was inhibited in transgenic mouse embryos by expressing a dominant-negative HIF-2 mutant in the developing the endothelium. These embryos developed severe cardiovascular defects, notably in vascular sprouting, remodeling and heart looping. These findings establish the essential role of hypoxia signaling in endothelial cells during cardiovascular development.

INTRODUCTION

Vascular endothelial growth factor (VEGF) is the major regulator of physiological and pathological angiogenesis. It is distinct from other endothelial growth factors such as PDGF and FGF by its ability to selectively stimulate endothelial cell growth. In addition, VEGF initiates endothelial procoagulant activity and vascular permeability, which led to its initial identification as a vascular permeability factor (VPF). Gene targeting experiments have shown that the signal transducing system consisting of VEGF (also named VEGF-A) and its high affinity receptors is essential for both the *de novo* formation of blood vessels from their mesenchymal progenitors (vasculogenesis) and the formation of new blood vessels from pre-existing ones (angiogenesis) (for reviews, see Breier, 2000; Ferrara, 2003). This signal transducing system is primarily active in the vascular system, since the high affinity VEGF receptors - VEGFR-1 (also known as Flt-1) and VEGFR-2 (also known as Flk-1) - are expressed predominantly by vascular endothelial cells while the ligand is produced by the tissue actually being vascularized. While the inactivation of VEGFR-2 in mouse embryos results in the complete lack of blood vessel differentiation, the role of VEGFR-1 in vascular development is less clear. Recent data suggest that VEGFR-1 may serve as a negative regulator of angioblast differentiation during vasculogenesis, and that this function does not require the intracellular tyrosine kinase domain. Obviously, the receptors for VEGF-A are not redundant in their function. Moreover, the severe haploid-insufficient phenotype observed in mice deficient for VEGF-A also indicates that the other members of the VEGF family are not capable of substituting VEGF activity, except possibly during early vasculogenesis. More recently, the neural cell receptor for type III semaphorins, neuropilin-1 (NP-1), was identified as co-receptor for the heparin-binding $VEGF_{165}$ isoform on endothelial cells. Consistently, NP-1 deficient mouse embryos show defective vascular development in addition to neuronal defects. However, in contrast to VEGFR-2, NP-1 is not involved in vasculogenesis.

While the gene targeting experiments in mouse embryos have clearly demonstrated that VEGF and the VEGF receptors are essential for the formation of the primordial vascular system (Carmeliet et al., 1996; Ferrara et al., 1996), we hypothesized, based on our analysis of the expression pattern of VEGF and the VEGF receptors in the embryonic and postnatal mouse, that the function of VEGF is not restricted to early stage vascular development (Breier et al., 1992; Breier et al., 1995). In the developing mouse brain, VEGF mRNA is expressed by neural progenitor cells in the ventricular zone, whereas the the high affinity VEGF receptors, VEGFR-1 and VEGFR-2, are detected primarily in the perineural vascular plexus and in capillaries that have invaded the neuroectoderm. Because VEGF stimulates both vascular sprouting and chemotaxis, this expression pattern suggested that VEGF stimulates brain angiogenesis in a paracrine fashion, by promoting the invasion of blood vessels from the perineural vascular plexus, and by directing their growth toward the ventricular zone along a gradient of VEGF protein. More recent evidence suggests that VEGF also has direct effects on neural cells (Carmeliet, 2003). In the first part of the project, we aimed at studying the function of the VEGF gene during brain development by using a conditional gene targeting approach based on the cre-lox system. This allowed the inactivation of the VEGF gene specifically in the developing central nervous system, without affecting its essential function in early stage vascular development.

Although VEGF was discovered based on its ability to induce proliferation of endothelial cells, the view that angiogenic functions mostly rely on endothelial cell growth is oversimplified. The ability to form sprouts from existing vessels and the differentiation of new blood vessels more likely requires several distinct activities of VEGF including proteolytic activities, migration and survival. Many of these activities have been dedicated to distinct intracellular signaling pathways (for review see Claesson-Welsh, 2003). For instance, VEGFR-2 induced Ras activation and initiation of the Ras-Raf-MAP-kinase pathway has been demonstrated to mediate endothelial proliferation. However, VEGF-

induced cell growth is not only due to its mitogenic potential but also linked to the ability of VEGF to act as a survival factor. This activity was demonstrated to be mediated by the PI_3-Kinase-AKT-signaling-pathway. In addition, the PI_3-Kinase-AKT-signaling-pathway was demonstrated to be involved in endothelial migration, indicating that this signaling pathway is of major importance for processes of angiogenesis. VEGFR-2 activation can also recruit PLC-γ, which, via increase of intracellular Ca^{2+} concentration, diacyglycerol generation and subsequent PKC-activation, effects migration and vascular permeability. Independently from PKC activation increased Ca^{2+} levels are involved in a series of cellular signaling events including eNOS activation. Both VEGF-induced vascular permeability as well as endothelial proliferation and/ or angiogenesis are also dependent on the generation of the free radical nitrogen monoxide (NO) by the endothelial NO-synthetase (eNOS) as well as the activation of the Src kinase, respectively. However, a clear distinction between VEGF-induced signaling events which are inducing angiogenesis from those responsible for vascular permeability have not been attempted yet. These aspects are of relevance for therapeutic pro- or antiangiogenic strategies based on the application or inhibition of VEGF. However, comparably little attention has been given to side effects of VEGF such as the induction of vascular permeability. In therapeutic angiogenesis with VEGF, increase in vascular permeability comprises a serious complication and may even be counteractive. The situation is different, however, for the treatment of tumors. Here, it may even be necessary to maintain vascular permeability for the purpose of optimal drug delivery when combining anti-angiogenic with chemotherapeutic approaches. Thus, further studies on signaling events of angiogenesis not only in terms of blocking or promoting angiogenesis, but also in exploring strategies which may avoid complications linked to VEGF were still in need. These aspects were addressed in the second part of the project.

VEGFR-2 is the first endothelial receptor tyrosine kinase known to be expressed during the differentiation of angioblasts from their precursors, and its function is strictly required for this process. The mechanisms that are responsible for the *de novo* expression of VEGFR-2 and which may also determine the conversion of pluripotent precursor cells towards an endothelial phenotype were largely unknown. Once initiated during vasculogenesis, VEGFR-2 expression is maintained during angiogenesis by the endothelium and is remarkably decreased when vascular growth slows down in postnatal life. During re-initiation of angiogenesis, for example in malignant tumors or during the female reproduction cycle, endothelial cells are re-activated to proliferate, a process which is initimately linked to the re-expression of abundant levels of VEGFR-2. Since the induction of VEGFR-2 is essential for vasculogenesis as well as for angiogenesis in many tumors, the study of the transcriptional regulation of the cognate *flk1* gene is of particular relevance for the understanding of the mechanisms of early stage vascular development and pathological angiogenesis.

VEGF itself is regulated in response to the metabolical demands of the tissue being vascularized. The oxygen tension and the concentration of glucose inside the cell are factors which reflect these demands. Hypoxia has been known as a driving force for angiogenesis for a long time. Consistently, low oxygen tension is a potent inducer of the angiogenesis factor VEGF. It is well established that a transcription factor named hypoxia inducible factor-1 (HIF-1) is important for the transcription of hypoxia sensitive genes such as VEGF or erythropoietin. HIF-1 is a heterodimer consisting of the basic helix-loop-helix/ PAS domain proteins HIF-1α and ARNT. HIF-1α protein is rapidly degraded by the proteasomal pathway, a process which is mediated by the von Hippel Lindau protein, the recognition component of the U3 ubiquitin ligase complex (Pugh and Ratcliffe, 2003). The HIF-1 heterodimer transactivates hypoxia responsive genes in the cell nucleus. Here it binds to a hypoxia responsive element (HRE) consisting of a 6 base pair consensus sequence, and becomes associated with other transcriptional regulators such as CBP/p300 and AP-1 which appears to be necessary for efficient transactivation.

Recently, a homologue of HIF-1α named HIF-2α was identified by several laboratories, including Ingo Flamme´s group at the Max Planck Institute in Bad Nauheim. In contrast to HIF-1α, which is expressed more or less ubiquitously, HIF-2α is expressed predominantly in the vascular system. We have found that HIF-2α transactivates the promoter of the *flk1* gene in reporter gene assays in vitro (Kappel et al., 1999). Since HIF-2α has been shown to also stimulate the promoter of the *tie2* gene, it seems to regulate crucial endothelial receptor tyrosine kinases during embryonic angiogenesis. However, HIF-2α gene targeting experiments largely failed to demonstrate an essential function for endothelial hypoxia-inducible factors in vascular development (see Licht et al., 2006, and references therein). The great variability in the phenotypes observed in these mice suggests that related factors, most likely HIF-1α, are capable of compensating partially for the loss of HIF-2α function.

Like HIF-2α, also the helix-turn-helix transcription factor ets-1 is expressed by endothelial cells during early ontogeny and is upregulated in endothelial sprouts invading tumors during tumor angiogenesis. Functional ets binding sites have been found in the regulatory sequences of genes encoding metalloproteinases, *flt1, tie2* and other angiogenesis relevant factors. However, like in the case of HIF-2α, the targeted inactivation of the *ets-1* gene in mouse embryos did not result in a vascular phenotype. Again, functional redundancy of other ets family members such as ets-2, Fli-1 or TEL can be the cause.

Although the available evidence strongly suggests that the above mentioned transcriptional regulators control the expression of VEGFR-2 and other key receptor tyrosine kinases involved in angiogenesis, direct evidence was lacking. We have used alternative strategies to study the function of candidate regulators of angiogenesis: (i) analysis of their role in the regulation of the *flk1* gene, and (ii) inhibition of their activity by dominant-negative transcription factor mutants. These studies constitute the third part of the project.

METHODS

Our lab has specific expertise in the generation and analysis of genetic mouse models for the study of regulators of angiogenesis, as well as on endothelial transcriptional regulators and receptors. Project Part 1 (Role of VEGF in brain angiogenesis) and Project Part 3 (The influence of endothelial transcriptional regulator on vasculogenesis and angiogenesis; Co-PI Ingo Flamme) are based on mouse molecular genetic approaches. Loss-of-function in mice was achieved by either conditional gene targeting (Role of VEGF in brain angiogenesis)(Licht et al., 2004; Raab et al., 2004) or by the transgenic expression of a dominant-negative HIF (dnHIF) transcription factor mutant (Elvert et al., 2003; Licht et al., 2006). In the latter case, tissue specific gene knockdown was achieved by expressing the dnHIF from gene regulatory sequences of the *flk1* gene that we have characterized in our laboratory (Kappel et al., 1999).

RESULTS AND DISCUSSION

1. The role of VEGF in brain angiogenesis (1st funding period)

Generation and characterization of of conditional VEGF-deficient mice

The cre-lox system was used to inactivate the VEGF gene specifically in the CNS of mice. Mice carrying a floxed VEGF allele (VEGF-lox) were cross-bred with transgenic mice that express the cre recombinase specifically in the CNS. Two different cre lines were used in order to achieve VEGF inactivation at different stages of development, and in different cell populations known to express VEGF: 1) Nestin-cre mice which express the cre from neural specific promoter/enhancer elements of

the rat nestin gene. These regulatory elements target the expresssion of the cre recombinase to neural stem and progenitor cells located in the ventricular zone of the developing neural tube, starting at around mid-gestation. Because Nestin positive cells differentiate into both neurons and astrocytes, recombined alleles are also transmitted to these neural cell types which represent the majority of cells in the brain. 2.) GFAP-cre mice which express the cre recombinase from the promoter of the gene encoding the green glial fibrillary acidic protein (GFAP). In these mice, the cre is expressed in astrocytes (and other GFAP-positive cells) which have been reported to express VEGF. The consequences of VEGF gene inactivaton in the brain should be studied in the resulting mice.

To generate conditional VEGF deficient mice, a targeting vector was designed in which the third exon was flanked by loxP sites. This exon encodes cysteine residues essential for proper dimerization of VEGF. The targeting vector was linearized and introduced by electroporation into R1 embryonic stem (ES) cells. ES cell clones were selected in G418 and analysed for homologous recombination. However, the resulting ES cell clones were not used further because in the meanwhile, VEGF-lox mice had been generated by Napoleone Ferrara (Genentech, South San Francisco) and were kindly provided for our studies.

Characterization of Nestin-cre und GFAP-cre mice

Nestin-cre mice (Tronche et al., 1999), kindly provided by Rüdiger Klein (Max Planck Institute, Martinsried) were characterized following cross-breeding with indicator mice. In the resulting double transgenic mice, the cre recombinase excises an inhibitory DNA fragment that prevents the expression of a lacZ reporter gene. As a consequence, lacZ expression is turned on in those cells that express the cre recombinase and can be identified by Xgal staining. Using this method, we observed that, in Nestin-cre mice, cre activity was first detected in the neural tube of embryonic day (E)10 mouse embryos (see Fig. 1 in Raab et al., 2004). Expression in the embryo was restricted to the neural tube. As expected, most cells of the adult brain showed lacZ expression. However, unexpectedly, lacZ expression was also observed in adult testis.

GFAP-cre mice were kindly provided by Anton Berns (NKI, Amsterdam). Analysis of these mice was performed as described for Nestin-cre mice and confirmed that the cre recombinase was expressed specifically in the brain (data not shown). The pattern of lacZ positive cells was consistent with cre expression in astrocytes and other GFAP positive cells. In conclusion, both cre lines showed expression of the cre recombinase in the CNS and were hence considered suitable for inactivating the the VEGF gene in the developing brain: in the case of the Nestin-cre mouse at the onset of brain angiogenesis, in the case of GFAP-cre mice around birth.

Retinal vasculature defects and absence of oxygen-induced proliferation of blood vessels in $VEGF^{lox/+}$/Nestin-cre mice

Cross-breeding of $VEGF^{lox/lox}$ mice with Nestin-cre mice resulted in heterozygous VEGF deficient ($VEGF^{lox/+}$/Nestin-cre) offspring. By Southern blot hybridization of DNA prepared from adult mouse brain, 42% of the VEGF alleles were deleted (the maximal rate being 50%). Thus, the floxed VEGF gene was efficiently deleted in the brain (see Fig. 1 in Raab et al., 2004). In contrast, most other adult organs tested showed only a relatively low deletion rate, with the exception of testis (35 %).

Heterozygous $VEGF^{lox/+}$/Nestin-cre were viable and fertile. Histological analysis of the brain of these mice did not reveal any obvious defects; notably, no differences in blood vessel density were observed between $VEGF^{lox/+}$/Nestin-cre and control mice. Thus it appears that the remaining intact

VEGF allele produces sufficient VEGF levels for normal vascular development. In collaboration with Hans-Peter Hammes (University of Mannheim-Heidelberg) we investigated the retina which like the brain is a neuroectodermal derivative, but becomes vascularized only after birth. We noticed that approximately 30% of adult eyes analysed showed pathologically reduced blood vessel density (see Fig. 2 in Raab et al., 2004). However, analysis of postnatal day 13 mice revealed that the vascularization of the juvenile retina was normal. This indicates that the vascular phenotype develops later on, in young adult mice, and that VEGF is required for the maintenance of the retinal vascular system.

Heterozygous $VEGF^{lox/+}$/Nestin-cre mice were also studied in a model for retinopathy of prematurity (ROP). In this model, 7-day old mice were transferred to atmosphere with elevated (75%) oxygen levels, and were returned to normal atmosphere 5 days later. During the incubation in hyperoxia, the development of the vascular system in the vitreous body is retarded. As a consequence, the retinal tissue becomes hypoxic after the mice had returned to normal atmosphere. Hypoxia in turn leads to the up-regulation of VEGF expression and, in consequence, induces the pathological proliferation of blood vessels (see Fig. 2 in Raab et al., 2004). This was observed in control mice, however, the proliferation of blood vessels was almost completely absent in $VEGF^{lox/+}$/Nestin-cre mice. This result is is remarkable because the pharmacological inhibition of VEGF signaling had led to only a 50% reduction of retinal blood vessels proliferation in this model. These results confirm the central role of VEGF in promoting ROP and show for the first time that VEGF acts in a dose dependent manner in this process.

Impaired brain development and angiogenesis in $VEGF^{lox/lox}$/Nestin-cre mice

Mice carrying a homozygous VEGF mutation ($VEGF^{lox/lox}$/Nestin-cre) were generated by cross-breeding of heterozygous $VEGF^{lox/+}$/Nestin-cre mice with $VEGF^{lox/lox}$ mice. These mice died shortly after birth. The head of these mice was abnormally flat. The brain of $VEGF^{lox/lox}$/Nestin-cre mice was considerably smaller than in control mice, most notably the telencephalon (see Fig. 3 in Raab et al., 2004). Moreover, the telencephalic hemispheres were not interconnected and showed central necrosis. PECAM-1/CD31 staining of the endothelium revealed that the vascularization of the brain was dramatically reduced, with the exception of the choroid plexus. TUNEL staining demonstrated massive apoptosis in the VEGF-deficient brain.

To study the development of the brain phenotype, homozygous VEGF-deficient ($VEGF^{lox/lox}$/Nestin-cre) mouse embryos were analysed at various stages. Analysis of E10.5 mouse embryos revealed that the invasion of capillaries from the perineural vascular plexus into the telencephalon which normally has started at this stage did not occur in the VEGF-deficient mice. This demonstrates that VEGF is required for the invasion of capillaries into the neuroectoderm. In the hindbrain, few capillaries had invaded the hindbrain of VEGF-deficient mice, however, they failed to penetrate the ventricular zone which normally is the site of strong VEGF production. This phenotype is consistent with our initial hypothesis that VEGF guides the growth of capillaries toward the ventricular zone (Breier et al., 1992). At E13.5, the first apoptotic cells were detected in the ventricular zone, and neural tissue degenerated progressively at later developmental stages.

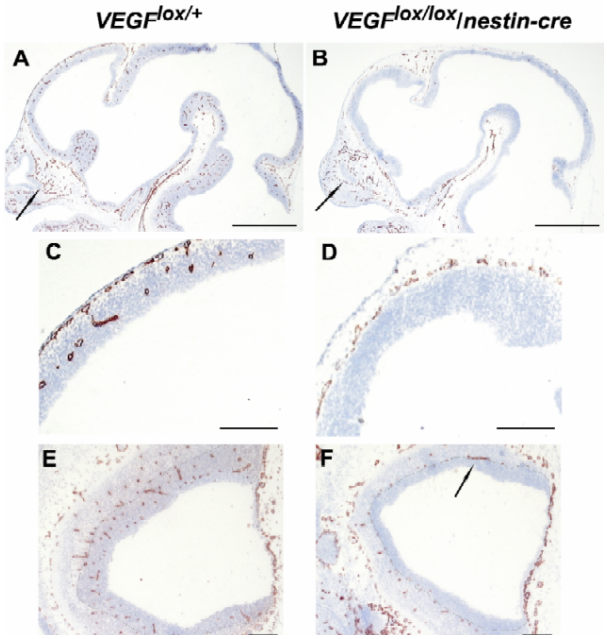


Fig. 1: Impaired brain angiogenesis induced by brain-specific inactivation of VEGF. Sections of E10.5 VEGF-deficient (D,F) and of control (C,E) mouse embryos were stained for PECAM-1 to detect blood vessels. Capillary invasion in the telencephalon was impaired in VEGF-deficient mouse embryos (D) as compared to control embryos (C). In the VEGF-deficient hindbrain (F), capillaries failed to penetrate into the ventricular zone (arrow). Control brain is shown in E. See Raab et al. (2004) for further details.

VEGF$^{lox/lox}$/GFAP-cre mice develop normally

VEGF$^{lox/lox}$/GFAP-cre mice were generated in an analogous way. These mice, in contrast to VEGF$^{lox/lox}$/Nestin-cre mice, developed normally, and were viable and fertile. No brain abnormalities were detected upon macroscopical or microscopical inspection. Brain sections were stained with PECAM-1/CD31 and subjected to morphometry. No differences in blood vessel density could be observed between VEGF$^{lox/lox}$/GFAP-cre mice and control mice.

Conclusions

Our results confirm impressively our initial hypothesis that VEGF is a key angiogenesis factor in the brain. VEGF is essential for the initial vascularization of the developing which in the mouse begins at E9. VEGF deficiency results in an almost complete avascularity in the brain. However, it remains to be determined whether the severe degeneration of neural tissue observed in VEGF deficient mice is entirely caused by tissue malperfusion, or whether VEGF deficiency directly affects the survival of neural stem/progenitor cells in the ventricular zone. To answer this question, it would be necessary to inhibit specifically VEGF activity on neural (stem and progenitor) cells, without interfering with the essential VEGF function in angiogenesis. This could be achieved by inactivating the VEGF receptors in neural cells. The inactivation of the VEGF gene in only a subset of neural cells (astrocytes and other GFAP-positive cells) around birth did however not affect the vascularization of the brain, suggesting that VEGF produced by other cell types can compensate for the loss of VEGF production in brain astrocytes.

2. VEGF-induced signaling in angiogenesis (2nd funding period, collaboration with Matthias Clauss)

In the second part of the project we intended to fill the gap in knowledge in this topic by in particular focusing on the following aims. In aim 1 (Identification of pro- and antiangiogenic signaling molecules) we analyzed signaling molecules in models of VEGF-induced angiogenesis and quickly identified the p38 MAPK as a novel antiangiogenic signaling molecule. We delineated these effects to the individual steps of angiogenesis including endothelial proliferation, survival, migration and protease activation. We identified erk-1/2 MAPK inactivation as the mechanism of the antiangiogenic effects of the p38 MAPK and demonstrated the p38alpha isoform responsible for this activity using adenoviral gene transfer. In aim 2 (Elucidation of unique VEGF signal transduction pathways) we compared VEGF with FGF-2 and EGF. We did not find significant differences in terms of sprout formation but identified egr-1 as a candidate molecule for VEGF effects downstream of erk-1/2. Furthermore, we addressed the upstream mechanism of p38 MAPK activation and analyzed VEGFR-2 mutants. In aim 3 (Effects of the identified signaling molecules in further functional assays) we assessed the role of the p38 MAPK and egr-1 in tumor angiogenesis and arteriogenesis. Finally in aim 4 (Elucidation of VEGF-specific signal transduction pathways for VEGF-caused side effects) we identified p38 MAPK as an essential inducer of vascular permeability. Based on its opposing activities in terms of angiogenesis and vascular permeability we suggest that this kinase is a molecular switch between angiogenesis and vascular permeability.

Specific aim 1: Identification of pro- and antiangiogenic signaling molecules.

We used the sprout formation assay for assessing angiogenic pathways. In this assay we employed endothelial-cell covered microbeads embedded into fibrinogen, which was polymerized to a fibrin gel by addition of thrombin. Sprouts induced by VEGF in presence and absence of inhibitors were counted after 24 hours. The PKC-inhibitor BIM I strongly reduced the ability of VEGF to induce sprout formation in vitro, which is in line with previous findings that this kinase is involved in angiogenesis (Yoshiji et al., 1999). In addition, we observed strong reduction of sprouting activity by using PD98059, a specific inhibitor of MEK-1/2, which is upstream of erk-1/2, an important player in angiogenesis (Tanaka et al., 1999). Also, we observed strong inhibition with wortmannin, which is in line with the hypothesis that VEGF-induced cell growth is not only due to its mitogenic potential but also linked to the ability of VEGF to act as a survival factor believed to be essentially mediated by the PI3-Kinase Akt-signaling-pathway. However, in contrast to previous reports showing that angiogenesis is also dependent on the generation of the free radical nitrogen monoxide (NO) by the endothelial NO-synthetase (eNOS)(Parenti et al., 1998) we found only little inhibition with the eNOS inhibitor L- NAME. In general, our findings so far are in line with other reports from in vitro proliferation and in vivo angiogenesis assays. However, when we applied two selective chemical inhibitors of p38 MAP kinase activation in the sprouting model of angiogenesis, an increase rather than a decrease in activity was seen, which was stronger in the presence than in the absence of VEGF (see Fig. 2 in Issbrucker et al., 2003). This negative regulatory role of the p38 MAP kinase in VEGF-induced angiogenesis was confirmed in the chorio-allantois-membrane (CAM) assay in the chicken embryo (see Figs. 4 and 5 in Issbrucker et al., 2003).

In order to explain these results on cellular mechanisms we tested whether this inhibition of p38 activity enhanced the VEGF effect on plasminogen activation, survival, and proliferation of endothelial cells. For measuring migration, human endothelial cells were grown to confluency, “wounds” were scratched with a pipette tip, and the temporal pattern of migration quantitated microscopically. Plasminogen activation, as the main fibrinolytic mechanism induced by VEGF, was assessed by mimicking the conversion of plasminogen to plasmin using a chromogenic substrate. To

measure survival, cells were grown in medium deprived of serum and growth factors for 24 hours in the presence/absence of hydrogen peroxide and apoptosis measured by the TUNEL assay. We found that proteolytic activity strongly, but also proliferation and survival were enhanced by inhibition of p38 activity in the presence of VEGF, whereas migration was rather inhibited, in concordance to a previous report (see Fig. 3A-C in Issbrucker et al., 2003). In conclusion, three essential mechanisms, i.e. proliferation, survival and plasminogen activation were demonstrated to be negatively regulated by VEGF-induced p38 MAPK activation.

Next we wanted to explore the molecular mechanisms of these findings. Based on the identified mechanism we hypothesized that the negative regulatory role of the p38 MAP kinase was caused by a deactivation of the erk-1/2 MAPK. Therefore, we probed erk-1/2 phosphorylation by using phospho-specific antibodies against erk/1/2 and Western blot analysis. p38 inhibition strongly enhanced VEGF-induced erk-1/2 phosphorylation up to 24 hours after stimulation with VEGF. This suggests that potent dephosphorylating pathways exist in endothelial cells, which are in control of erk/1/2 and are induced and activated by p38 MAP kinase. We have new evidence that p38 MAP kinase activated the dual specific phophatase-1 (DUSP1) and the protein phosphatase A2, which deactivates erk-1/2 and thus comprise a potent anti-angiogenic mechanism.

Finally, we wanted to identify the p38 MAPK isoform being responsible for these effects. In endothelial cells, both alpha- and beta-isoforms out of the four known isoforms were found to be expressed. To address this question we employed adenoviral vectors (kindly provided by Shuang Wang, Scripps Institute, La Jolla) encoding these isoforms together with their upstream activators, MKK3 (which activates p38 alpha) and MKK6 (activates all p38 isoforms). Transduction of HUVECs with adenoviral vectors encoding for p38alpha in combination with MKK3 but not with the p38beta isoform in combination with MKK6 caused inhibition of erk-1/2 MAPK activation by VEGF (data not shown). This identification of the p38alpha isoform as the likely candidate for our observed effects is relevant in light of diverse functions for these two p38 isoforms described in cardiomyocytes. Whereas hypertrophy was attributed to the alpha form, the beta form was linked to induction of apoptosis.

Specific aim 2: Elucidation of unique VEGF signal transduction pathways

First we compared FGF and VEGF in terms of regulation of angiogenesis by p38. In fact, sprout formation induced by FGF displayed a similar enhancement of the effect as observed with VEGF (see Fig. 2 in Issbrucker et al, 2003). This finding is in line with a parallel publication also demonstrating a negative regulatory role of this kinase in FGF-induced angiogenesis in vitro and in vivo (Matsumoto et al., 2002). Together, it suggests that the negative regulatory role of p38 MAPK is a general phenomenon and could be applied for an antiangiogenic therapy approach.

VEGF can bind and activate two receptors on endothelial cells, VEGFR-1 and VEGFR-2. Although we have previously identified the VEGFR-2 as the main signaling receptor in endothelial cells, we wanted to confirm that also sprout formation as well as the inhibitory effects of the p38 MAPK are mediated by this receptor. Therefore we compared VEGF with the VEGFR-1-specific ligand placenta growth factor (PlGF) and the VEGFR-2-specific ligand VEGF-E. We found that sprout formation is mediated by the VEGFR-2 because VEGF-E can almost as potently as VEGF induce angiogenesis in this assay. Furthermore, the effect of VEFG-E can be enhanced by p38 MAPK inhibitors to a similar extent as with VEGF. However, PlGF failed to induce vessel formation in this assay. Interestingly, even combinations of high concentrations of PlGF with VEGF or VEGF-E cannot further enhance the effects of this factor, indicating that the two VEGF-receptors do not cooperate in this system.

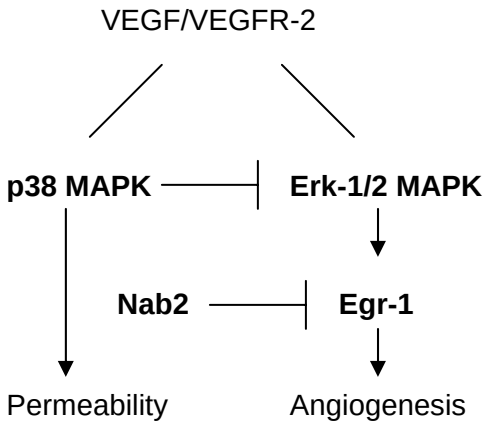


Fig. 2: Schematic overview over the signaling pathways addressed in this project. See text for a description.

Based on our previous finding that erk-1/2 MAPK is essential for VEGF induced serum response factor egr-1 induction by VEGF, we tested the hypothesis that this pathway would comprise a mechanism by which VEGF-induced erk-1/2 is negatively regulated by p38 MAPK. We approached this by using an adenoviral vector encoding for Nab2, a naturally occurring inhibitor of egr-1. As shown in Figure 6 in Lucerna et al. (2003), VEGF-induced sprout formation is strongly reduced when the microvascular endothelial cell were transduced with Nab2 adenovirus in comparison to endothelial cells treated with no insert containing control virus. In addition, we observed reduced proliferation and plasminogen activator expression in Nab2 overexpressing cells. These findings are in line with our demonstration from above that proliferation and plasminogen activation are main targets in p38 MAPK-induced inhibition of sprout formation. However, this pathway is not unique for VEGF but appears to also mediate EGF- and FGF-induced angiogenesis. In conclusion, we have identified the proangiogenic VEGFR-2 – erk-1/2 – egr-1 pathway in this study. Importantly, we demonstrated two natural inhibitory mechanisms for this pathway, which comprise the p38 MAPK for the erk-1/2 and Nab2 for egr-1 (See schematic overview in Fig. 2).

Specific aim 3: Effects of the identified signaling molecules in further functional assays

Having identified p38 MAPK and Nab2 as two novel regulators of angiogenesis in assays for physiological angiogenesis (CAM) sprout formation, we wanted to explore their role in pathophysiological settings of angiogenesis, i.e. tumor angiogenesis and arteriogenesis. For assessment of tumor angiogenesis we used the fibrosarcoma model established in our laboratory. In this model, cells of a fibrosarcoma cell line generated by intradermal treatment of C57Bl/6 mice with methylcholanthren (kindly provided by Daniela Männel, University of Regensburg, Germany) were inoculated in the skin of C57Bl/6 mice. Starting with the time of maximal angiogenesis (day 4 to 6 after inoculation) mice were daily injected i.p. with 20 μg/animal/day SB203580 for 5 days before analyzed. Tumor angiogenesis was determined by immunohistological analysis of vessels using CD31 antibodies. However, systemic treatment did not result in the expected enhancement in angiogenesis. In fact, the results were highly variable and there was rather a tendency towards inhibition of angiogenesis in presence of the MAPK inhibitor. These results may reflect the highly proinflammatory status of tumor angiogenesis. Of note, tumor vessels are highly activated and TNF expressed in the tumor endothelium accounts for increased vascular permeability often seen in tumors. In line with the overall feature of an activated endothelium, tumor endothelial cells express

increased levels of adhesion molecules such as ICAM-1, which have been described to be proangiogenic. In this context, suppression of p38 MAPK activity, which is upstream of ICAM-1 expression may interfere with angiogenesis resulting in proangiogenic and antiangiogenic activities of the p38 MAPK in tumors. Next, we tested the proposed antiangiogenic role of Nab2 in angiogenesis using the Matrigel plug assay in vivo. GFP or NAB2 expressing adenoviruses were mixed into the Matrigel solution with or without VEGF. These mixtures were injected subcutaneously into mice and analyzed six days later. As seen in the hematoxylin staining of sections (Fig. 8A in Lucerna et al, 2003), a high number of cells invaded the Matrigel plug and vessel-like structures formed by CD31 positive endothelial cells were visible only when VEGF was present. Quantification of cells in the Matrigel plugs demonstrated that the presence of adenoviral vectors encoding for Nab2 reduced the number of total cells in sections of the plugs supplemented with VEGF to levels seen with control virus and no vessel-like structures formed by endothelial cells could be observed inside the Matrigel plug in Nab2 adenoviral vectors containing plugs. These inhibitory effects suggest an antiangiogenic role for Nab2 in vivo. To further support an important role of egr-1 in angiogenesis we injected adenovirus encoding for egr-1 in the C57BL/6 tumor model described above. Surprisingly, we found inhibition of tumor angiogenesis rather than the expected increase. We explain this that egr-1 overexpression leads to the induction of antiangiogenic molecules, which, when exceeding a certain threshold level, destabilize the otherwise tightly regulated angiogenesis balance. In conclusion, more studies are required to address the role of the erk-1/2 MAPK – egr-1 axis and of their inhibitors p38 MAPK and Nab2 in tumor angiogenesis.

Our next aim was to address the role of the p38 MAPK in an *in vivo* model for the formation of collateral vessels (arteriogenesis). While angiogenesis involves sprouting of new vessels from existing vessels, arteriogenesis improves perfusion by conversion of small arteriolar anastomotic vessels into functional collateral arteries. To address arteriogenesis researchers use the model of femoral artery ligation to search for arteriogenic factors. In this model, one to two weeks after ligation with/without intra-arterial infusion of growth factor at the site of occlusion, the formation of new collateral vessels in the hind limb is assessed by angiography and the recovery in blood perfusion is evaluated by invasive ultrasound probes or by noninvasive laser Doppler measurements. VEGF-induced arteriogenesis is inhibited by the systemic application of a chemical p38 MAPK inhibitor (data not shown). This is in contrast to our finding from above that the p38 MAPK is an inhibitor of in *vitro* and *in vivo* angiogenesis. A possible solution of these contradicting results could be that in arteriogenesis proinflammatory cytokines and nonendothelial cells contribute stronger as it may be the case in angiogenesis. For instance, we have previously reported a critical role of monocytes in arteriogenesis, and p38 MAPK has been identified to be important for monocyte migration and activation. In line with the demonstration that monocytes play a pivotal role in angiogenesis is the monocyte activating activity of VEGF. Therefore, it is possible that our observation of increased arteriogenesis by p38 MAPK inhibition is caused by an effect of VEGF on monocytes rather than endothelial cells.

Specific aim 4: Elucidation of VEGF-specific signal transduction pathways for VEGF-caused side effects

Given the negative regulatory role of the p38 MAPK in angiogenesis, we also wanted to know whether another cognate activity of VEGF, namely to induce vascular permeability, was regulated by this kinase. First, we tested the effect of p38 MAPK inhibition in the CAM. We have observed increased angiogenesis in this assay after treatment with the SB203580 inhibitor of p38 MAPK activity. By prestimulation with SB203580 we found dramatically decreased hyperpermeability in the CAM as tested by FITC-dextran injection measurement of tissue leakage (see Fig. 6A in Issbrucker

et al., 2003). Next, we examined the effect of p38 MAPK on VEGF-induced hyperpermeability in endothelial monolayers grown on polycarbonate filter membranes. In fact, VEGF-induced vascular permeability was completely blocked by addition of p38 MAPK inhibitor (see Fig. 6C in Issbrucker et al., 2003), which suggests an involvement of p38 MAPK in the control of endothelial monolayer permeability.

In another model, vascular permeability changes were studied in the brain under hypoxic conditions. Hypoxia, which induces the expression of VEGF, was achieved by substituting oxygen with nitrogen after a gradual adaptation time of 1 h. Mice were intraperitoneally injected with 20 µg SB203580 or vehicle and were exposed to normobaric hypoxia at 8% oxygen for 24 h or were kept at room air. To quantify vascular permeability of brain vessels, 200 µl sodium-fluorescein (6 mg/ml in PBS) was injected through the tail vein and the fluorescence of the brain parenchyma was determined. Mice exposed to normobaric hypoxia at 8% oxygen for 24 h showed a 2 times higher vascular permeability in the brain. Furthermore, intraperitoneal application of the SB203580 inhibitor of the p38 MAPK completely abolished the hypoxia-induced vascular permeability in the brain (see Fig. 6B in Issbrucker et al., 2003). Vascular permeability caused by VEGF has implications both for pathophysiology and therapeutic design. VEGF-induced vascular permeability is a chief candidate mediator for stroke-related edema formation in the brain based on animal studies and for high altitude cerebral edema formation. These findings have considerable clinical implication because they support the hypothesis that endothelial activation and edema causes secondary tissue damage in stroke patients. Thus, the use of anti-VEGF therapy may not be practical in these settings, based on animal studies showing that blocking VEGFR-2 signaling can lead to pulmonary emphysema. This may be due to interference with survival or proangiogenic effects of VEGF, promoting ongoing vascular injury and regression. In addition, endothelial permeability due to VEGF hampers its potential usefulness for treating ischemic disorders in heart and other organs. For these reasons, it may be beneficial to identify molecular mechanisms which could permit selective interference with VEGF–induced vascular permeability, rather than VEGF-mediated survival. However, most VEGF-induced signaling molecules induce vascular permeability as well as endothelial survival and/or angiogenesis, including triggering of signaling via activation of PKC and NO. Therefore, our demonstration that the p38 MAPK inhibitor SB203580 abrogates VEGF-induced vascular permeability is of significance. Altogether, these data support the hypothesis that the p38 MAP-kinase is a molecular switch that can be used to separate angiogenesis from vascular permeability.

3. The influence of transcriptional regulator on angiogenesis in vivo (1^{st}-3^{rd} funding periods, collaboration with Ingo Flamme)

The induction of VEGFR-2 expression in mesodermal cells is the key event in the differentiation of the hemangioblastic lineage. In the adult organism, VEGFR-2 is remarkably down-regulated which leads to the cessation of endothelial cell growth. Both physiological and pathological angiogenesis in the adult organism, however, is dependent on the re-induction of VEGFR-2 in endothelial cells. The importance of VEGFR-2 induction in host and tumor endothelium was demonstrated in a study performed in collaboration with Peter Vajkoczy (University of Mannheim-Heidelberg). In a rat glioma model, angiogenesis was initiated by simultaneous induction of VEGFR-2 and Angiopoietin-2 in host blood vessels (Vajkoczy et al., 2002). This induction preceded the up-regulation of VEGF in hypoxic tumors, indicating that tumor cells may induce angiogenesis already before they become hypoxic. Based on the central role of VEGFR-2 in angiogenesis, upstream regulators of VEGFR-2 can be considered as key regulators of angiogenesis and vasculogenesis. In the third part of the project, we aimed at identifying such crucial endothelial transcriptional regulators of the *flk1* gene and at studying their role in embryonic angiogenesis. We have previously characterized promoter and enhancer

elements of the mouse *flk1* gene which were sufficient for endothelial cell-specific lacZ reporter gene expression in transgenic mice *in vivo* (Kappel et al., 1999; Kappel et al, 2000). An enhancer element located in the first intron was essential for endothelial cell specificity. The expression pattern and time course of expression of the lacZ reporter gene in these transgenic mice recapitulated the expression of the endogenous *flk1* gene, i.e. the transgene was strongly and ubiquitously expressed in the developing vasculature, was down-regulated in most vascular beds in the adult organism when angiogenesis has ceased, but was strongly re-induced in the neovasculature of tumors.

A detailed functional analysis of the *flk1* promoter/enhancer (p/e) was carried out in the second funding period in a collaboration with Shin-Ichi Nishikawa (University of Kyoto)(Hirai et al., 2003). ES cell clones were stably transfected with the *flk1* p/e-driven *lacZ* gene construct that is strongly active in transgenic mouse embryos, or a *flk1* p/e-driven green fluorescent protein (GFP) reporter construct. The activity of the *flk1* promoter was studied in a two-dimensional ES cell differentiation model. In this system, embryonic stem (ES) cells are cultured under conditions that promote their differentiation into endothelial cells which is accompanied by the induction of VEGFR-2. These experiments showed that the *flk1* p/e is inactive in VEGFR-2 positive hemangioblasts (which have the capacity to form both blood cells and endothelial cells), but becomes strongly activated when the cells differentiate into definitive endothelium, as indicated by an onset of endothelial VE-cadherin expression. Blood cells did not express the reporter gene, consistent with our previous observation that the *flk1* p/e is active selectively in endothelial cells of transgenic reporter mice, but – in contrast to the endogenous *flk1* gene – not in hematopoietic cells. Moreover, it was found that *flk1* p/e activity can be used to distinguish hemogenic from non-hemogenic endothelium. And finally, endothelium-specificity of the *flk1* p/e *in vivo* was confirmed in transgenic mice that expressed the GFP reporter gene under the control of these sequences.

Our initial *in vivo* analyses of the *flk1* p/e addressed the mechanisms of endothelial cell restriction (Kappel et al., 1999; Kappel et al., 2000). The analysis of the regulatory sequences of both genes provided evidence that a minimal promoter together with enhancer sequences from the first intron of the genes is sufficient to drive endothelial cell specific gene expression. This has been demonstrated by lacZ reporter gene studies in transgenic mice. In the respective promoters and enhancers, a series of putative transcription factor binding sites have been identified and some of them have been shown to be functional and even necessary for the expression of the transgene in the mouse embryo.

Next, we focused on transcriptional activators of the *flk1* promoter which mediates strong endothelial cell-specific transcription. Our *in vitro* and *in vivo* analyses showed that c-ets1 strongly activates the *flk1* promoter via two binding sites (Elvert et al., 1999). The *flk1* promoter is also stimulated by HIF-2α, but only weakly by HIF-1α. Two HIF-2α binding sites (differing in sequence from the consensus HRE) were identified in the immediate vicinity of the functional ets binding sites in the *flk1* promoter. Analysis of protein-DNA interaction at these sites revealed that HIF-2α is the predominant endothelial HIF, at least in cultured bovine aortic endothelial cells. By GST pull-down experiments, ets-1 was shown to interact directly with HIF-2α. The selective activity on the *flk1* promoter of HIF-2α versus HIF-1α could be allocated to the carboxy-terminal half of the proteins by the analysis of HIF-1/2α chimeras. In another series of constructs, either transactivation domain or the DNA binding domain or both were deleted. As expected, the deletion constructs exerted dominant negative activity over the wild type HIF-2α in reporter assays employing *flk1* p/e-based luciferase constructs. Transfection experiments showed that HIF-2 and ets-1 cooperate to stimulate the *flk1* promoter. To investigate the relevance of the ets/HIF-2 motifs *in vivo*, we performed a mutational analysis by lacZ reporter gene analyses in transgenic mouse embryos. The mutation of a single ets/HIF-2 motif only slightly reduced the promoter activity, whereas the mutation of both motifs

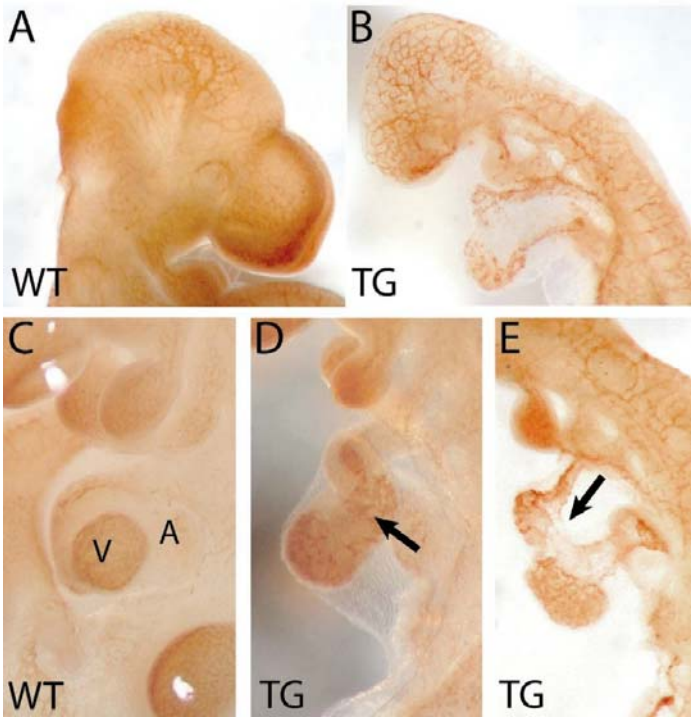


Fig. 3: Inhibition of HIF activity in endothelial cells disrupts embryonic cardiovascular development. A dominant-negative HIF mutant was expressed from the endothelium-specific flk1 gene regulatory elements in transgenic mouse embryos. PECAM-1 whole mount staining of E10.5 embryos is shown. A: wild-type embryo. B: dnHIF transgenic embryo displaying defective vascular remodelling in the head region. C: heart of a wild-type embryo with atrium (A) and Ventricle (V). D, E: defective heart looping in dnHIF embryo. See Licht et al. 2006 for further details.

rendered the promoter completely inactive in mid-gestation mouse embryos. Taken together, these results provided clear evidence that the combinatorial activity of HIF-2 and ets-1 is the major stimulus of *flk1* transcription during embryonic angiogenesis.

In the second and third funding periods, we performed a functional analysis of endothelial HIF function in transgenic mouse embryos by expressing a dominant negative HIF-2α (dnHIF) mutant specifically in the developing vascular system. This mutant lacks both transactivation domains and is likely to compete for the binding of HIF-2α and other HIF-α subunits (including HIF-1α) to their dimerization partner ARNT. The dnHIF mutant inhibited the transactivation activity of both HIF-1 and HIF-2 (Licht et al., 2006). To interfere specifically with HIF activity in endothelial cells, a cDNA encoding the dnHIF mutant was fused to *flk1* p/e elements, and transgenic mouse embryos resulting from microinjected oocytes were generated and analysed at various stages of development. A total number of 49 transgenic mouse embryos was obtained from 512 embryos examined. One third (n=15) of transgenic embryos showed severe developmental abnormalities. They were growth retarded and died around E12 due to severe malformation of the cardiovascular system (Fig. 3). The remodeling of the yolk sac and head vasculature was defective, the perineural vascular plexus failed to sprout inti the neuroectoderm, and the heart displayed an enlarged pericardial cavity and lacked trabeculation. These vascular defects are reminiscent of those in mice lacking Tie2 or Angiopoietin-1. The occurence of the phenotype was correlated with the expression of the transgene, as only transgenic mice that expressed the transgene (as detected by RT-PCR or immunohistochemistry) developed this phenotype whereas all transgenic embryos examined that did not develop the phenotype also did not express the transgene.

Analysis of endothelial receptor expression by RT-PCR revealed a partial down-regulation of VEGFR-2, and an almost complete absence of VEGFR-1 and Tie2 mRNA expression, whereas VE-Cadherin mRNA levels were unaffected (see Fig. 5 in Licht et al, 2006). This result demonstrates that the transgenic expression of the dnHIF mutant in mouse embryos caused a selective down-regulation of specific endothelial target genes (VEGFR-1 and Tie2), but did not abolish expression of all endothelial genes. Tie2 had already previously been proposed to be a HIF-2α target. It is unclear at present whether the reduced VEGFR-1 expression is a result of the loss of HIF-2α activity or of HIF-1α activity. The *flt1* promoter contains a consensus HRE and is stimulated by HIF-1. However, it cannot be excluded that *flt1* is primarily a HIF-2α target gene because HIF-2α also binds to and transactivates via the HRE. As expected, the expression of the HIF-1 target gene *VEGF* was unaltered in the dnHIF transgenic mice because primarily non-endothelial cells express VEGF.

Role of endothelial HIFs in vascular sprouting

To study whether the impaired vascular sprouting observed in the brain of transgenic dnHIF mouse embryos were a direct consequence of inhibition of HIF activity in endothelial cells, we performed an in vitro sprouting assay (Korff and Augustin, 1999). We decided to introduce the dnHIF mutant via virus-mediated gene transfer because in these experiments, it was essential to achieve a high transduction rate. Ecotropic retroviruses encoding the dnHIF mutant were generated and used to transduce mouse MS1 endothelial cells. Cells were grown to spheroids and embedded a in three-dimensional collagen matrix. Cells transduced with dnHIF showed a reduced sprouting activity (Fig. 4). In particular, the number of sprouts (rather than the length of individual sprouts) was reduced.

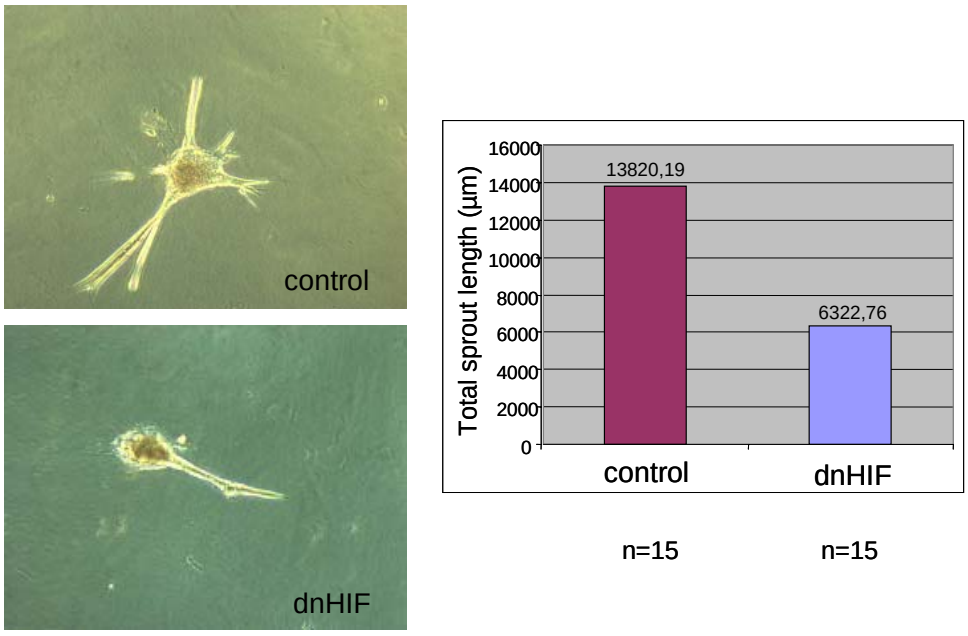


Fig. 4: Inhibition of HIF activity in endothelial cells causes impaired endothelial sprouting. Mouse MS1 endothelial cells were transduced with retrovirus encoding a dnHIF mutant of empty vector as a control. Spheroids were formed and embedded in collagen. Cumulative sprout length was determined one day later using NIH image software.

Conclusions

Taken together, our results show for the first time that HIF signaling in endothelial cells is essential for angiogenesis and blood vessel morphogenesis. Although HIFs regulate a number of genes, Tie2 appears to be a particularly critical HIF target gene in endothelial cells. Interestingly, the inactivation of the HIF-1 gene in endothelial cells of mice disrupted (pathological) angiogenesis in adult mice but did not affect embryonic (vascular) development (Tang et al., 2004). This indicates that the loss of endothelial HIF-1 activity does not account for the vascular phenotype that we observed in dnHIF transgenic mice. Furthermore, if one considers that HIF-2α knockout mice do not reproducibly develop a vascular phenotype, it is also unlikely that HIF-2 alone is responsible for the cardiovascular phenotype observed in dnHIF transgenic mice. In conclusion, HIF-1 and HIF-2 appear to exert partially overlapping functions in the developing endothelium.

It remains to be determined whether HIF signaling in the developing endothelium reflects a true hypoxia response. It has been proposed that hypoxia stimulates blood vessel growth in the embryo, and even though endothelial cells are normally not considered to be hypoxic, it may well be that the oxygen levels in the embryo are low enough to trigger the hypoxia response (Lee et al., 2001). On the other hand, it has been described that growth factor signaling also modulates HIF activity and stability in a hypoxia-independent fashion (Pugh and Ratcliffe, 2003).

Another important question to be addressed in the future is whether endothelial HIF signaling is serving similarly important functions in tumors. As described above, Tang et al. (2004) have already shown that the loss of HIF-1 in endothelial cells results in impaired tumor angiogenesis. However, it remains to be determined whether HIF-2 has similar functions, and what the effects of inhibiting both HIF-1 and HIF-2 would be. Moreover, based on our observation that HIF and Ets-1 cooperate to activate the *flk1* promoter, analysis of the role of the HIF-Ets interaction in tumor endothelium would be desirable. Finally, the recent discovery of HIF hydroxylases which act as cellular oxygen sensors (Pugh and Ratcliffe, 2003) opens a new important field in research on endothelial hypoxia signaling in physiological and pathological angiogenesis.

REFERENCES

Breier G, Albrecht U, Sterrer S, Risau W: Expression of vascular endothelial growth factor during embryonic angiogenesis and endothelial cell differentiation. *Development* 114: 521-532, 1992.

Breier G, Clauss M, Risau W: Coordinate expression of VEGF receptor-1 (flt-1) and its ligand suggests a paracrine regulation of murine vascular development. *Dev Dyn* 204: 228-239, 1995.

Breier G: Functions of the VEGF/VEGF receptor system in the vascular system. *Semin Thromb Hemost* 26: 553-559, 2000.

Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, Moons L, Collen D, Risau W, Nagy A: Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. *Nature* 380: 435-439, 1996.

Carmeliet P: Blood vessels and nerves: common signals, pathways and diseases. *Nat Rev Genet* 4: 710-720, 2003.

Claesson-Welsh L: Signal transduction by vascular endothelial growth factor receptors. *Biochem Soc Trans* 31: 20-24, 2003.

Elvert G, Lanz S, Kappel A, Flamme I: mRNA cloning and expression studies of the quail homologue of HIF-2alpha. *Mech Dev* 87: 193-197, 1999.

Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, Acker T, Rauter M, Plate K, Sieweke M, Breier G, Flamme I: Cooperative interaction of hypoxia inducible factor (HIF)-2α and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *J Biol Chem* 278: 7520-7530, 2003.

Ferrara N, Gerber HP, LeCouter J: The biology of VEGF and its receptors. *Nat Med* 9: 669-676, 2003.

Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW: Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. *Nature* 380: 439-442, 1996.

Hirai H, Ogawa M, Suzuki N, Yamamoto M, Breier G, Mazda O, Imanishi J, Nishikawa S: Hemogenic and nonhemogenic endothelium can be distinguished by the activity of fetal liver kinase (Flk)-1 promoter/enhancer during mouse embryogenesis. *Blood* 101: 886-893, 2003.

Issbrucker K, Marti HH, Hippenstiel S, Springmann G, Voswinckel R, Gaumann A, Breier G, Drexler HC, Suttorp N, Clauss M: p38 MAP kinase - a molecular switch between VEGF-induced angiogenesis and vascular hyperpermeability. *Faseb J* 17: 262-264, 2003.

Kappel A, Ronicke V, Damert A, Flamme I, Risau W, Breier G: Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice. *Blood* 93: 4284-4292, 1999.

Kappel A, Schlaeger TM, Flamme I, Orkin SH, Risau W, Breier G: Role of SCL/Tal-1, GATA, and ets transcription factor binding sites for the regulation of flk-1 expression during murine vascular development. *Blood* 96: 3078-3085, 2000.

Korff T, Augustin HG: Tensional forces in fibrillar extracellular matrices control directional capillary sprouting. *J Cell Sci* 112: 3249-3258, 1999.

Lee YM, Jeong CH, Koo SY, Son MJ, Song HS, Bae SK, Raleigh JA, Chung HY, Yoo MA, Kim KW: Determination of hypoxic region by hypoxia marker in developing mouse embryos in vivo: a possible signal for vessel development. *Dev Dyn* 220: 175-186, 2004.

Licht AH, Muller-Holtkamp F, Flamme I, Breier G: Inhibition of hypoxia-inducible factor activity in endothelial cells disrupts embryonic cardiovascular development. *Blood* 107: 584-90, 2006.

Licht AH, Raab S, Hofmann U, Breier G: Endothelium-specific Cre recombinase activity in flk-1-Cre transgenic mice. *Dev Dyn* 229: 312-318, 2004.

Lucerna M, Mechtcheriakova D, Kadl A, Schabbauer G, Schafer R, Gruber F, Koshelnick Y, Muller HD, Issbrucker K, Clauss M, Binder BR, Hofer E: NAB2, a corepressor of EGR-1, inhibits vascular endothelial growth factor-mediated gene induction and angiogenic responses of endothelial cells. *J Biol Chem* 278: 11433-11440.

Matsumoto T, Turesson I, Book M, Gerwins P, Claesson-Welsh L: p38 MAP kinase negatively regulates endothelial cell survival, proliferation, and differentiation in FGF-2-stimulated angiogenesis. *J Cell Biol* 156: 149-160, 2002.

Mitola S, Brenchio B, Piccinini M, Tertoolen L, Zammataro L, Breier G, Rinaudo MT, den Hertog J, Arese M, Bussolino F: Type I Collagen limits VEGFR-2 signaling by a SHP2 protein-tyrosine phosphatase-dependent mechanism. *Circ Res* 98: 45-54, 2006.

Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, Ledda F, Ziche M: Nitric oxide is an upstream signal of vascular endothelial growth factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. *J Biol Chem* 273: 4220-4226, 1998.

Pugh CW, Ratcliffe PJ: Regulation of angiogenesis by hypoxia: role of the HIF system. *Nat Med* 9: 677-684, 2003.

Raab S, Beck H, Gaumann A, Yuce A, Gerber HP, Plate K, Hammes HP, Ferrara N, Breier G: Impaired brain angiogenesis and neuronal apoptosis induced by conditional homozygous inactivation of vascular endothelial growth factor. *Thromb Haemost* 91: 595-605, 2004.

Tanaka K, Abe M, Sato Y: Roles of extracellular signal-regulated kinase 1/2 and p38 mitogen-activated protein kinase in the signal transduction of basic fibroblast growth factor in endothelial cells during angiogenesis. *Jpn J Cancer Res* 90: 647-654, 1999.

Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber HP, Ferrara N, Johnson RS: Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. *Cancer Cell* 6: 485-495, 2004.

Tronche F, Kellendonk C, Kretz O, Gass P, Anlag K, Orban PC, Bock R, Klein R, Schutz G: Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. *Nat Genet* 23: 99-103, 1999.

Vajkoczy P, Farhadi M, Gaumann A, Heidenreich R, Erber R, Wunder A, Tonn JC, Menger MD, Breier G: Microtumor growth initiates angiogenic sprouting with simultaneous expression of VEGF, VEGF receptor-2, and angiopoietin-2. *J Clin Invest* 2002: 109:777-785, 2002.

Yoshiji H, Kuriyama S, Ways DK, Yoshii J, Miyamoto Y, Kawata M, Ikenaka Y, Tsujinoue H, Nakatani T, Shibuya M, Fukui H: Protein kinase C lies on the signaling pathway for vascular endothelial growth factor-mediated tumor development and angiogenesis. *Cancer Res* 59: 4413-4418, 1999.

The Role of Hypoxia inducible Transcription Factors (HIF) in Tumor Growth

Prof. Dr. Karl H. Plate
Dr. Till Acker

Edinger Institute, Dept. of Neuropathology
University of Frankfurt
Deutschordenstr. 46, 60528 Frankfurt, Germany

Member of the SPP 1069 from 1999 to 2005

SUMMARY

High proliferating tumors frequently outstrip their vascular supply leading to a tumor microenvironment characterized by low PO_2, low glucose levels and an acidic pH. The ability to initiate homeostatic responses such as induction of angiogenesis and to adapt to hypoxia represents an important and crucial aspect in solid tumor growth. The mechanisms by which tumors recruit their vasculature have been subject to intense investigations. The acquisition of a functional blood supply is rate limiting for the tumor's ability to grow beyond a certain size and metastasize to other sites. Accumulating evidence indicates that hypoxia and the key transcriptional system, HIF (hypoxia-inducible factor)-1α and HIF-2α, are the major triggers for new blood vessel growth in malignant tumors. Recent insight into the cellular and molecular crosstalk suggests a model in which hypoxia, HIF and several HIF-target genes participate in the coordinate collaboration between tumor and endothelial cells to enhance and promote tumor vascularization. However, their distinct and cell specific role in the hypoxia response in tumors remains elusive.

Interestingly, the HIF pathway has a dual role in tumor growth encompassing both tumor growth promoting and inhibiting effects of which, as we could recently show, critically depends on HIF protein levels. By modulating HIF function in three different experimental models we provided genetic evidence that HIF overexpression though increasing tumor vessel density resulted in stunted tumor growth partly by inducing apoptosis. In contrast, HIF inhibition or loss of function decreased tumor angiogenesis but paradoxically accelerated tumor growth. Based on our findings careful consideration of HIF inhibitors as cancer therapeutic strategies is warranted, as HIF inhibition may select for individual tumor clones resistant to stress-induced apoptosis, which would promote – instead of suppress - tumor growth. Tight regulation of HIF levels is thus essential for tumor survival and growth. We could further demonstrate that metabolic parameters such as pH or glucose, reciprocal regulation via HIF-α subunits as well as prolyl hydroxylases, central regulators of HIF-α activity, control HIF levels and function in glioblastomas and may thus be critical in setting the balance between pro- and anti-tumorigenic effects.

Mounting evidence suggests that HIF may act as endothelial master switches to regulate endothelial (EC) function in angiogenesis. We could show that in settings of physiological (brain development) or pathological (vascular malformations, tumor growth) angiogenesis elevated HIF-α levels correlate with endothelial cell activity. To functionally analyze the endothelial cell intrinsic function of HIF we generated transgenic mice with inducible endothelial cell specific HIF modulation.

In consequence, our findings help to elucidate the complex role of HIF in tumor growth. An integrated understanding of the dual function of HIF, i.e. the regulation of pro- and antitumorigenic

mechanisms, as well as the cell specific role of HIF in the intricate tumor microenvironment is indispensable to reveal novel targets for therapeutic intervention to efficiently inhibit tumor growth.

INTRODUCTION

Tumor growth and progression occurs as a result of cumulative acquisition of genetic alterations affecting oncogenes or tumor suppressor genes selecting for tumor cell clones with enhanced proliferation and survival potential. However, high proliferating tumors frequently outstrip their vascular supply leading to a tumor microenvironment characterized by low oxygen tension, low glucose levels and an acidic pH. Regions of low oxygen tension are indeed common findings in malignant tumors (Brown and Giaccia, 1998) being associated with increased frequency of tumor invasion and metastasis and a poor therapy outcome. The ability to initiate homeostatic responses and adapt to hypoxia represents an important and crucial aspect in solid tumor growth. In particular activation of the HIF (hypoxia inducible factor) system has been identified as an important mediator of these processes (Acker and Plate, 2002). To date more than 60 genes have been identified as potential HIF-target genes providing further evidence for HIF as a key regulatory system of adaptive mechanisms.

The Transcription Factor System HIF

Since its discovery in 1995 the HIF transcriptional complex has emerged as the key regulatory system of adaptive mechanisms in response to reductions in oxygen tension. The HIF transcriptional complex is a heterodimer composed of HIF-α and HIF-β subunits belonging to the bHLH (basic helix loop helix)-PAS family of transcription factors. Specificity to hypoxia-mediated responses is conferred by HIF-α subunits. At least two mammalian α subunits, HIF-1α and HIF-2α, are regulated by oxygen in a similar fashion (Wiesener et al., 1998). Transcriptional activity of HIF-α subunits by cellular O_2 is mainly regulated by protein levels and transactivation domain function. Under normoxic conditions HIF-α are subject to rapid ubiquitination and proteosomal degradation requiring binding of pVHL (von-Hippel-Lindau protein) to specific hydroxylated prolyl residues within the HIF-α subunits. Hydroxylation is conferred by a distinct class of 2-oxoglutarate-dependent oxygenases termed HIF-prolyl-hydroxylase (HPHD) (Epstein et al., 2001). A second oxygen dependent switch involves a hydroxylation of an asparagine residue within the C-TAD of HIF- α subunits by factor-inhibiting HIF (FIH-1) (Lando et al., 2002) resulting in reduced transcriptional activity. Apart from oxygen tension additional mechanisms have been identified which influence HIF function including tumor specific alterations of oncogenes and tumor suppressor genes as well as various growth factor signalling pathways. Induction by these pathways is generally less intense than that mediated by reductions in oxygen tension (Acker and Plate, 2002).

The precise role of HIF-1α and/or HIF-2α in the hypoxia response is only beginning to be elucidated and an emerging field of research (Hu et al., 2003). The differences in target specificity and oxygen dependent regulation of the two orthologues HIF-1α and HIF-2α may add a further level of complexity to oxygen signaling though recent studies argue for a limited HIF-2α function in hypoxia-induced signaling (Sowter et al., 2003). However, specific target genes regulated independently of HIF-1α do exist as we showed for VEGFR-2 (Elvert et al., 2003), pneumocyte type II dependent VEGF expression (Compernolle et al., 2002) or was shown for TGFα (transforming growth factor) (Smith et al., 2005). Indeed, studies analyzing the tissue specific expression pattern of HIF-1α (Stroka et al., 2001) and HIF-2α (Wiesener et al., 2003) suggest rather complementary than

redundant functions of both proteins. This is further evidenced by the diverse phenotypes reported that were obtained by targeted deletion of HIF-1α or HIF-2α rev. in (Acker and Acker, 2004).

HIF in tumor progression

In comparison to adjacent tissue widespread HIF activation is observed in tumors, correlating with tumor growth and progression (Zhong et al., 1999; Zagzag et al., 2000). HIF overexpression in tumors is related both to hypoxia-dependent as well as hypoxia-independent mechanisms, such as oncogene activation and growth factor signaling pathways. The relevance of the HIF system to tumor growth and progression is further highlighted by the variety of mechanisms regulated by HIF-target genes ranging from angiogenesis to increase tissue oxygenation over glycolysis and pH regulation allowing for energy generation when oxygen is scarce to cell proliferation and survival pathways. This has lead to the assumption that HIF acts as a tumor-promotor. The recent insight into the precise mechanisms of oxygen sensing and signaling may help to develop novel anti-tumor strategies which specifically target the PHD-HIF-VHL pathway. Given the widespread HIF activation in tumors, the role of HIF in transactivating angiogenic factors and the role of angiogenic factors in tumor growth, interfering with this pathway is particularly appealing. Its rationale lies in depriving the tumor cell of oxygen and nutrients by inhibiting angiogenesis while at the same time disabling adaptive mechanisms that help the cell to survive in this microenvironment. The feasibility of this approach has been confirmed in different reports (Maxwell et al., 1997; Ryan et al., 1998; Kung et al., 2000; Kim et al., 2001). However, given their key regulatory role in various complex physiological pathways stretching from metabolism, proliferation, differentiation to apoptosis general manipulation of the HIF system is likely to show variable outcome depending on the cellular context e.g. tumor cell type (Carmeliet et al., 1998; Blancher et al., 2000) or tumor microenvironment (Blouw et al., 2003) and should for this reason be employed cautiously. Indeed, we could recently demonstrate that HIF-1a and HIF-2a can act as a tumor suppressor partly by inducing apoptosis (Acker et al., 2005).

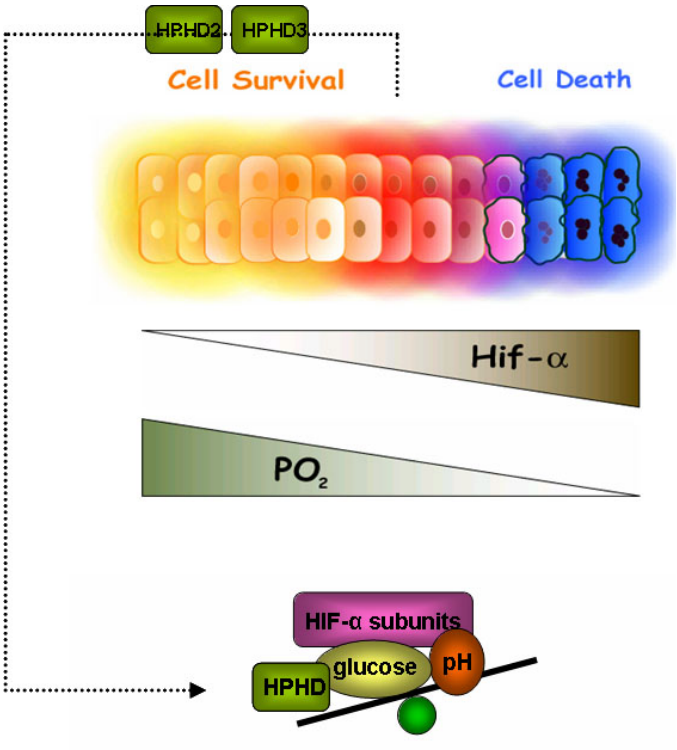


Fig.1: Model of the dual function of HIF in tumor growth The HIF system transactivates an extended physiological pathway which encompasses a wide array of physiological responses to hypoxia ranging from mechanisms that increase cell survival to those inducing cell cycle arrest or even apoptosis. Depending on the degree and duration of hypoxia quantitative and qualitative changes in the hypoxia response occur which are regulated by concomitant changes in HIF-α protein levels, modifications and HIF-α subunit expression. We propose that HIF-protein levels/activity (moderate vs. high) as a function of the PO_2 regulate the qualitative response of the tumor cell (pro-tumorigenic vs. anti-tumorigenic). Central regulators of HIF activity such as metabolic parameters (glucose, pH), prolyl hydroxylases (PHD) and the reciprocal regulatory effect of the distinct HIF-α subunits dictate the balance between pro- and antitumorigenic mechanisms. PHD2 and PHD3 as HIF target genes could act a part of a negative feedback loop to prevent HIF-α from reaching toxic concentration upon decreases in oxygen levels.

Moreover, HIF itself may directly influence the outcome of present therapies such as chemotherapy and radiotherapy. HIF-1α deficiency in fibroblasts resulted in increased apoptotic cell death in response to agents such as carboplatin and ectoposide or ionizing radiation known to induce double-strand breaks. This suggests that HIF-1α may induce the expression of genes involved in the repair of DNA double-strand breaks. (Unruh et al., 2003). Thus, HIF may act as a tumor promoter as well as a tumor suppressor. How these opposing responses are regulated is unclear but may, as we suggested, critically depend on HIF activity/levels with high HIF levels favoring the induction of apoptotic responses (Acker and Acker, 2004)(Fig. 1). As a consequence central regulators of HIF function such as PHD or FIH may have a crucial role in determining the qualitative response of the cells toward hypoxia by setting the balance between HIF induced pro- and antitumorigenic mechanisms.

It is worth to remember that the HIF pathway exerts its effects not only on the tumor cell but also on the stromal microenvironment to enhance and promote tumor vascularization and growth. In fact, the activation of the HIF system elicits a cellular and molecular crosstalk leading to the coordinated collaboration between tumor, endothelial, inflammatory/hematopoietic, and circulating endothelial precursor cells rev. in (Acker and Plate, 2003).

HIF as master regulators of endothelial cell function

Hypoxia and HIF-mediated induction of angiogenesis is thought to be mainly conferred by transactivation of VEGF in surrounding cells, thus acting in a paracrine fashion on EC (extrinsic pathway). However, exposure of EC to intermittent and chronic hypoxic conditions has been shown to occur in vivo as a result of the structural and functional abnormal tumor vasculature (Helmlinger et al., 1997; Kimura et al., 1996). Recent studies suggest, that hypoxia may operate as an intrinsic regulator of EC growth and function by stimulating receptor and ligand expression. HIF-α subunits have been reported to induce VEGFR-1, VEGFR-2 and Tie2 (Gerber et al., 1997; Kappel et al., 1999; Tian et al., 1997; Favier et al., 2001; Elvert et al., 2003). Stabilization of HIF-1α by the peptide regulator (PR) 39 in different EC lines resulted in VEGF upregulation and accelerated formation of vascular structures. Moreover, in vivo expression of PR39 targeted to the myocardium increased myocardial vascularization, though it was not clear from that study how relevant HIF pathway activation in EC as opposed to activation in the surrounding tissue was (Li et al., 2000). Interestingly, hypoxic VEGF induction in EC was also shown to promote network formation in vitro (Helmlinger et al., 2000). In contrast, transgenic endothelial specific HIF inhibition with a dominant negative HIF mutant resulted in embryonic lethality due to vascularization remodeling and cardiac defects (Licht et al., 2006). Moreover, targeted HIF-1α deficiency in endothelial cells reduced tumor growth as a result of decreased angiogenesis clearly supporting the significance of the endothelial cell specific HIF function in tumor growth (Tang et al., 2004).

Thus, hypoxia-driven autocrine stimulation of endothelial cells may enhance the angiogenic pathway and participate in the formation and reorganization of the vascular network. Taken together, several lines of evidence suggest that HIF-1α and HIF-2α act as both intrinsic and extrinsic regulators of EC growth and function in tumor growth by stimulating receptor and ligand expression (Acker and Plate, 2003). This system, thus, represents an interesting target to use as a master switch for therapeutic angiogenesis or anti-angiogenesis.

METHODS

The laboratories of Till Acker and Karl Heinz Plate have a strong background in molecular and cell biology techniques as well as in vivo models to study the regulation and function of hypoxia inducible gene expression in different physiological and pathophysiological settings. We use a panel of well-characterized human glioblastoma (Gl55, Gl120, G 121, Gl123, Gl141, Gl142, Gl270, U87, 229, GBM, U343, U251, U118, SNB19) as well as rat glioma cell lines (C6, GS9l) for our studies on the effect of hypoxia induced mechanisms in tumor growth. Glioblastoma are particularly well vascularized tumors with regions of hypoxia and necrosis. They constitute one of the most hypoxic tumor entities and therefore serve as prototype tumors to analyze the function of adaptive mechanisms to changes in oxygen concentration. We cloned and constructed HIF-1α, HIF-2α, HIF-1αΔPP and HIF-2αΔPP as well as a dominant negative HIF construct. To modulate HIF function we further employ RNAi techniques. To analyze hypoxia/HIF mediated mechanisms we use Northern Blot, qPCR, Promotor-Luciferase assays and Western Blot, to determine mRNA and protein expression, respectively, of different HIF target genes including angiogenic factors (VEGF), glycolytic enzymes (LDH, Glut-1), pH regulators (CAIX). Functional assays included Caspase 3 ELISA, TUNEL and oligonucleosome to analyze apoptotic induction as well as BrdU incorporation to measure cell proliferation. Further, cell transfection and selection of stable clones, retroviral infection, production of retrovirus producer cells are established techniques in our lab. The tet-on and tet-off expression systems have been successfully employed. The in vivo growth of tumors is analyzed by different transplantation models such as the subcutaneous as well as the intracranial tumor growth model. We have established various histological techniques including immunohistochemistry as well as in situ hybridization to analyze vessel density (IHC-immunohistochemistry with the endothelial cell marker CD-34), vessel branching and morphology in comparison to normal brain blood vessels, endothelial (EC) proliferation (co-labeling of CD34 and the proliferation marker Ki-67, tumor proliferation (Ki-67 labeling index) and apoptosis (TUNEL, activated caspase 3, oligonucleosome). HIF-1α, HIF-2α, transgene expression and different HIF target genes such as VEGF, LDH, Glut-1 are further analyzed by in situ hybridization, IHC, qPCR and/or Western Blot analysis. Using a hypoxyprobe (Molecular Probes) we assess their relation to hypoxic areas within the tumor. Histological quantification of the parameter mentioned is routinely performed using the Soft-Imaging analysis system and is well established in our laboratory.

RESULTS AND DISCUSSION

During the funding period of the SPP 1069 (1999-2005), we have focused on the (differential and cell specific) function and regulation of the key regulators HIF-1α and HIF-2α in mediating adaptive responses to hypoxia during mouse development and in tumor growth and angiogenesis.

HIF in mouse development

Both, hypoxia and HIF have been implicated in regulating developmental vascularization. Within the VEGF-promotor binding of HIF to the hypoxia-response element (HRE) transactivates VEGF-expression in response to decreased oxygen tensions. In collaboration with P. Carmeliet (Leuven) we further analyzed the relevance of hypoxia and HIF in VEGF function by generating "knock-in" mice with a deletion of the HRE in the VEGF promotor (Oosthuyse et al., 2001). Concurrent with reduced normoxic and hypoxic expression of VEGF in neural tissue, mice homozygous for the deletion developed an adult-onset progressive motor neuron degeneration with muscle weakness, reminiscent of the clinical and neuropathological changes observed in amyotrophic lateral sclerosis. The results indicate that chronic vascular insufficiency as well as insufficient $VEGF_{165}$ mediated

neuroprotection via binding to VEGFR-2 and Neuropilin-1 cause selective degeneration of motor neurons. The findings established a new, previously unknown function of VEGF in the pathogenesis of neurodegenerative diseases and represented a novelty in the field.

In obstetrics respiratory distress syndrome (RDS) due to insufficient production of surfactant presents a common and severe complication of preterm delivery. In collaboration with P. Carmeliet (Leuven) HIF-2α deficient neonatal mice were found to develop fatal RDS due to insufficient surfactant production by alveolar type 2 cells coinciding with reduced levels of the HIF-2α target gene VEGF (Compernolle et al., 2002), thus representing yet another function of HIF-2α in embryonic development (see above). Several experiments demonstrated the decisive role of VEGF in fetal lung maturation. Mice with a deficiency of $VEGF_{164}$ or $VEGF_{188}$ isoforms or deletion of the HRE in the VEGF promotor demonstrated severe signs of RDS. A comparable RDS phenotype was induced by intrauterine delivery of anti-VEGFR-2 antibodies while intrauterine delivery or postnatal intratracheal instillation of VEGF which stimulated conversion of glycogen to surfactant protected preterm mice against RDS. In line with these findings, VEGF administration was shown to induce production of surfactant proteins in cultured type 2 pneumocytes. The results point to an important role of HIF-2α target genes such as VEGF in the pathogenesis but also the treatment of RDS in preterm infants.

HIF in brain tumor growth

Adaptation to hypoxia and hypoxic induction of angiogenesis represent important aspects in solid tumor growth (Acker and Plate, 2002; Acker and Acker, 2004; Acker and Plate, 2003). Several lines of evidence suggest that hypoxia inducible transcription factors play a decisive role in tumor physiology and progression. HIF-1α and HIF-2α mediate cellular and adaptive responses to hypoxia and hypoglycaemia, including angiogenesis, regulation of pH and energy metabolism. Findings of our lab showed that important regulators of pathological angiogenesis such as VEGF, VEGFR-2, angiopoietin-2 and PlGF are regulated by HIF/hypoxia and have an impact on tumor growth (Elvert et al., 2003; Beck et al., 2000; Stratmann et al., 2001; Beck et al., 2002; Carmeliet et al., 2001). Interestingly, VEGFR-2 was specifically regulated by HIF-2α (independently of HIF-1α) via a HIF-2α binding site in the VEGFR-2 promotor further pointing to a non-redundant role of the HIF-α subunits in hypoxia inducible target gene expression (Elvert et al., 2003). We could show that widespread HIF activation is observed particularly in hypoxic regions in a variety of malignant tumors (Kunz et al., 2001) but also in VHL-deficient tumors (Krieg et al., 2000). Using different VHL-deficient renal cell carcinoma (RCC) cell lines we demonstrated upregulation of HIF-1α and –2α protein and putative target genes by VHL-loss of function, which was abolished by reintroduction of wildtype VHL (Krieg et al., 2000) clearly establishing the VHL-dependency of HIF-a protein. In addition, we could similarly show that VHL deficient hemangioblastomas and RCCs reveal a constitutive upregulation of HIF-1/2α in vivo.

However, the role of HIF in tumorigenesis remains controversial, as both tumor growth-promoter and -suppressor activities have been ascribed to HIF-1α, whereas the role of HIF-2α remains largely unknown. Very recently we could demonstrate that the HIF pathway is far more complex, activating both tumor growth promoting and inhibiting components. Overexpression of HIF-2α in rat gliomas enhanced angiogenesis but reduced tumor growth (Fig. 2) in part due to increased tumor cell apoptosis. siRNA mediated knock-down of HIF-1α and HIF-2α abrogated hypoxia mediated apoptosis in human glioblastoma cells. In contrast, HIF inhibition by a dominant negative HIF mutant in rat gliomas or by HIF-2α knock-out in teratomas accelerated tumor growth and reduced tumor angiogenesis. Compared to control tumors, apoptosis was increased 5.1 ± 0.6 fold in HIF-2α tumors (P<0.005; N=6). The increased apoptosis of HIF-2α overexpressing tumors was confirmed by

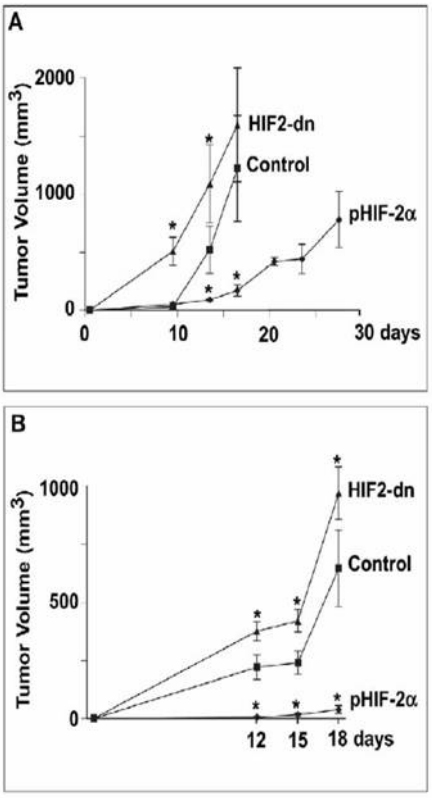


Fig. 2: HIF-2α reduces tumor growth, whereas HIF inhibition promotes tumor growth A and B: Growth rate of tumors derived from GS9L cells, stably transfected with control vector (pcDNA3) or with constructs expressing HIF-2α (*pHIF-2α*) or a dominant-negative HIF (pHIF-dn). **A** and **B** display two separate experiments, using the same control and *pHIF-dn* tumor clone but different individual HIF-2α-overexpressing tumor clones. Data represent mean ± SEM (n = 10). *p < 0.05 versus pcDNA3.

measuring the tumor cell area, immunoreactive for activated caspase-3 (aCasp-3), an executer of apoptosis. In control tumors, aCasp-3 positive tumor cells were detected only occasionally, while numerous aCasp-3-positive cells were present in the perinecrotic area in tumors overexpressing HIF-2α (aCasp-3$^+$ area/optical field: 11.4 ± 1.9%; *N*=21). Similar findings were obtained for HIF-1α tumors. Protein levels of HIF-2α, both of the endogenous protein as well as of the transgene product, varied in individual cells, both in cultured tumor cells *in vitro* as well as in tumors *in vivo* – presumably because of cellular differences in post-translational stabilization. We took advantage of this heterogeneous expression pattern to examine whether those cells that expressed the highest HIF-2α levels were also labeled the strongest for aCasp3, as this might suggest that HIF-2α would switch on apoptosis in these cells. In both mock-transfected (not shown) and HIF-2α-transfected tumor cells, the highest HIF-2α levels were detected in cultured cells, containing the highest aCasp-3 levels (Fig. 3A-C). When grown as tumors *in vivo*, aCasp-3 levels were maximal in a subset of glioma cells in the perinecrotic area, which co-expressed abundant HIF-2α (Fig. 3D-F). These findings were not a peculiarity of rodent glioma tumors, as HIF-2α protein levels were also maximal in pseudopallisading cells in the perinecrotic area in human glioblastoma multiforme tumors (Fig. 3G). Importantly, induction of apoptosis, assayed by TUNEL or aCasp-3 immunostaining, was also maximal in the pseudopallisading cells, expressing maximal HIF-2α protein levels (Fig. 3H-I). This study was published in Cancer Cell in August last year (Acker et al., 2005).

In summary, we propose the working hypothesis that the decision between survival versus apoptosis may critically depend on HIF protein or activity levels with high levels of HIF favoring induction of cell death (Acker and Acker, 2004) (Fig. 1). Tight regulation of HIF levels is, thus, essential for tumor survival and growth. As a consequence regulators of HIF activity may crucially determine the balance between pro- and antitumorigenic mechanisms and thus the outcome of HIF function on tumor growth.

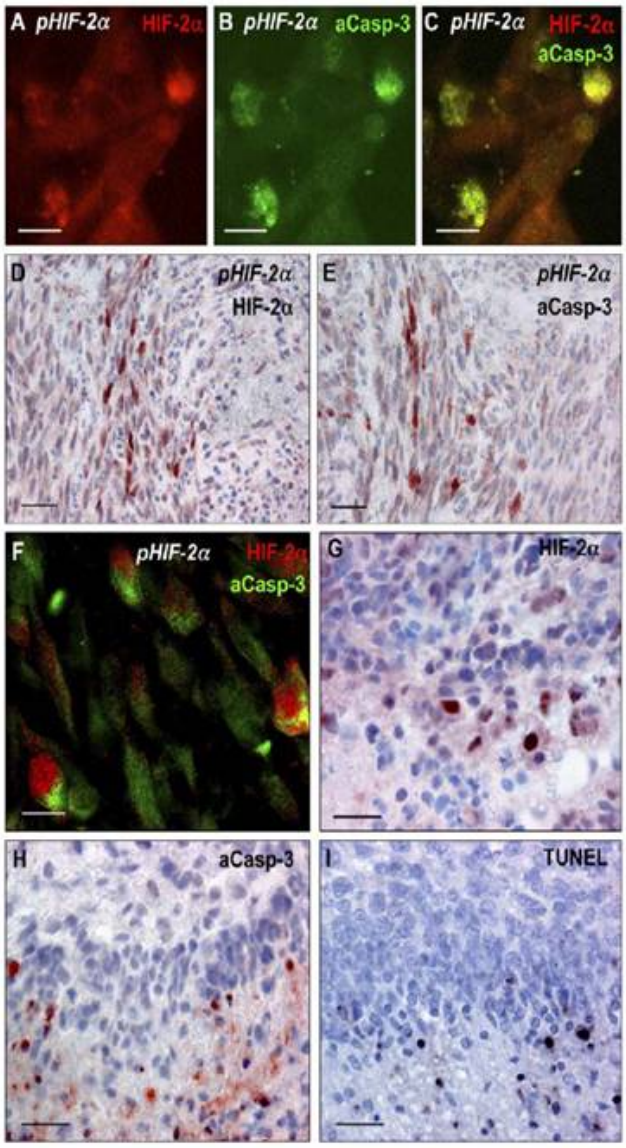


Fig. 3: HIF-2α regulates tumor cell apoptosis A–C: Coexpression of HIF-2α and activated caspase-3 in vitro. **D and E:** Tumors derived from GS9L cells, stably transfected with a construct expressing HIF-2α (*pHIF-2α*), showed excessive staining (red) of HIF-2α (**D**) and activated caspase-3 (**E**) in perinecrotic regions. **F:** Double immunostaining of GS9L tumors, stably transfected with a construct expressing HIF-2α (*pHIF-2α*), for aCasp-3 (green) and HIF-2α (red) reveals coexpression in the same tumor cells. **G–I:** HIF-2α protein levels were highest in the pseudopalisading cells in the perinecrotic area (red staining), which showed maximal induction of apoptosis, as assayed by activated caspase-3 immunostaining (red) or TUNEL (brown/black staining), thereby confirming the colocalization of HIF-2α and apoptosis

Regulation of HIF function by PHD (work in progress)

Tight regulation of HIF levels is thus essential for tumor survival and growth. As recent studies suggest, HIF activity is exquisitely controlled by an oxygen sensing cascade comprising a subfamily of 2-oxoglutarate dependent dioxygenases termed HIF prolyl-hydroxylase (HPHD). So far, four othologues of HPHD have been described with distinct subcellular localization either in the nucleus (HPHD1), the perinuclear space (HPHD2, 4) or more widely distributed within the cell (HPHD3) suggesting non redundant functions. All PHD orthologues were expressed in a panel of glioblastoma cell lines as assessed by qPCR and Western Blot analysis. Interestingly, both PHD2 and PHD3 demonstrated a hypoxia dependent upregulation. We could show that PHD2 was specifically regulated by HIF-1α whereas PHD-3 expression was dependent on both HIF-1α and HIF-2α. Forced overexpression of PHD1-4 induced a clear decrease in HIF-1/2α protein levels and transactivation activity both under normoxic and hypoxic conditions.

In summary, our data indicate that PHDs could, as a part of a negative feedback loop, help to control HIF-α levels under hypoxia preventing the induction of pro-apoptotic mechanisms. Thus, PHD could have an essential function in setting the balance between pro- and anti-tumorigenic effects.

Given the widespread physiological importance of the HIF system and broad spectrum of processes influenced by the HIF pathway this system constitutes an intriguing novel target for therapeutic intervention. Manipulation of the putative oxygen sensor system HPHD offers yet another new and challenging strategy to analyze and influence tumor biology (Riedel et al. publication in preparation).

Regulation of HIF function by metabolic parameter (work in progress)

HIF controls fundamental metabolic changes that are favourable for tumor growth by inducing a shift from oxidative to glycolytic pathways. The concomitant increase in lactate production by glycolysis is thought to be the major source of protons within the tumor, defining the microenviroment of the tumor. We were interested to analyze to what extent other metabolic parameters apart from PO_2 regulate HIF levels and function in glioblastomas. To this end we found that both low PO2 and an acidic pH synergistically increased HIF-1/2α protein levels with a concomitant induction of HIF-target genes. Interestingly, in contrast to previous findings, we observed HIF regulation by pH to be independent of PHD/VHL function.

Taken together, our results suggest that the additive induction of HIF by the metabolic parameters PO_2 and pH via PHD dependent and independent mechanisms may provide means to fully activate the HIF system, helping to efficiently and synergistically induce homeostatic responses in a hostile microenvironment (Diem et al. publication in preparation).

EC-intrinsic HIF-function (physiological and pathological aspects)

Angiogenesis requires a precisely controlled temporal and spatial gene expression in EC. Several transcription factors such SCL/Tal-1, GATA-2, c-ETS1 and HIF-2α are predominantly expressed in endothelial cells. In a collaboration with I. Flamme (Wuppertal, former SPP1069 member) and G. Breier (Bad Nauheim, SPP1069 member) we could show that HIF-2α (but not its close relative HIF-1α) cooperates with Ets-1 in activating transcription of VEGFR-2 (Elvert et al., 2003). VEGFR-2 is indispensable for angiogenesis with its expression being closely regulated during development. Consistent with the hypothesis that HIF-2α controls VEGFR-2 expression coordinated downregulation of HIF-2α and VEGFR-2 in ECs could be observed in postnatal mouse brain development correlating with declining angiogenic activity. A tandem HIF-2α /Ets binding site was identified within the VEGFR-2 promoter acting as a strong enhancer element. Based on transgenic mouse and heterologous promoter studies, these were found to be essential for EC-specific reporter gene expression. Similarly to the glioma experiments HIF-dn suppressed transactivation by the wild type HIF-2α protein and further failed to synergize with Ets-1. Indeed, physical interaction of HIF-2α with Ets-1 could be localized to the HIF-2α carboxyl terminus and the auto-inhibitory exon VII domain of Ets-1, respectively. The results identify HIF-2α as an important regulator of EC-function by inducing decisive angiogenic receptors such as VEGFR-2. Moreover, they suggest that interaction between HIF-2α and endothelial Ets factors is required for full transcriptional activation of VEGFR-2 in EC representing a putative target for manipulation of angiogenesis.

Given the increasing evidence that HIF may act as master switches of EC function with decisive roles in physiological angiogenesis, we were interested to analyze their involvement in settings of pathological blood vessel growth. Aberrant expression of angiogenic factors has been implicated in the genesis and maintenance of vascular malformations. Cutaneous and leptomeningeal vascular malformations are hallmarks of the Sturge-Weber Syndrome (SWS). Interestingly, immunohistochemical analysis revealed elevated HIF-1α and HIF-2α protein levels in ECs of leptomeningeal SWS blood vessels of affected brain tissue as compared to intraparenchymal blood vessels of the same patient and leptoemingeal/intraparenchymal vessels in control brains. HIF-2α

upregulation is commonly seen in ECs in different settings of ongoing physiological or pathological angiogenesis. This may suggest that ECs in SWS are constitutively activated and susceptible for different angiogenic signals. In line with this hypothesis, we found increased expression of VEGF and VEGFR-2 protein in leptomeningeal SWS blood vessels. Moreover, up to 5% of leptomeningeal SWS blood vessel proliferated, as assessed by MIB-1 and CD-34 co-immunostaining, while no co-localization could be detected in intraparenchymal blood vessels of the same patient and leptoemingeal/intraparenchymal vessels in control brains.

Taken together increased expression of angiogenic factors in leptomeningeal vascular malformations of SWS was associated with an increased proliferative response in ECs. Thus, in terms of angiogenesis vascular malformations in SWS are not static lesion, but constitute dynamic structures. Our data implicate EC-specific HIF activation in providing a setting which supports and sustains angiogenesis (A. Comati et al. publication in preparation). It is of great interest to analyze whether dysregulation of HIF in EC is cause or consequence in this disease, i.e. whether EC specific HIF expression may induce a cell-autonomous proliferative response without the influence of paracrine factors as was recently suggested in embryonic development (Licht et al., 2006) and tumor growth (Tang et al., 2004). We approached this working hypothesis by generating transgenic mice to inducibly modulate EC intrinsic HIF function in vitro and in vivo.

EC-intrinsic HIF-function (generation of transgenic mouse lines)

For this purpose we generated three transgenic mouse lines carrying as a transgene 1.) murine (m) HIF-1α, 2.) mHIF-2ą 3.) a dominant negative mutant (HIF-dn; lacking the DNA-binding domain and both transactivation domains), under the control of a tet-responsive element (TRE). Expression of the transgene was initially targeted to the EC using a Tet-off approach, by crossbreeding TRE-transgenics with tie2-tTA mice, obtained from U. Deutsch (Münster, SPP1069 member). Transgenic mice were generated in collaboration with G. Breier (Bad Nauheim, SPP1069 member) by microinjection of TRE-HIF-1α, -HIF-2α, -HIF-dn constructs, respectively, in fertilized mouse oocytes. Fertilized mouse oocytes were isolated from superovulated C57BL/6 mice. Genotyping was performed by PCR analysis using construct specific primers. Four rounds of microinjections yielded 9 HIF-1α, 11 HIF-2α and 15 HIF-dn founders. Double transgenics were generated by crossbreeding with tie2-tTA mice. 5 HIF-1α, 6 HIF-2α and 8 HIF-2dn transgenic founders transmitted their transgene to the F1-Generation. To proof functional EC-specific induction doxycycline was withdrawn from a proportion of animals at the age of 6-8 weeks. These experiments were repeated at least three times with each founder animal. Tissue was harvested from induced and non-induced animals and transgene expression analyzed and compared by RT-PCR, revealing induced expression in double transgenics of 2 HIF-1α, 2 HIF-2α and 3 HIF-2dn transgenic founders. To facilitate detection of expression all transgene expression constructs harbor a carboxyterminal flag-tag. EC-specific transgene induction was further analyzed by flag-tag specific immunohistochemistry. Yet, EC-specific, inducible flag-tag protein expression could only be observed in double transgenics of the HIF-1α founder (21978). However, though having tested flag antibodies of four different suppliers we experienced substantial difficulties in visualizing specific antibody staining due to a high background. Thus, also other founders might have shown an EC specific expression. In summary, so far one founder (HIF-1α) gave an inducible and a clearly EC-restricted expression of the transgene (Fig. 4A).

Potential drawbacks with an inducible system consist in leakiness in inducible transgene expression, i.e. continuing expression with doxycycline supplementation or expression in absence of the Tet inducer. As depicted in Fig. 4C in a number of founder animals (34685, 34688, 21916) we observed leaky transgene expression in single transgenics, i.e. in the absence of the tet-inducer. In addition, we observed substantial transgene expression variations among animals of the same and

of different litters using double-transgenics of the same founder e.g. HIF-1α founder 21978, HIFdn founder 34685. To further investigate transgene induction we crossed the tie2-tTA transgenic with a TRE-LacZ reporter mice. Transgene induction upon doxycyclin withdrawal was seen in less than 50% of all brain capillaries and even in a significant smaller proportion of tumor capillaries of BFS-1 fibrosarcoma, varying among different animals of the same litter (Fig. 4B). Interestingly, transgene induction was markedly higher in medium-sized blood vessels. To analyze whether the variability in transgene expression was related to the inducer mice tie2 we switched to the transgenic mouse line with tTA under the control of tie1 promotor elements.

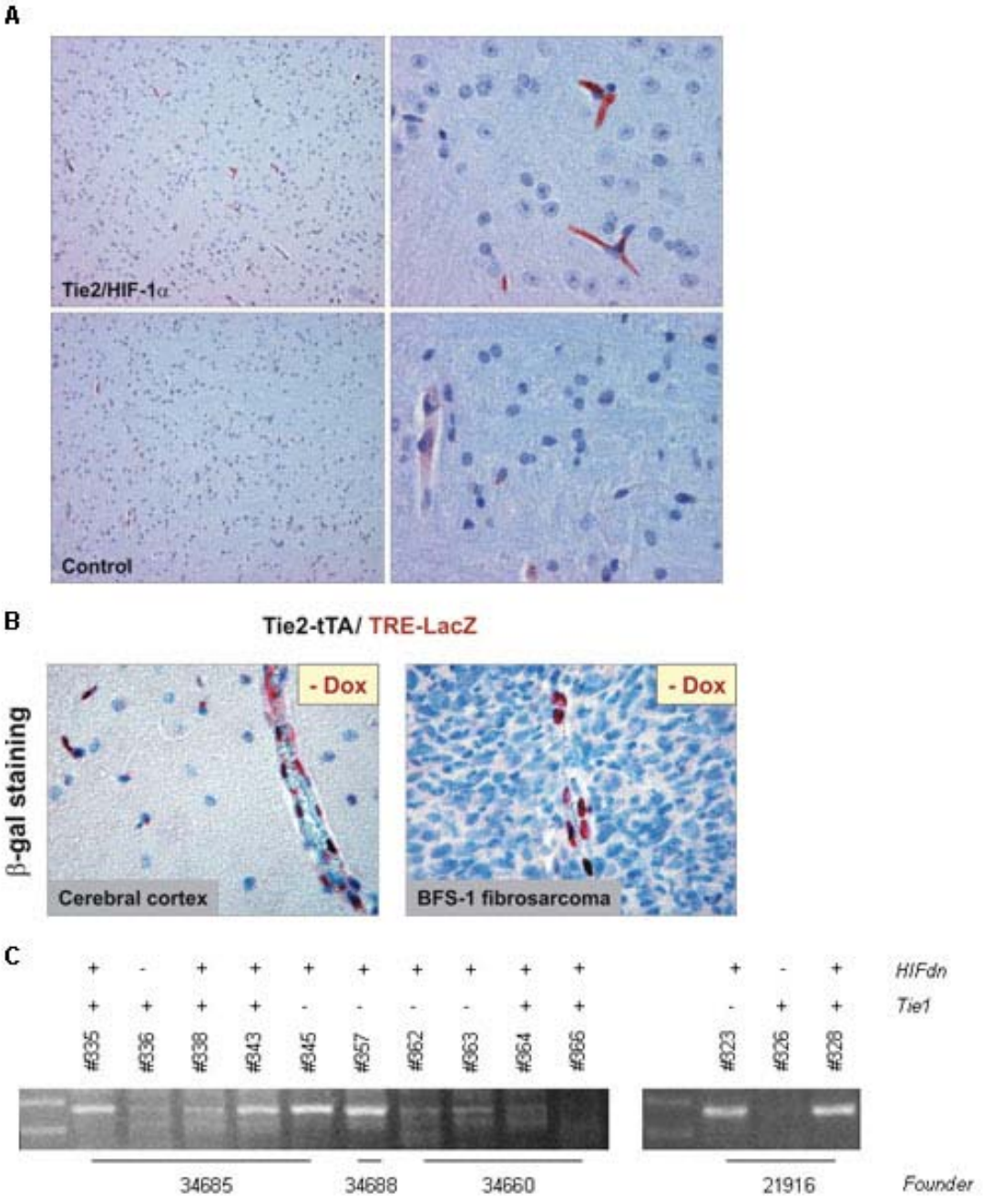


Fig. 4: Endothelial cell specific transgene expression in transgenic mice under physiological and pathological conditions A: Induction of transgene expression in brain capillaries as evidenced by flag-immunoreactivity in Tie2/HIF-1α transgenics (founder 21978) upon Dox-withdrawal as compared to control. **B:** Induction of reporter gene expression in Tie2-tTA/TRE-Lacz as assessed by β-gal immunostaining in a proportion of brain capillaries and tumor capillaries of subcutaneously transplanted BFS-1 fibrosarcomas **C:** Characterization of transgene induction upon Dox withdrawal in HIFdn/Tie1-transgenic animals by RT-PCR reveals variability in HIFdn expression in HIFdn/Tie1 double transgenics and leakiness in HIFdn single transgenics (see e.g. founder 34685, 21916)

These have been successfully used with other TRE responder transgenics and reveal noticeably higher expression levels postnatally than mice with tTA under the control of tie2 promotor elements when crossed to TRE-Lacz reporter mice (N. Ward, D. Dumont; Toronto, personal communications). However, similarly to the tie2 double transgenics tie1 double transgenics revealed a striking variability in transgene expression when assessing different founder lines. Thus, the screening of a much higher number of founder mice might be necessary to obtain founders with a satisfactory and reliable induction. As an efficient screening of the mice was hampered by the difficulty in detecting transgene expression we plan on employing the following alterations. To improve detection of the transgene we plan on tagging the constructs with a V5-His tag. Furthermore, we will use bidirectional vectors (Clontech) with concomitant induction of GFP. The constructs have already been generated.

This will allow us to easily and efficiently analyze and quantify cell type specific transgene induction, helping in the efficient screening of functional responder mice.

REFERENCES

Acker T, Acker H: Cellular oxygen sensing need in CNS function: physiological and pathological implications. *J Exp Biol* 207: 3171-3188, 2004.

Acker T, Diez-Juan A, Aragones J, Tjwa M, Brusselmans K, Moons L, Fukumura D, Moreno-Murciano MP, Herbert JM, Burger A, Riedel J, Elvert G, Flamme I, Maxwell PH, Collen D, Dewerchin M, Jain RK, Plate KH, Carmeliet P: Genetic evidence for a tumor suppressor role of HIF-2alpha. *Cancer Cell 8*: 131-141, 2005.

Acker T, Plate KH: Role of hypoxia in tumor angiogenesis – molecular and cellular angiogenic crosstalk. *Cell Tissue Res 80*: 562-575, 2003.

Acker T, Plate KH: A role for hypoxia and hypoxia-inducible transcription factors in tumor physiology. *J Mol Med 80*: 562-575, 2002.

Appelhoff RJ, Tian YM, Raval RR, Turley H, Harris AL, Pugh CW, Ratcliffe PJ, Gleadle JM: Differential function of the prolyl hydroxylases PHD1, PHD2, and PHD3 in the regulation of hypoxia-inducible factor. *J Biol Chem* 279: 38458-38465, 2004

Beck H, Acker T, Puschel AW, Fujisawa H, Carmeliet P, Plate KH: Cell type-specific expression of neuropilins in an MCA-occlusion model in mice suggests a potential role in post-ischemic brain remodeling. J. Neuropathol. *Exp Neurol* 61: 339-350, 2002.

Beck H, Voswinckel H, Wagner S, Ziegelhoeffer T, Heil M, Schaper W, Acker T, Hatzopoulos AK, Plate KH. Participation of bone marrow-derived cells in long-term repair processes after experimental stroke. *J Cereb Blood Flow Metab* 23: 709-717, 2003.

Beck H, Acker T, Wiessner C, Allegrini PR, Plate KH: Expression of angiopoietin-1, angiopoietin-2, and tie receptors after middle cerebral artery occlusion in the rat. *Am J Pathol* 157: 1473-1483, 2000.

Birner P, Preusser M, Gelpi E, Berger J, Gatterbauer B, Ambros IM, Acker T, Plate KH, Harris AL, Hainfellner JA. Expression of hypoxia-related tissue factors correlates with diminished survival of adjuvantly treated patients with chromosome 1p-aberrant oligodendroglial neoplasms: therapeutic implications. *Clin Cancer Res* 10: 6567-71, 2004.

Blancher C, Moore JW, Talks KL, Houlbrook S, Harris AL.: Relationship of hypoxia-inducible factor (HIF)-1alpha and HIF-2alpha expression to vascular endothelial growth factor induction and hypoxia survival in human breast cancer cell lines. *Cancer Res* 60: 7106-7113, 2000.

Blouw B, Song H, Tihan T, Bosze J, Ferrara N, Gerber HP, Johnson RS, Bergers G: The hypoxic response of tumors is dependent on their microenvironment. *Cancer Cell* 4: 133-146, 2003.

Brown JM, Giaccia AJ: The unique physiology of solid tumors: opportunities (and problems) for cancer therapy. *Cancer Res* 58: 1408-1416, 1998.

Carmeliet P, Dor Y, Herbert JM, Fukumura D, Brusselmans K, Dewerchin M, Neeman M, Bono F, Abramovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D, Keshet E: Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature* 394: 485-490, 1998.

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG: Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 7: 575-583, 2001.

Compernolle V, Brusselmans K, Acker T, Hoet P, Tjwa M, Beck H, Plaisance S, Dor Y, Keshet E, Lupu F, Nemery B, Dewerchin M, Van Veldhoven P, Plate K, Moons L, Collen D, Carmeliet P: Loss of HIF-2alpha and inhibition of VEGF impair fetal lung maturation, whereas treatment with VEGF prevents fatal respiratory distress in premature mice. *Nat Med* 8: 702-710, 2002.

Distler O, Distler J, Scheid A, Acker T, Hirth A, Rethage J, Michel BA, Gay RE, Müller-Ladner U, Matucci-Cerinic M, Plate KH, Gassmann M, Gay S. Vascular endothelial growth factor and its receptors in systemic sclerosis -Regulation by hypoxia and hypoxia inducible factor-1α. *Circ Res* 95: 109-16, 2004.

Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, Acker T, Rauter M, Plate K, Sieweke M, Breier G, Flamme I: Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and Ets-1 in the

transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *J Biol Chem* 278: 7520-7530, 2003.

Epstein AC, Gleadle JM, McNeill LA, Hewitson KS, O'Rourke J, Mole DR, Mukherji M, Metzen E, Wilson MI, Dhanda A, Tian YM, Masson N, Hamilton DL, Jaakkola P, Barstead R, Hodgkin J, Maxwell PH, Pugh CW, Schofield CJ, Ratcliffe PJ: C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* 107: 43-54, 2001.

Favier J, Kempf H, Corvol P, Gasc JM: Coexpression of endothelial PAS protein 1 with essential angiogenic factors suggests its involvement in human vascular development. *Dev Dyn* 222: 377-388, 2001.

Gerber HP, Condorelli F, Park J, Ferrara N: Differential transcriptional regulation of the two vascular endothelial growth factor receptor genes. Flt-1, but not Flk-1/KDR, is up-regulated by hypoxia. *J Biol Chem* 272: 23659-23667, 1997.

Helmlinger G, Endo M, Ferrara N, Hlatky L, Jain RK: Formation of endothelial cell networks. *Nature* 405: 139-141, 2000.

Helmlinger G, Yuan F, Dellian M, Jain RK: Interstitial pH and pO2 gradients in solid tumors in vivo: high-resolution measurements reveal a lack of correlation. *Nat Med* 3: 177-182, 1997.

Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC: Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Mol Cell Biol* 23: 9361-9374, 2003.

Kappel A, Ronicke V, Damert A, Flamme I, Risau W, Breier G: Identification of vascular endothelial growth factor (VEGF) receptor-2 (Flk-1) promoter/enhancer sequences sufficient for angioblast and endothelial cell-specific transcription in transgenic mice. *Blood* 93: 4284-4292, 1999.

Kim MS, Kwon HJ, Lee YM, Baek JH, Jang JE, Lee SW, Moon EJ, Kim HS, Lee SK, Chung HY, Kim CW, Kim KW: Histone deacetylases induce angiogenesis by negative regulation of tumor suppressor genes. *Nat Med* 7: 437-443, 2001.

Kimura H, Braun RD, Ong ET, Hsu R, Secomb TW, Papahadjopoulos D, Hong K, Dewhirst MW: Fluctuations in red cell flux in tumor microvessels can lead to transient hypoxia and reoxygenation in tumor parenchyma. *Cancer Res* 56: 5522-5528, 1996.

Krieg M, Haas R, Brauch H, Acker T, Flamme I, Plate KH: Up-regulation of hypoxia-inducible factors HIF-1alpha and HIF-2alpha under normoxic conditions in renal carcinoma cells by von Hippel-Lindau tumor suppressor gene loss of function. *Oncogene* 19: 5435-5443, 2000.

Kung AL, Wang S, Klco JM, Kaelin WG, Livingston DM: Suppression of tumor growth through disruption of hypoxia-inducible transcription. *Nat. Med*. *6*: 1335-1340, 2000.

Kunz M, Ibrahim S, Koczan D, Thiesen HJ, Kohler HJ, Acker T, Plate KH, Ludwig S, Rapp UR, Brocker EB, van Muijen GN, Flory E, Gross G: Activation of c-Jun NH2-terminal kinase/stress-activated protein kinase (JNK/SAPK) is critical for hypoxia-induced apoptosis of human malignant melanoma. *Cell Growth Differ* 12: 137-145, 2001.

Lando D, Peet DJ, Gorman JJ, Whelan DA, Whitelaw ML, Bruick RK: FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev* 16: 1466-1471, 2002.

Li J, Post M, Volk R, Gao Y, Li M, Metais C, Sato K, Tsai J, Aird W, Rosenberg RD, Hampton TG, Sellke F, Carmeliet P, Simons M: PR39, a peptide regulator of angiogenesis. *Nat Med* 6: 49-55, 2000.

Licht AH, Muller-Holtkamp F, Flamme I, Breier G: Inhibition of hypoxia-inducible factor activity in endothelial cells disrupts embryonic cardiovascular development. *Blood* 107: 584-590, 2006.

Maxwell PH, Dachs GU, Gleadle JM, Nicholls LG, Harris AL, Stratford IJ, Hankinson O, Pugh CW, Ratcliffe PJ: Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc Natl Acad Sci USA* 94: 8104-8109, 1997.

Mekhail K, Gunaratnam L, Bonicalzi ME, Lee S: HIF activation by pH-dependent nucleolar sequestration of VHL. *Nat Cell Biol* 6: 642-647, 2004.

Oosthuyse B, Moons L, Storkebaum E, Beck H, Nuyens D, Brusselmans K, Van Dorpe J, Hellings P, Gorselink M, Heymans S, Theilmeier G, Dewerchin M, Laudenbach V, Vermylen P, Raat H, Acker T, Vleminckx V, Van Den Bosch L, Cashman N, Fujisawa H, Drost MR, Sciot R, Bruyninckx F, Hicklin DJ, Ince C, Gressens P, Lupu F, Plate KH, Robberecht W, Herbert JM, Collen D, Carmeliet P: Deletion of the hypoxia-response element in the vascular endothelial growth factor promoter causes motor neuron degeneration. *Nat Genet* 28: 131-138, 2001.

Preusser M, Wolfsberger S, Haberler C, Breitschopf H, Czech T, Slavc I, Harris AL, Acker T, Budka H, Hainfellner JA. Vascularization and expression of hypoxia-related tissue factors in intracranial ependymoma and their impact on patient survival. *Acta Neuropathol* 109: 211-6, 2005.

Raval RR, Lau KW, Tran MG, Sowter HM, Mandriota SJ, Li JL, Pugh CW, Maxwell PH, Harris AL, Ratcliffe PJ: Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Mol Cell Biol* 25: 5675-5686: 2005.

Ryan HE, Lo J, Johnson RS: HIF-1 alpha is required for solid tumor formation and embryonic vascularization. *EMBO J* 17: 3005-3015, 1998.

Smith K, Gunaratnam L, Morley M, Franovic A, Mekhail K, Lee S: Silencing of epidermal growth factor receptor suppresses hypoxia-inducible factor-2-driven VHL-/- renal cancer. *Cancer Res* 65: 5221-5230, 2005.

Sowter HM, Raval RR, Moore JW, Ratcliffe PJ, Harris AL: Predominant role of hypoxia-inducible transcription factor (Hif)-1alpha versus Hif-2alpha in regulation of the transcriptional response to hypoxia. *Cancer Res* 63: 6130-6134, 2003.

Stratmann A, Acker T, Burger AM, Amann K, Risau W, Plate KH: Differential inhibition of tumor angiogenesis by tie2 and vascular endothelial growth factor receptor-2 dominant-negative receptor mutants. *Int J Cancer* 91: 273-282, 2001.

Stroka DM, Burkhardt T, Desbaillets I, Wenger RH, Neil DA, Bauer C, Gassmann M, Candinas D: HIF-1 is expressed in normoxic tissue and displays an organ-specific regulation under systemic hypoxia. *FASEB J* 15: 2445-2453, 2001.

Tang N, Wang L, Esko J, Giordano FJ, Huang Y, Gerber HP, Ferrara N, Johnson RS: Loss of HIF-1alpha in endothelial cells disrupts a hypoxia-driven VEGF autocrine loop necessary for tumorigenesis. *Cancer Cell* 6: 485-495, 2004.

Tian H, McKnight SL, Russell DW: Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev* 11: 72-82, 1997.

Unruh A, Ressel A, Mohamed HG, Johnson RS, Nadrowitz R, Richter E, Katschinski DM, Wenger RH: The hypoxia-inducible factor-1alpha is a negative factor for tumor therapy. *Oncogene* 22: 3213-3220, 2003.

Wiesener MS, Jurgensen JS, Rosenberger C, Scholze CK, Horstrup JH, Warnecke C, Mandriota S, Bechmann I, Frei UA, Pugh CW, Ratcliffe PJ, Bachmann S, Maxwell PH, Eckardt KU: Widespread hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs. *FASEB J* 17: 271-273, 2003.

Wiesener MS, Turley H, Allen WE, Willam C, Eckardt KU, Talks KL, Wood SM, Gatter KC, Harris AL, Pugh CW, Ratcliffe PJ, Maxwell PH: Induction of endothelial PAS domain protein-1 by hypoxia: characterization and comparison with hypoxia-inducible factor-1alpha. *Blood* 92: 2260-2268, 1998.

Zagzag D, Zhong H, Scalzitti JM, Laughner E, Simons JW, Semenza GL: Expression of hypoxia-inducible factor 1alpha in brain tumors: association with angiogenesis, invasion, and progression. *Cancer* 88: 2606-2618, 2000.

Zhong H, De Marzo AM, Laughner E, Lim M, Hilton DA, Zagzag D, Buechler P, Isaacs WB, Semenza GL, Simons JW: Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. *Cancer Res* 59: 5830-5835, 1999.

Angiogenic signaling

Identification of VEGF-induced signaling events involved in angiogenesis and vascular permeability

Dr. Matthias Clauss

Dept. of Cellular and Integrative Physiology
Indiana Center for Vascular Biology and Medicine
975 W. Walnut Street IB 433
Indianapolis, IN 46202, USA

Member of the SPP 1069 from 2001 until 2003

This project has been transferred in the course of its funding period. See project of Prof. Dr. Georg Breier (page 205).

Overexpression of CEACAM1 in Vascular Vells and its Role in Angiogenesis

PD Dr. Süleyman Ergün
Dr. Nerbil Kilic

SE: Institute of Anatomy, University Hospital Essen, Hufelandstr. 55, 45147 Essen, Germany
NK: Medical Clinic II, Dept. of Hematology/Oncology and Bone Marrow Transplantation, Unversity Hospital Hamburg-Eppendorf, Martinistraße 52, 20246 Hamburg,Germany

Member of the SPP1069 from 2001 until 2003

SUMMARY

Angiogenesis is regulated by a net balance of angiogenic activators and inhibitors. Cell adhesion molecules are also involved in the different stages of angiogenesis, in particular of vascular morphogenesis. Prior to the application of our proposal we characterized soluble CEACAM1 (CEA-related cell adhesion molecule 1) as a pro-angiogenic factor and morphogenic effector of VEGF. One of the remarkable findings was the increased expression of CEACAM1 in endothelial cells of new blood vessels. Thus, the aim of this study was to explore the role of membrane-bound CEACAM1 in angiogenesis using the following experimental models:

1. Adenoviral overexpression of CEACAM1 in vascular endothelial and smooth muscle cells which were subsequently used a) for gene array analyses regarding the expression of known potent angiogenic factors, b) migration and *in vitro* wound assays and c) endothelial tube formation assay.

2. Local microinjection of CEACAM1-adenovirus in chicken chorioallantoic membrane to study the effects of gene delivered CEACAM1 in angiogenesis and vascular leakage. The major part of the supposed experiments has been carried out successfully. The obtained results demonstrate clearly that CEACAM1 overexpression in vascular endothelial cells results in up-regulation of potent pro-angiogenic and pro-lymphangiogenic factors such as VEGF-A, -C, -D and their receptors VEGFR-2 and -3, angiogenin, angiopoietin-1, angiopoietin-2 and Interleukin-8 while the expression of collagen 18, the maternal substance of the angiogenesis inhibitor endostatin, was significantly down-regulated. Detailed analyses of tumor tissues revealed that CEACAM1 is up-regulated in tumor associated blood vessels prior to the switch of tumor tissue to an invasive phenotype. This is obviously accompanied by vascular destabilization indicating activated angiogenesis. Interestingly, CEACAM1 is detectable in endothelial cells of lymphatics in close proximity to the early tumor cells such CIS in testis and PIN in prostate. Since at this state of tumor development the vascular endothelial cells are still negative for CEACAM1 these findings indicate an activation of lymphatics by presence of single non-invasive tumor cells. In summary, these results clarified for the first time the effects of membrane-bound CEACAM1 in vascular and lymphatic endothelial cells and demonstrate an essential role of CEACAM1 in the early morphogenesis of blood vessels, in particular of tumor blood vessels. Future studies will be focused on the angiogenesis-relevant signaling pathways in endothelial versus epithelial cells induced or influenced by CEACAM1. Further, we want to study the effect of endothelial knock-down of CEACAM1 via adenoviral delivery of siRNA in vivo on the tumor vascularization using an experimental bladder carcinoma model.

INTRODUCTION:

Angiogenesis is defined as sprouting of new vessels from preexisting blood vessels. It is a prerequisite for tumor growth and metastasis and is regulated by angiogenic activators and inhibitors (Hanahan and Folkman, 1996; Carmeliet, 2000). The structural formation and maturation of blood vessels during vasculogenesis and angiogenesis are very complex processes which run in successive steps including proliferation and tube formation of endothelial cells, construction of basement membrane, integration of peri-endothelial cells into the vascular wall and embedding of blood vessels into the perivascular tissue (Folkman et al., 1989; Risau, 1991; Folkman and D'Amore, 1996; Hanahan, 1997)(Hanahan, 1997). These processes seem to be region-specific during tumor growth (Kilic et al., 1999). Numerous angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), angiopoietin-1 (Ang1), angiopoietin-2 (Ang2) (Sato et al., 1995) (Suri et al., 1996; Maisonpierre et al., 1997) and their receptors, which belong to the receptor tyrosine kinase family, are involved in several steps of these processes (Maisonpierre et al., 1997). The effects of angiogenesis activators are apparently balanced by angiogenesis inhibitors (Hanahan and Folkman, 1996) including fibronectin (Homandberg et al., 1995), thrombospondin-1 (Good et al., 1990; Homandberg et al., 1995), platelet factor-4 (Gupta et al., 1995), angiostatin and endostatin (O'Reilly et al., 1994; O'Reilly et al., 1997).

Also cell adhesion molecules play a crucial role for the capillary morphogenesis and for functional modulation of blood vessels such as regulation of vascular permeability (Carmeliet et al., 1999). Prior starting with this project we showed that the human carcinoembryonic antigen-related cell adhesion molecule (CEACAM1), formerly known as biliary glycoprotein (BGP) or CD66a, exhibits angiogenic properties and functions as a morphogenic effector for VEGF (Ergun et al., 2000). CEACAM1 is expressed in the newly formed immature blood vessels of different tumors as well as in new vessels of physiological angiogenesis such as occur in wound healing and endometrial proliferation (Ergun et al., 2000). CEACAM1 is a member of the carcinoembryonic antigen family and can bind homophilically to itself as well as heterophilically to the other CEA family members (Rojas et al., 1990). Up to now, 11 alternative splicing forms of the CEACAM1 gene are known (Kuroki et al., 1991; Barnett et al., 1993). CEACAM1 is expressed in epithelia and leukocytes in addition to endothelia. It has been shown that the mRNA expression of CEACAM1 is down regulated in some tumors such as colorectal and prostate carcinomas (Nollau et al., 1997a; Lin and Pu, 1999). Based on such results a tumor suppressive role has been postulated. It has been reported that the tumor inhibitory function of CEACAM1 depends on the cis-determinants in its cytoplasmic domain (Izzi et al., 1999). Recently, it could be demonstrated that CEACAM1 is differently expressed in proliferating and quiescent epithelial cells and that this influenced their proliferation activity (Singer et al., 2000).

The expression of CEACAM1 in endothelial cells of developing rat central nervous system was reported by Öbrink and coworkers (Sawa et al., 1994). We showed that two soluble CEACAM1 forms at 120 and 50 kDa are detectable in conditioned media of endothelial cells after stimulation with vascular endothelial growth factor (VEGF) (Ergun et al., 2000) and that these forms exhibit also angiogenic properties similar to CEACAM1 purified from human granulocytes. In endothelial cells of angiogenic blood vessels, such as in tumors or in angiogenic tissues, the expression of membrane-bound CEACAM1 is significantly increased and it is detectable in both luminal and basal surface of endothelial cells. Most of the data showing the effects of membrane-bound CEACAM1 have been derived from epithelial or tumor cells. At the beginning of this project, there were no data demonstrating the role of membrane-bound CEACAM1 in vascular endothelial cells regarding the expression of known angiogenic activators and inhibitors. We expected that constitutive overexpression of CEACAM1 in endothelial cells will have regulating effects on the expression of known potent angiogenic factors such as VEGF and FGF-2, factors influencing stability of vascular

wall such as Ang1 and Ang2 and angiogenic inhibitors such as collagen XVIII/Endostatin. Additionally, we expected also that the endothelial overexpression of CEACAM1 will influence capillary morphogenesis and the interaction of endothelial cells with peri-endothelial cells of vascular wall such as pericytes or smooth muscle cells.

METHODS

Morphological techniques:

Light microscopy, electron microscopy for ultrastructural analyses, immune electron microscopy, immunohistochemistry and immunocytochemistry. These techniques were already established in our group at the planning of this project. Using these techniques we performed extensive expression studies on tissues and vascular endothelial cells regarding the expression of CEACAM1 and/or co-localisation of CEACAM1 with other angiogenic factors such as VEGF and VEGF receptors. Also studies regarding the relation between ultrastructural construction of vascular wall and CEACAM1 expression have been performed.

Proteinbiochemical analyses:

Immunoblotting, immunoprecipitation and overlay binding studies are performed. These methods were established in our group prior starting with the project. Using these techniques, we controlled the efficiency of CEACAM1 overexpression versus silencing in endothelial versus epithelial cells at protein level. Also in protein extracts of tissues and cells the protein levels of CEACAM1 alone or in comparison to that of other angiogenesis relevant factors were determined via these methods.

In vitro, ex vivo and *in vivo* angiogenesis assays:

Endothelial migration using modified Boyden chamber and *in vitro* wound assay, endothelial tube assay using 3-D collagen gel or matrigel assay and endothelial survival assay on collagen gel matrix were used in our studies. Endothelial survival assay was established during this project while the other assays were established prior starting with this project. We also established during this project a human adult arterial ring assay using fragments of human internal thoracic artery as an *ex vivo* assay. Additionally, *in vitro* tubes and *ex vivo* sprouting capillaries from arterial rings were studied further by immunohistochemistry after embedding of these tissues in paraffin.

As *in vivo* assays we performed CAM- (chick chorionallantoic membrane) and matrigel plug assay.

Molecular biological techniques:

CEACAM1 overexpression in endothelial versus epithelial cells was performed using the adenoviral technique and/or an expression vector. CEACAM1 silencing was performed using the siRNA technique. From CEACAM1-overexpressing and CEACAM1-silenced cells RNA was extracted, reverse transcribed in cDNA which was subsequently used in gene arrays and/or quantitative RT-PCR analyses using LightCycler System. While the adenoviral overexpression of CEACAM1 was established prior starting this project, the vector based overexpression and CEACAM1 silencing via siRNA were established in our laboratory during this project. CEACAM1-overexpressing versus –silenced endothelial cells were used in *in vitro* endothelial tube, wound and survival assays.

RESULTS

The role of CEACAM1 in endothelial cells and tumor angiogenesis

Detailed immunohistochemical studies on tumor tissues such as prostate cancer and glomus caroticum revealed that CEACAM1 is expressed in endothelial cells of tumor associated blood vessels prior to tumor invasion and in endothelial cells of sprouting capillaries but not in those of mother blood vessels. Particularly, studies on prostate cancer showed a strong endothelial expression of CEACAM1 in blood vessels at the pre-cancerous stage of prostate cancer, high-grade PIN (prostate intraepithelial neoplasia). Approximately, more than 80% of blood vessels of the PIN area expressed CEACAM1 while only 5% of blood vessels in the neighbouring normal area were positive for CEACAM1 (Fig.1A-D). Electron microscopic studies from the corresponding normal and PIN areas demonstrated that the majority of PIN-associated blood vessels, particularly capillaries within the wall of the PIN-glands, showed extensive endothelial fenestration, trans-endothelial gaps, opening of inter-endothelial contacts and degradation of basal lamina indicating a destabilization of vascular wall (Fig.1E-F). Occasionally, invasion of PIN cells into such destabilized blood vessels was observed.

To determine the relation between expression of CEACAM1 in endothelial cells and capillary morphogenesis we performed in vitro endothelial tube formation assay on 3D-collagen gel. Immunohistochemical staining on sections obtained from paraffin embedded collagen gels after performing of tube assay revealed that human primary microvascular endothelial cells (HDMEC) invading collagen gel and forming endothelial tubes stained strongly positive for CEACAM1 whereas endothelial cells not involved into tube formation and remaining at the top of the gel were negative or exhibited only a very weak staining. Higher light microscopic magnification from tube formation areas

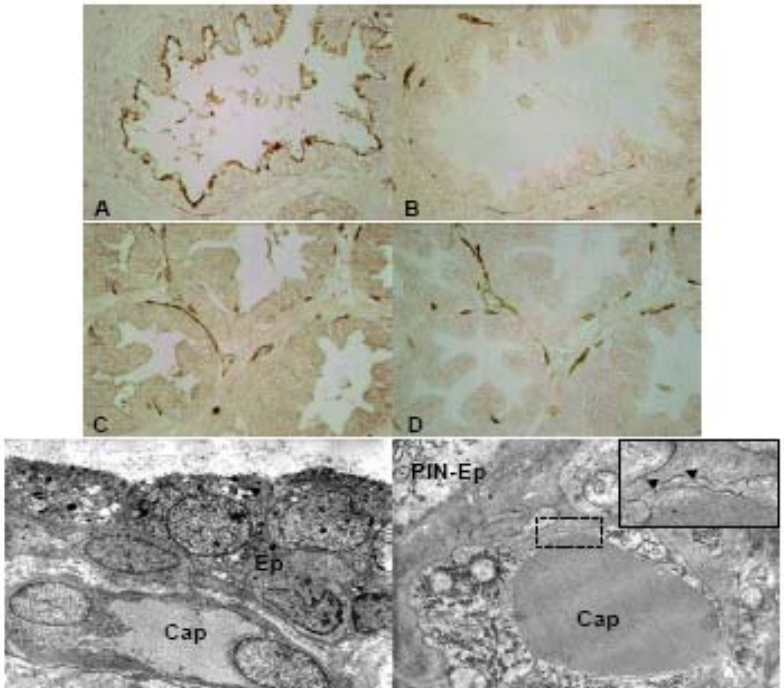


Figure 1A-F: In normal prostate, CEACAM1 is detectable at the luminal surface of the prostate epithelium using the antibody 4D1/C2 (A) but not in the adjacent blood vessels visualized by CD34-staining on a serial section (B). In high grade PIN, the epithelial CEACAM1 is almost disappeared while the majority of the adjacent blood vessels (C) exhibit CEACAM1 when compared with the immunostaining for the endothelial marker CD34 on the serial section (D). Electron microscopic studies show normal ultrastructure of capillaries (*Cap*) in the wall of a normal prostate gland (E) while extensive endothelial fenestrations (dotted quadrangle, arrow heads in insert) are detectable in capillaries of PIN area (F). *Ep*: normal epithelium; *PIN-Ep*: High grade PIN-epithelium

demonstrated the presence of CEACAM1 immunostaining at the luminal as well as the basal side of endothelial cells.

To mimic the up-regulation of CEACAM1 in endothelial cells during angiogenic activation we performed CEACAM1 overexpression in HDMECs via *in vitro* transfection using adenoviral technique or expression vector. Immunoblotting analyses confirmed the efficient overexpression of CEACAM1 in HDMECs at the protein level (Fig. 2A). Adenoviral transfection caused a transfection rate of more than 80% leading to a very high amount of CEACAM1 protein in HDMECs. These cells were then used for RNA extraction with subsequent reverse transcription in cDNA which was used in gene array and/or quantitative real time RT-PCR analyses. *LacZ*-transfected and/or non-transfected endothelial cells were used as control. The overview of the gene array membranes demonstrated clear differences between CEACAM1- (Fig. 2B) and *LacZ*-transfected (Fig. 2C) HDMECs regarding the expression of factors from which the cDNA was immobilized in these membranes. The densitometric analyses of these non-radioactive gene array studies as well as real-time RT-PCR studies using the LightCycler System revealed a significant up-regulation of pro-angiogenic factors such as VEGF (Fig. 2D), angiopoietin-1 (Fig. 2E), angiopoietin-2 (Fig. 2F), and Tie-2 (Fig. 2G). The expression of VEGFR-1 (Flt-1) was increased in tendency but not significantly (not shown), while the expression of VEGFR-2 (KDR) was enhanced in CEACAM1-overexpressing HDMECs, particularly in LightCycler analyses (Fig. 2H). Additionally, the expression of the pro-angiogenic factors angiogenin (Fig. 2I) and interleukin-8 (IL-8) (Fig. 2J) was significantly increased in gene array analyses. In contrast, the expression of collagen 18, the maternal substance of the angiogenesis inhibitor endostatin, was significantly down-regulated in CEACAM1-overexpressing HDMECs (Fig. 2K). Also the expression of Tie-1, a tyrosine kinase receptor with unknown ligand yet was strongly suppressed by CEACAM1 overexpression in HDMECs (Fig. 2L). Western blot analyses confirmed the results obtained in quantitative RT-PCR studies and revealed that the amount of the most potent angiogenic factor VEGF is significantly increased but that of the angiogenic inhibitor endostatin is decreased at

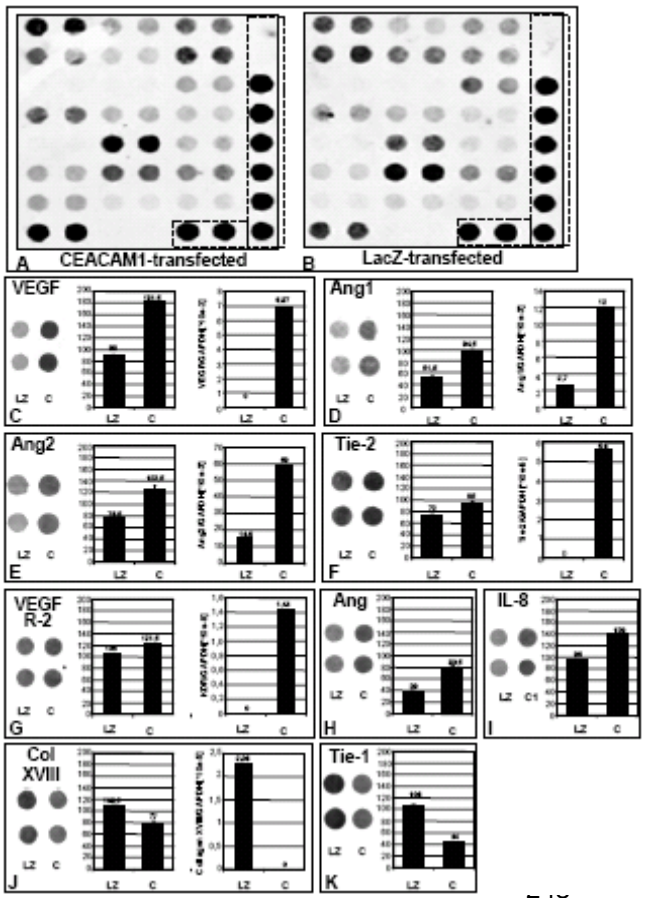


Figure 2A-K: Gene array analyses (A and B) show differences between CEACAM1-overexpressing (*C*) and Lac-Z (*LZ*)-transfected HDMECs regarding the expression of angiogenesis related factors (controls: dotted quadrangles). CEACAM1 overexpression increases VEGF (C), Ang1 (D), Ang2 (E), Tie-2 (F). VEGFR-2-expression was significantly increased in quantitative RT-PCR (G) analysis. Also the expression of angiogenin (Ang) (H) and IL-8 (I) are enhanced by CEACAM1 overexpression. The gene array results are confirmed by the findings obtained from quantitative RT-PCR studies (right diagrams in the figure panels C-G and J). In contrast, collagen 18 (J, right diagram represents quantitative RT-PCR), the maternal substance of the angiogenesis inhibitor endostatin, and Tie-1 (unknown ligand until now) (K) are down-regulated after CEACAM1 overexpression in HDMECs.

the protein level in CEACAM1 overexpressing HDMECs (not shown).

We also wanted to clarify whether CEACAM1 up-regulation in endothelial cells has any effect on the survival of these cells, particularly when they were cultured on the collagen gel as a matrix. To this aim HDMECs overexpressing CEACAM1 and *LacZ*-transfected HDMECs were cultured under hunger conditions (only 2% FCS). In comparison to the non-transfected or *LacZ*-transfected (not shown) HDMECs, CEACAM1-overexpressing HDMECs showed a significantly prolonged survival time. CEACAM1-overexpressing HDMECs remained confluent after 5-8 days of culture whereas a major part of *LacZ*-transfected or non-transfected HDMECs detached from culture dishes within 3-4 days of culture and became apoptotic. To avoid any artificial effects caused by the very high level of CEACAM1 after adenoviral transfection, we used HDMECs transfected for CEACAM1 using the expression vector pcDNA3.1/CEACAM1 versus wild type HDMECs in survival assay. In contrast to the wild type, CEACAM1-overexpressing HDMECs remained confluent for at least two days longer and the sum of the areas of cell detachment was nearly two times larger in wild type. The prolonged survival of CEACAM1-overexpressing HDMECs was significantly reduced and the sum of the areas of cell detachment was close to that of wild type HDMECs alone when a polyclonal VEGF antibody was added to CEACAM1-overexpressing HDMECs.

To gain further insights into the function of CEACAM1 in vascular morphogenesis, we established for the first time *in vitro* knock-down of CEACAM1 in endothelial cells via siRNA. The effectiveness of CEACAM1 overexpression versus CEACAM1 silencing in HDMECs was confirmed by Western blot analyses demonstrating a clearly enhanced CEACAM1 protein amount after CEACAM1 overexpression but a significantly decreased protein level when HDMECs were treated with CEACAM1-specific siRNA (Fig. 3A). Subsequently, we used CEACAM1-overexpressing (transfected with the vector pcDNA3.1) versus CEACAM1-silenced HDMECs in a migration assay using Boyden chamber and in tube formation assay using type-1-collagen gel. While no significant differences between both CEACAM1-overexpressing and CEACAM1-silenced HDMECs were observed in the migration assay for both, basal and VEGF-induced chemotaxis (not shown), the VEGF-induced endothelial tube formation was influenced by CEACAM1-overexpression versus CEACAM1-silencing in HDMECs. In contrast to the control, where HDMECs were exposed to basal media and where no tubes were observed (Fig. 3B) the application of VEGF induced the formation of endothelial tubes as expected (Fig. 3C). Simultaneous application of VEGF and the CEACAM1 neutralizing antibody 4D1/C2 reduced the length and the network of the VEGF-induced endothelial tubes (Fig. 3D). In contrast, the length of the VEGF-induced endothelial tubes was enhanced in CEACAM1 overexpressing HDMECs for approximately 42% (Fig. 3E) in comparison to VEGF alone. Also, the network between the tubes was increased and tighter organized as shown in higher magnification (Fig. 3F). The simultaneous application of VEGF and the CEACAM1 antibody 4D1/C2 reduced the number and the length of the tubes for more than 75% (Fig. 3G). The tube formation of CEACAM1-silenced HDMECs (Fig. 3H) in response to VEGF was reduced for approximately 83% in comparison to CEACAM1-overexpressing HDMECs. The VEGF-induced tube formation of CEACAM1-silenced HDMECs was blocked completely when VEGF and the CEACAM1 antibody 4D1/C2 were added simultaneously (Fig. 3I). The luciferase silencing in HDMECs used as control for siRNA technique did not affect the VEGF-induced endothelial tubes (Fig. 3J). The morphometric quantification of the length of endothelial tubes described above revealed the significant differences in VEGF-induced endothelial tubes depending on the presence or cellular knock-down of CEACAM1 (Fig. 3K). These findings have been confirmed by light microscopic studies on sections obtained from paraffin embedded collagen gels after tube formation assay. The networking and the length of endothelial tubes induced by application of VEGF on CEACAM1 overexpressing HDMECs were reduced when VEGF was simultaneously applied with CEACAM1 antibody 4D1/C2. The VEGF-induced endothelial tubes were abolished completely when VEGF was applied to CEACAM1-silenced HDMECs.

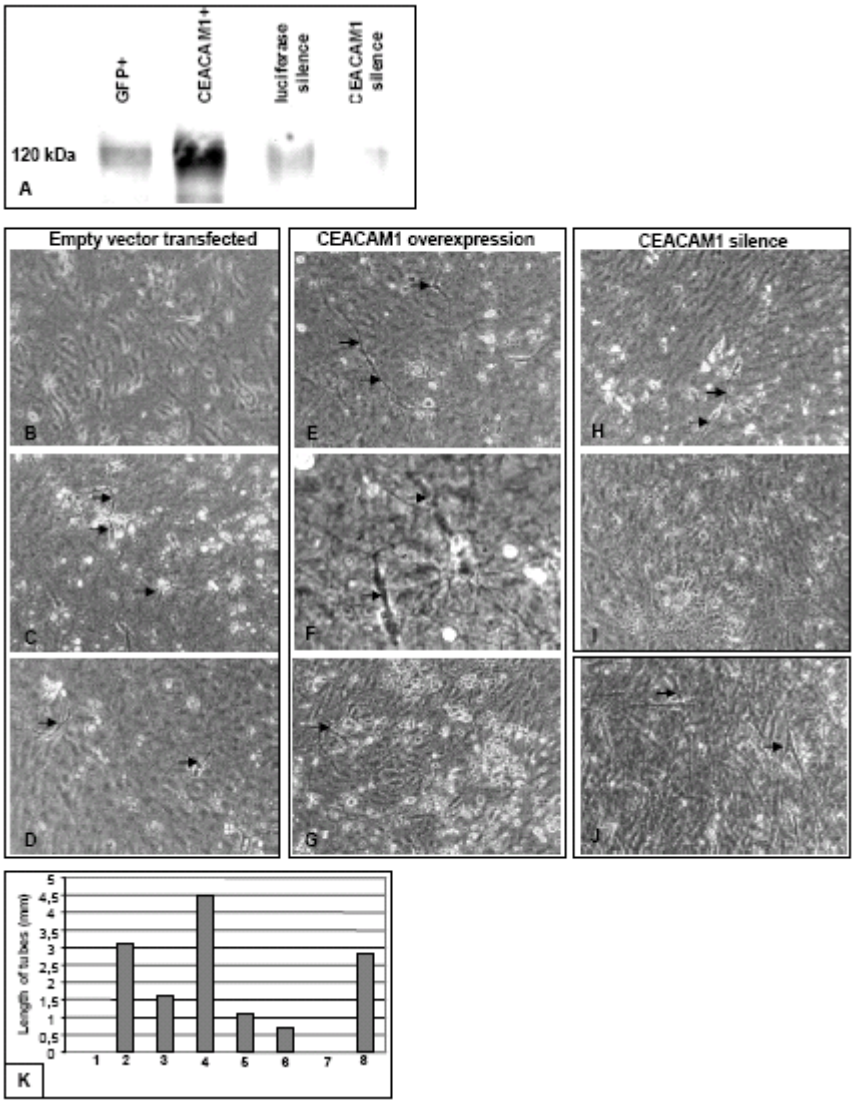


Figure 3A-K: CEACAM1 overexpressing versus CEACAM1-silenced (siRNA technique) HDMECs were used in endothelial tube formation assay. Empty vector pcDNA3.1 and luciferase siRNA-transfected HDMECs were used as control. Western blot analysis demonstrates the efficiency of CEACAM1 overexpression (A, lane CEACAM1+) versus CEACAM1 silencing (A, lane CEACAM1 silence) in HDMECs at the protein level. GFP-transfection and luciferase silencing were used as control. Non-treated and empty vector transfected HDMECs do not form tubes (B) but their stimulation with VEGF induces endothelial tube formation (arrows) (C) as expected. In contrast, co-stimulation of them with VEGF plus CEACAM1 specific antibody 4D1/C2 reduces the VEGF-induced tubes (arrows) significantly (D). The length and the networking of endothelial tubes (arrows) are significantly increased when VEGF was applied to HDMECs overexpressing CEACAM1 (E, and higher magnification in F) in comparison to empty vector transfected HDMECs (C). The combined application of VEGF and CEACAM1 antibody 4D1/C2 reduced the tube inducing effect (arrow) of VEGF on CEACAM1-overexpressing HDMECs (G). CEACAM1-silenced HDMECs do not form tubes (H) when they are exposed to basal medium only. Remarkably, the tube forming effect of VEGF is abolished when it was applied to CEACAM1-silenced HDMECs (I). The tube forming effect (arrows) of VEGF was not altered when it was applied to endothelial cells silenced for luciferase used as control (J). The quantification of tubular length (K): 1: control, 2: empty vector transfected HDMECs plus VEGF stimulation, 3: empty vector transfected HDMECs plus VEGF stimulation plus CEACAM1-antibody (4D1/C2), 4: CEACAM1 transfected HDMECs plus VEGF stimulation, 5: CEACAM1 transfected HDMECs plus VEGF stimulation plus 4D1/C2, 6: CEACAM1-silenced HDMECs plus VEGF stimulation, 7: CEACAM1-silenced HDMECs plus VEGF stimulation plus 4D1/C2, 8: luciferase-silenced HDMECs plus VEGF stimulation.

Furthermore, we performed immunohistochemistry on paraffin sections using the CEACAM1 antibody 4D1/C2. In CEACAM1 overexpressing HDMECs, a strong CEACAM1 immunostaining was found in a part of these cells. Also VEGF-stimulated HDMECs and particularly those involved in the tube formation exhibited a strong CEACAM1 staining. In contrast, CEACAM1 staining was not or only very weakly detectable in CEACAM1-silenced HDMECs, also even after VEGF-treatment confirming the efficiency of CEACAM1 silencing performed here.

Lymphangiogenic properties of CEACAM1

First evidences showing CEACAM1 expression in lymphatic endothelial cells were obtained from immunohistochemical studies on human testicular seminoma. Lymphatic vessels surrounding the tumor areas and mostly localized near to large arteries or veins exhibited CEACAM1 staining whereas large and small blood vessels remained negative. Often, the CEACAM1 positive lymphatics were filled with tumor cell clusters once tumor cells invaded testicular interstitium. Corresponding controls with the secondary antibody showed no such immunostaining. Such activated lymphatics expressing CEACAM1 were also found in the tumor surrounding area of prostate and urinary bladder. For further characterization we performed single and double immunostaining for CEACAM1 and VEGFR-3 (Flt-4) which is recognized to bind VEGF-C and VEGF-D in high affinity. Similar to the CEACAM1 staining as shown in figure 4E also VEGFR-3 immunostaining was found in cells lining lymphatic spaces and angiogenic small blood vessels. The endothelial origin of these cells was confirmed by immunostaining for CD34 (Fig. 4G). The double immunostaining for CEACAM1 and VEGFR-3 revealed a co-localization of both in the same cells. Further immunohistochemical studies on human testicular and prostate tissues containing CIS (Carcinoma-in-Situ) of testis and PIN (prostate intraepithelial neoplasia) of prostate, both are considered as early non-invasive tumor forms and need a relative long period to transform to an invasive phenotype, revealed a clear presence of CEACAM1 in lymphatics in the interstitium before invasion of tumor cells into the interstitium, and more interestingly before CEACAM1 detection in vascular endothelia as exemplarily shown for CIS.

According to the studies regarding CEACAM1 effects in angiogenesis we performed gene array and quantitative real-time RT-PCR analyses focused on the expression of lympangiogenic factors. These studies revealed a significant up-regulation of VEGF-C and –D in HDMECs overexpressing CEACAM1 after adenoviral transfection when compared with wild type or *LacZ*-transfected HDMECs (not shown). Also at the protein level, a two fold enhanced amount of VEGF-D protein was detected in HDMECs overexpressing CEACAM1 in comparison to the wild type (not shown). We were not able to detect VEGF-C protein neither in CEACAM1-overexpressing nor in wild type HDMECs, possibly because of a low protein level or some specificity/affinity problems with the VEGF-C antibody.

To study whether VEGF-C and –D are involved in the CEACAM1-mediated endothelial cell survival we treated CEACAM1-overexpressing HDMECs with antibodies against VEGF-C and –D. Confirming the previously published results mentioned above, CEACAM1-overexpressing HDMECs showed a prolonged survival in comparison to those transfected with empty vector (not shown). In comparison to untreated CEACAM1-overexpressing HDMECs which remained nearly confluent and adherent under hunger conditions particularly those treated with anti-VEGF-C or anti-VEGF-D showed large areas with cell detachment from the culture well. The cell detachment was most prominent when empty-vector transfected HDMECs were treated with anti-VEGF-C or anti-VEGF-D (not shown) supporting the essential role of CEACAM1 in endothelial survival. Furthermore, in a reverse experimental approach we silenced CEACAM1 in HDMECs and subsequently treated them with VEGF-A ($VEGF_{165}$), VEGF-C and VEGF-D. First of all, the survival of CEACAM1-silenced HDMECs was significantly reduced in comparison to luciferase-silenced HDMECs confirming the results recently published by Kilic et al. (Kilic et al., 2005). While VEGF-A alone was able to restore

the survival of both CEACAM1- and luciferase-silenced HDMECs and to keep them in a confluent state, the treatment with VEGF-C and VEGF-D could only improve the survival of luciferase-silenced but not that of CEACAM1-silenced HDMECs as exemplarily shown for VEGF-C treatment.

Since the application of VEGF-A but not that of VEGF-C and VEGF-D was able to restore the reduced endothelial survival after CEACAM1 silencing in HDMECs, we suspected an interaction between endothelial CEACAM1 and VEGFR-3 (Flt-4). Indeed we found an 8.1 fold increase of the VEGFR-3 protein in the cell extract of CEACAM1-overexpressing HDMECs using Western blot analyses in comparison to wild type HDMECs. The detection of vimentin was used to control the equal loading of protein amount. Conformingly, we found a stronger staining for VEGFR-3 in CEACAM1-overexpressing HDMECs immunocytochemically in comparison to wild type or empty vector transfected HDMECs. CEACAM1-overexpressing HDMECs showed also significantly stronger immunostaining for further lymph endothelial markers such as podoplanin6, Prox1 and LYVE-1 in comparison to corresponding empty vector transfected HDMECs.

Further detailed studies revealed that after invasion of tumor cells into the interstitium of the studied organs tumor cell clusters were frequently found in dilated lymphatic vessels as shown here for bladder cancer. In some small lymphatic vessels also single CEACAM1 expressing tumor cells were found. A strong CEACAM1 staining was localized at the contact areas between tumor cells and lymphatic endothelial cells expressing CEACAM1. In terms of large tumor cell clusters within the lymphatic vessels, particularly the tumor cells of the outer cell row expressed CEACAM1.

CEACAM1 is expressed during angiogenic mobilization of vascular wall resident endothelial pre-cursor cells

During this project we established a new human adult arterial ring assay, in which rings of the human internal thoracic artery (HITA) were cultured between two layers of collagen gel until capillary-like sprouting (Fig. 4A-B). Surprisingly, we found that in a zone of vascular wall between smooth muscle and adventitial layers CD34-positive cells reside and called this zone a “vasculogenic zone” of the vascular wall. In ring assays we observed that these cells are involved in the capillary sprouting from the HITA wall. In further studies we could demonstrate that these cells are endothelial pre-cursors and can differentiate into mature endothelial cells. A manuscript containing these novel findings is recently accepted for publication in “*Development*”. We wanted to explore whether CEACAM1 is involved in the capillary sprouting from HITA wall. Detailed immunohistochemical studies on sections

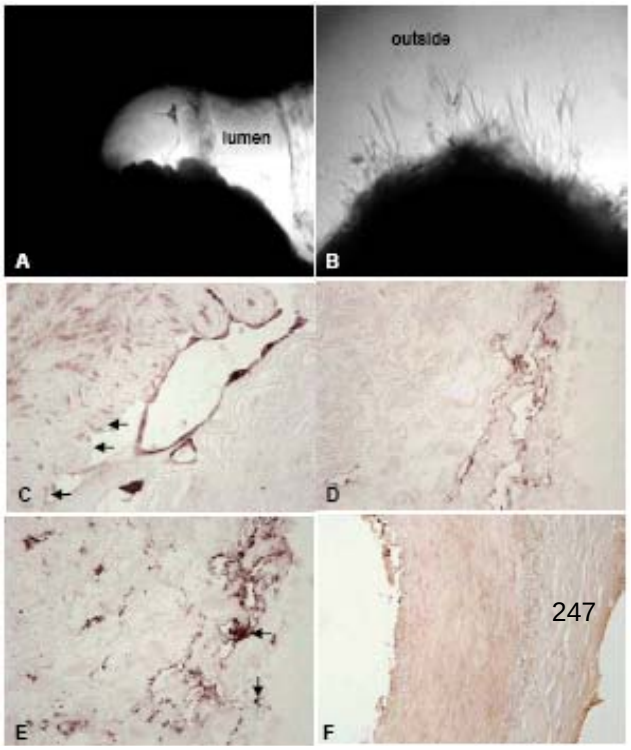


Figure 4A-F: HITA rings embedded in collagen gel or in matrigel show extensive capillary sprouting into the lumen (A) as well as outside the rings (B). Immunostaining for CEACAM1 on sections of embedded HITA rings demonstrate the presence of CEACAM1-positive cells within the lumen (C) as well as in the outside of HITA rings (D). Note that mature endothelial cells lining HITA-lumen and not involved in the sprouting remain negative for CEACAM1 (arrows). CD34 immunostaining of a serial section following the section in panel D confirms the involvement of $CD34^{+}$ cells (arrows) in formation of these new capillaries at the border between vascular wall and the collagen gel (E). CEACAM1 is not present in the quiescent endothelial cells lining HITA lumen and also not in the vasculogenic zone of HITA when ring assay was not performed (F).

obtained from HITA-tissues embedded in paraffin after being used in ring assay revealed that CEACAM1 is expressed in angiogenicly activated endothelial cells but not in quiescent endothelial cells lining the HITA lumen (Fig. 4C). More interestingly, cells involved in capillary sprouting from the "vasculogenic zone" expressed CEACAM1 (Fig. 4D) as confirmed by CD34 immunostaining on a serial section (Fig. 4E). Quiescent CD34-positive cells of the vasculogenic zone (without ring assay) were negative for CEACAM1 (not shown). No immunostaining for CEACAM1 was seen in the vasculogenic zone prior to ring assay (Fig. 4F). Double immunostaining for CEACAM1 and VEGFR-2 on cells isolated from the collagen gel after capillary sprouting from HITA wall revealed a co-expression of both in the majority of these cells (not shown). These data suggest that CEACAM1 is up-regulated during differentiation of the endothelial pre-cursor cells to angiogenicly activated endothelial cells. We currently focused on the role of CEACAM1 in this process.

DISCUSSION

The present results demonstrate for the first time the role of membrane-bound CEACAM1 in angiogenesis and lymphangiogenesis. Moreover, CEACAM1 seems to be involved in the differentiation of vascular wall resident endothelial pre-cursor cells and their participation on capillary sprouting. Interestingly, CEACAM1 is up-regulated in endothelial cells of new or angiogenicly activated blood vessels and activated lymphatic endothelial cells prior to the switch of tumor tissue from a superficial non-invasive to an invasive phenotype. The expression of CEACAM1 in tumor associated lymphatics is detectable earlier than that in tumor associated blood vessels. The overexpression of CEACAM1 HDMECs mimicking its up-regulation in vascular and lymphatic endothelial cells during tumor development results in the up-regulation of potent angiogenic factors such VEGF-A, -C, -D, VEGFR-2 and -3, angiogenin, angiopoietin-1 and -2 and IL-8 while collagen 18/endostatin and Tie-1 are down-regulated. Furthermore, we silenced CEACAM1 in endothelial cells via siRNA and found that this affects VEGF-induced endothelial tube formation *in vitro*. CEACAM1-knock-down decreases the endothelial survival.

Our results demonstrate that endothelial overexpression of CEACAM1 potentates the angiogenic, particularly the morphogenic effects of VEGF, increases angiogenesis and endothelial survival while the overexpression of the same molecule in tumor cell lines of epithelial origin such cells of urinary bladder and prostate cancer resulted in suppression of the expression of angiogenic factors and angiogenic activity (Kilic et al., 2005; Oliveira-Ferrer et al., 2004; Tilki et al., 2006). This dual role of CEACAM1 in the controlling of angiogenesis serves a major contribution clarifying why and how the epithelial down-regulation of CEACAM1 is related to tumor development as it has previously been shown for many cancer types (Hixson et al., 1985; Lin and Pu, 1999; Nollau et al., 1997b; Pu et al., 1999) and why CEACAM1 has been called a tumor suppressor. Superficially observed paradox to these findings is the concurrent up-regulation of CEACAM1 in tumor associated blood vessels and the here demonstrated angiogenic effects induced by endothelial CEACAM1 alone or in combination with VEGF (Ergun et al., 2000; Kilic et al., 2005; Tilki et al., 2006). In summary, these results demonstrate contrary effects induced by CEACAM1 regarding the controlling of angiogenic activity depending on which cell type is expressing it. This ping pong like expression of CEACAM1 in epithelial versus endothelial cells seems to be essential in the early stages of tumor development and at beginning of angiogenic activation. These findings are underlined by our recent results in prostate cancer (Tilki et al., 2006) showing that CEACAM1 up-regulation in PIN associated blood vessels correlates with vascular destabilization, an initial step of angiogenic activation. This process of vascular destabilization is obviously supported by epithelial down-regulation of CEACAM1 leading to a switch in the expression of Ang1 and Ang2, where Ang1 expression is significantly suppressed but that of Ang2 is enhanced.

Supporting the previous findings our experimental induced CEACAM1 knock-down in endothelial cells via siRNA results in the blocking of VEGF-induced endothelial tube formation *in vitro* (Kilic et al., 2005). This is an interesting finding for therapeutic strategies targeting endothelial CEACAM1, but we still do not know, whether the CEACAM1-mediated cell-cell adhesion between endothelial cells or the CEACAM1-induced signalling pathways are crucially involved in this process of vascular morphogenesis. Based on these findings our current work is focused a) on CEACAM1-induced signalling pathways in epithelial versus endothelial cells and b) on CEACAM1-based anti-angiogenic therapy in a rat bladder carcinoma model where we want to target endothelial CEACAM1 in an early stage of tumor development via shRNA using an endothelial specific adenoviral vector.

Further interesting and novel aspect of CEACAM1 is its involvement in lymphangiogenesis. First evidence suggesting such a role is that experimental knock-down of CEACAM1 in bladder cancer cell lines resulted in up-regulation of VEGF-C and- D, which are known to be the most potent pro-lymphangiogenic factors (Oliveira-Ferrer et al., 2004). Remarkably, it could be shown that CEACAM1 is among that factors which are up-regulated during reprogramming of vascular to lymphatic endothelial cells, e.g. by infection with Kaposi-sarcoma herpes virus (Hong et al., 2002). Our unpublished results presented here demonstrate clearly that CEACAM1 overexpression in HDMECs results in significantly enhanced expression of VEGF-C, –D and their receptor VEGFR-3 at mRNA as well as at the protein level. Also immunostaining for other lymphatic markers such LIVE-1 and Prox1 is significantly increased in HDMECs overexpressing CEACAM1. In situ, the co-localization of CEACAM1 and VEGFR-3 in tumor associated lymphatics of bladder carcinoma suggests a potential

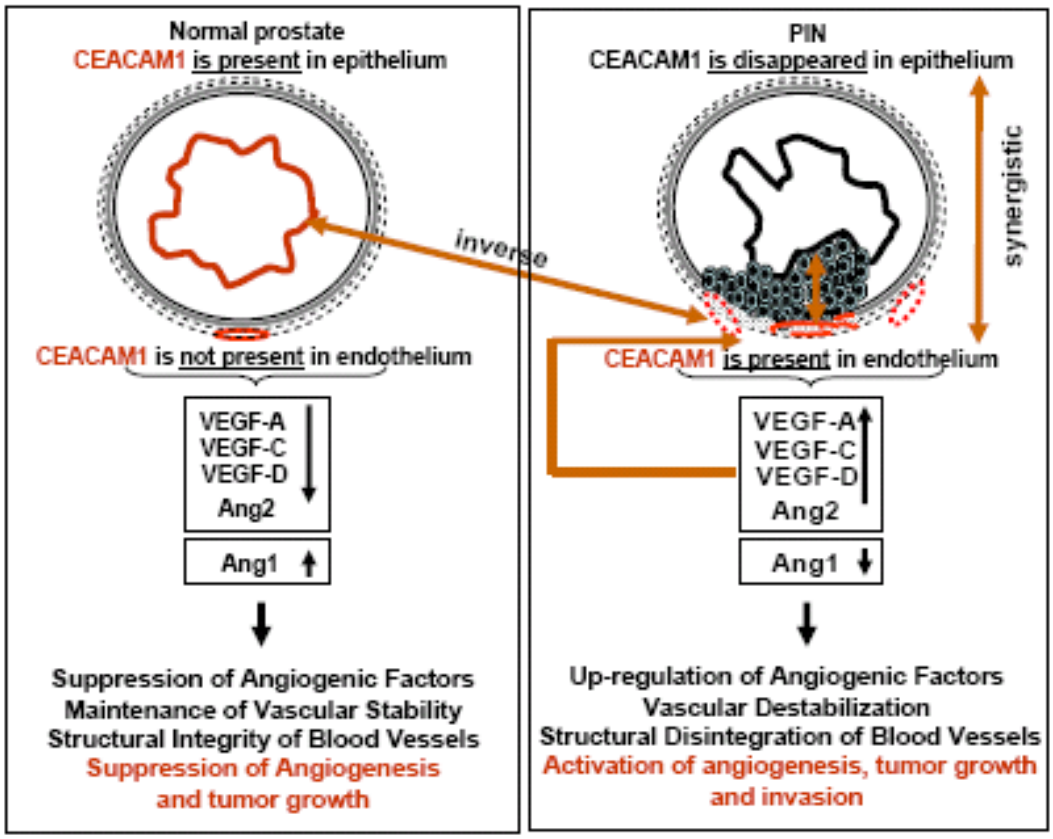


Figure 5: The presence of CEACAM1 in the normal prostate epithelium suppresses the expression of potent angiogenic factors such as VEGF-A, -C, and –D, and Ang2 but increases the expression of Ang1, the natural Ang2 antagonist. Via this mechanism CEACAM1 may be involved in the maintenance of vascular stability and integrity, a process probably preventing the angiogenic switch. CEACAM1 down-regulation, as it occurs in PIN, is obviously accompanied by an up-regulation of angiogenic factors mentioned above but by a down-regulation of Ang1. Concurrently, CEACAM1 is up-regulated in endothelial cells of angiogenicly activated blood vessels adjacent to PIN. This switch is apparently accompanied by vascular destabilization, a process marking the initiation of angiogenesis. In summary, CEACAM1 seems to play a dual role in the regulation of angiogenesis depending on which cell type is expressing it. While epithelial presence of CEACAM1 functions inverse to the endothelial expression of CEACAM1, the epithelial down- but the concurrent endothelial up-regulation act synergistically and promote angiogenesis, tumor vascularization, and probably tumor invasion.

interaction of CEACAM1 and VEGFR-3 in regulation of tumor lymphangiogenesis. Currently, we are working on this issue.Finally, the up-regulation of CEACAM1 in VW-EPC (vascular wall resident endothelial precursor cells) (Zengin and Chalajour et al., 2006) during their mobilization for capillary sprouting from human adult arterial wall indicates a possible role of CEACAM1 in differentiation and maturation of these cells. The co-localization of CEACAM1 with VEGFR-2 in these cells supports this interpretation. These findings are also in line with those we found during capillary formation in an experimental SCID-mouse tumor model where human circulating AC133-postive cells have been co-implanted with the tumor cells and formed tumor blood vessels (Gehling et al., 2000).

Taken together these data suggest that epithelial as well as endothelial expression of CEACAM1 is involved in the controlling of tumor angiogenesis. Epithelial down- but endothelial up-regulation of CEACAM1 act synergistically and activate angiogenesis explaining why the epithelial presence of CEACAM1 functions as tumor suppressor as visualized by the cartoon in figure 5 exemplarily for prostate cancer. The up-regulation of CEACAM1 in a certain time frame of vascular development but its absence in normal blood vessels are making this molecule an interesting candidate for anti-angiogenic tumor therapy targeting the endothelial expression of CEACAM1. The pre-requisite for successful development of such a strategy is to understand CEACAM1-induced signalling pathways and to characterize the CEACAM1 interaction partners during the short time frame of vascular development in which CEACAM1 is up-regulated in vascular endothelial cells.

REFERENCES

Barnett TR, Drake L, Pickle W: Human biliary glycoprotein gene: characterization of a family of novel alternatively spliced RNAs and their expressed proteins. *Mol Cell Biol* 13: 1273-1282, 1993.

Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6: 389-395, 2000.

Carmeliet P, Lampugnani MG, Moons L, Breviario F, Compernolle V, Bono F, Balconi G, Spagnuolo R, Oostuyse B, Dewerchin M, Zanetti A, Angellilo A, Mattot V, Nuyens D, Lutgens E, Clotman F, de Ruiter MC, Gittenberger-de Groot A, Poelmann R, Lupu F, Herbert JM, Collen D, Dejana E: Targeted deficiency or cytosolic truncation of the VE-cadherin gene in mice impairs VEGF-mediated endothelial survival and angiogenesis. *Cell* 98: 147-157, 1999.

Ergun S, Kilic N, Ziegeler G, Hansen A, Nollau P, Gotze J, Wurmbach JH, Horst A, Weil J, Fernando M, Wagener C: CEA-related cell adhesion molecule 1: a potent angiogenic factor and a major effector of vascular endothelial growth factor. *Mol Cell* 5: 311-320, 2000.

Folkman J, Watson K, Ingber D, Hanahan D: Induction of angiogenesis during the transition from hyperplasia to neoplasia. *Nature* 339: 58-61, 1989.

Gehling UM, Ergun S, Schumacher U, Wagener C, Pantel K, Otte M, Schuch G, Schafhausen P, Mende T, Kilic N, Kluge K, Schafer B, Hossfeld DK, Fiedler W: In vitro differentiation of endothelial cells from AC133-positive progenitor cells. *Blood* 95: 3106-3112, 2000.

Good DJ, Polverini PJ, Rastinejad F, Le Beau MM, Lemons RS, Frazier WA, Bouck NP: A tumor suppressor-dependent inhibitor of angiogenesis is immunologically and functionally indistinguishable from a fragment of thrombospondin. *Proc Natl Acad Sci USA* 87: 6624-6628, 1990.

Gupta SK, Hassel T, Singh JP: A potent inhibitor of endothelial cell proliferation is generated by proteolytic cleavage of the chemokine platelet factor 4. *Proc Natl Acad Sci USA* 92: 7799-7803, 1995.

Hanahan D: Signaling vascular morphogenesis and maintenance [comment]. *Science* 277: 48-50, 1997.

Hanahan D, Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86: 353-364, 1996.

Hixson DC, McEntire KD, Obrink B: Alterations in the expression of a hepatocyte cell adhesion molecule by transplantable rat hepatocellular carcinomas. *Cancer Res* 45: 3742-3749, 1985.

Homandberg GA, Williams JE, Grant D, Schumacher B, Eisenstein R: Heparin-binding fragments of fibronectin are potent inhibitors of endothelial cell growth. *Am J Pathol* 120: 327-332, 1995.

Hong YK, Harvey N, Noh YH, Schacht V, Hirakawa S, Detmar M, Oliver G: Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn* 225: 351-357, 2002.

Izzi L, Turbide C, Houde C, Kunath T, Beauchemin N: cis-Determinants in the cytoplasmic domain of CEACAM1 responsible for its tumor inhibitory function. *Oncogene* 18: 5563-5572, 1999.

Kilic N, Lauke H, Fiedler W, Pottek T, Kilic E, Freeman M, Ergun S: Angiogenic switch and vascular stability in human Leydig cell tumours. *Angiogenesis* 3: 231-240, 1999.

Kilic N, Oliveira-Ferrer L, Wurmbach JH, Loges S, Chalajour F, Vahid SN, Weil J, Fernando M, Ergun S: Pro-angiogenic signaling by the endothelial presence of CEACAM1. *J Biol Chem* 280: 2361-2369, 2005.

Kuroki M, Arakawa F, Matsuo Y, Oikawa S, Nakazato H, Matsuoka Y: Three novel molecular forms of biliary glycoprotein deduced from cDNA clones from a human leukocyte library. *Biochem Biophys Res Commun* 176: 578-585, 1991.

Lin SH, Pu YS: Function and therapeutic implication of C-CAM cell-adhesion molecule in prostate cancer. *Semin Oncol* 26: 227-233, 1999.

Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, Sato TN, Yancopoulos GD: Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277: 55-60, 1997.

Nollau P, Prall F, Helmchen U, Wagener C, Neumaier M: Dysregulation of carcinoembryonic antigen group members CGM2, CD66a (biliary glycoprotein), and nonspecific cross-reacting antigen in colorectal carcinomas. Comparative analysis by northern blot and in situ hybridization. *Am J Pathol* 151: 521-530, 1997a.

Nollau P, Scheller H, Kona-Horstmann M, Rohde S, Hagenmuller F, Wagener C, Neumaier M: Expression of CD66a (human C-CAM) and other members of the carcinoembryonic antigen gene family of adhesion molecules in human colorectal adenomas. *Cancer Res* 57: 2354-2357, 1997b.

Oliveira-Ferrer L, Tilki D, Ziegeler G, Hauschild J, Loges S, Irmak S, Kilic E, Huland H, Friedrich M, Ergun S: Dual role of carcinoembryonic antigen-related cell adhesion molecule 1 in angiogenesis and invasion of human urinary bladder cancer. *Cancer Res* 64: 8932-8938, 2004.

O'Reilly,M.S., Boehm,T., Shing,Y., Fukai,N., Vasios,G., Lane,W.S., Flynn,E., Birkhead,J.R., Olsen,B.R., Folkman,J: Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. *Cell* 88: 277-285, 1997.

O'Reilly MS, Holmgren L, Shing Y, Chen C, Rosenthal RA, Moses M, Lane WS, Cao Y, Sage EH, Folkman J: Angiostatin: a novel angiogenesis inhibitor that mediates the suppression of metastases by a Lewis lung carcinoma. *Cell* 79: 315-328, 1994.

Pu YS, Luo W, Lu HH, Greenberg NM, Lin S.H: Differential expression of C-CAM cell adhesion molecule in prostate carcinogenesis in a transgenic mouse model. *J Urol* 162: 892-896, 1999.

Rojas M, Fuks A, Stanners CP: Biliary glycoprotein, a member of the immunoglobulin supergene family, functions in vitro as a Ca2(+)-dependent intercellular adhesion molecule. *Cell Growth Differ* 1: 527-533, 1990.

Risau W: Vasculogenesis, angiogenesis and endothelial cell differentiation during embryonic development. In: The Development of the Vascular System. Feinberg RN, Shere GK, Auerbach R (Eds.),Basel, Karger, pp 58-68, 1991.

Sato TN, Tozawa Y, Deutsch U, Wolburg-Buchholz K, Fujiwara Y, Gendron-Maguire M, Gridley T, Wolburg H, Risau W, Qin Y: Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376: 70-74, 1995.

Sawa H, Kamada K, Sato H, Sendo S, Kondo A, Saito I, Edlund M, Obrink B: C-CAM expression in the developing rat central nervous system. *Brain Res Dev Brain Res* 78: 35-43, 1994.

Singer BB, Scheffrahn I, Obrink B: The tumor growth-inhibiting cell adhesion molecule CEACAM1 (C-CAM) is differently expressed in proliferating and quiescent epithelial cells and regulates cell proliferation. *Cancer Res* 60: 1236-1244, 2000.

Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD: Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87: 1171-1180, 1996.

Tilki D, Irmak S, Oliveira-Ferrer L, Hauschild J, Miethe K, Atakaya H, Hammerer P, Friedrich MG, Schuch G, Galalae R, Kilic E, Huland H, Ergun S: CEA-related cell adhesion molecule-1 (CEACAM1) is involved in angiogenic switch in prostate cancer. *Oncogene 25: 4965-74*, 2006.

Zengin E, Chalajour F, Gehling UM, Ito W, Treede T, Lauke H, Weil J, Reichenspurner H, Kilic N, Ergun S: Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development* 133: 1543-51, 2006.

Interleukin-6 as an *N-myc* regulated angiogenesis inhibitor: mechanisms and clinical implications

Prof. Dr. Lothar Schweigerer

Pediatrics I, Children's Hospital
University of Göttingen
Robert-Koch-Stasse 40, 37075 Göttingen, Germany

Member of SPP 1069 from 2000 until 2004 (thereafter see: project: Wilting/Schweigerer)

SUMMARY

Patients with neuroblastomas of advanced stages have a poor prognosis. Enhanced expression of the *N-myc* oncogene within the neuroblastomas appears to contribute essentially, but the mechanisms involved have remained unclear. It appeared possible that enhanced *N-myc* expression stimulates angiogenesis and thereby permits tumor progression. We have therefore examined cultured neuroblastoma cells for the presence of inhibitors of endothelial cell proliferation, an essential partial process of angiogenesis. We have identified a previously unknown, potent and specific inhibitor of endothelial cell proliferation (SI.2) in neuroblastoma cells with normal, but not enhanced, *N-myc* expression, suggesting that the inhibitor is down-regulated by enhanced *N-myc* expression, thereby permitting neuroblastoma progression. We have previously demonstrated that N-Myc facilitates angiogenesis by down-regulating an angiogenesis inhibitor identified as the inhibin βA homodimer activin A. We have therefore defined the molecular, biological and clinical consequences of activin A expression in human neuroblastoma. We report that enhanced activin A expression suppresses proliferation and colony formation of human neuroblastoma cells with amplified *MYCN in vitro*; that it inhibits neuroblastoma growth and angiogenesis *in vivo*; that it is highly expressed in differentiated, but not undifferentiated human neuroblastomas; and that it correlates with favourable outcome of neuroblastoma patients. Our results indicate that high activin A expression plays an important beneficial role in human neuroblastoma.

Since tumor angiogenesis is governed by alterations of tumor suppressor or oncogenes operant in a broad range of tumors, we have further addressed this issue in neuroblastoma, characterized by the near-exclusive amplification and over-expression of the N-Myc oncogene. We observed that N-Myc over-expression results in down-regulation of interleukin-6 (IL-6) and that IL-6 is an inhibitor of endothelial cell proliferation and VEGF-induced rabbit corneal angiogenesis. STAT3 is instrumental for IL-6 activity as infection with adenoviruses expressing a phosphorylation deficient STAT3 mutant renders endothelial cells insensitive to the anti-proliferative action of IL-6. Finally, though IL-6 does not influence neuroblastoma cell growth, IL-6-expressing xenograft tumors in mice exhibit reduced neovascularization and suppressed growth. Our data shed new light on the mechanisms by which N-myc oncogene amplification enhances the malignant phenotype in neuroblastomas.

INTRODUCTION

Neuroblastoma is a malignant tumor derived from immature sympathetic precursor cells of the neural crest. Neuroblastomas can be classified into three groups addressing their clinical and biological properties: the first group is characterized by hyperdiploid karyotype, normal chromosomes, low stage and a favourable prognosis, the second by near-diploid or tetraploid karyotype, structural chromosomal anomalies, normal *MYCN* and an intermediate, highly variable prognosis and the third

group by diploidy, chromosome 1p deletion, high TrkB expression, amplified *MYCN*, and poor prognosis (Brodeur, 2003). Despite aggressive multimodal therapy, survival rates have remained in the range of 60 or 25 percent for patients of all disease stages or of the advanced stage 4, respectively (Brodeur, 2003).

Although the mechanisms leading to the progression of human neuroblastoma have remained unclear, amplification of the MYCN oncogene appears to contribute significantly (Westermann and Schwab, 2002). We have demonstrated that *MYCN* over-expression stimulates angiogenesis by the concomitant down-regulation of three angiogenesis inhibitors (Fotsis et al., 1999), one of which was identified as activin A (Breit et al., 2000). Within the large superfamily of transforming growth factor-ß members, activin A is a member of the inhibin / activin family of growth modulators (Chen et al., 2002; Mather et al., 1997). Activin A is a homodimeric Mr ≈ 25 kD protein composed of two inhibiņ βA chains whereas other members of the inhibin / activin family including activin B, activin AB, inhibin A or inhibin B are composed of inhibin βB homodimers, inhibiņ βA/ inhibin βB heterodimers, inhibin α/ inhibiņ βA heterodimers or inhibin α / inhibin βB heterodimers, respectively. Activin A is evolutionary conserved and controls various functions (Mather et al., 1997).

Amplification of N-*myc* oncogene is a frequent event in advanced stages (III and IV) of human neuroblastomas. N-*myc* amplification correlates with poor prognosis and enhanced vascularisation of human neuroblastomas, suggesting that N-*myc* oncogene could stimulate tumor angiogenesis and thereby enhance neuroblastoma progression. Indeed, stable transfection and 100-fold over-expression of N-Myc in a neuroblastoma cell line (SH-EP) resulted in an enhanced malignant phenotype of the transfectants (WAC2) and the ability to form well vascularised tumors in nude mice (Schweigerer et al., 1990). We have previously screened conditioned media from SH-EP007 and WAC2 cells reasoning that differentially expressed angiomodulators would strongly suggest that these molecules are regulated by N-*myc* oncogene providing information about the molecular mechanisms linking oncogene activation with initiation of tumor angiogenesis. We were able to demonstrate that three EC proliferation inhibitors present in SH-EP007 supernatants (SI.1, SI.2, and SI.3) were completely down-regulated in WAC2 cells (Fotsis et al., 1999). In a further study, we have identified SI.3 as being activin A and documented antiangiogenic properties for this TGF-family member (Breit et al., 2000). The present study deals with the structural and functional characterisation of SI.2.

METHODS

Cloning of inhibin βAand cell transfection

Inhibin βA cDNA was inserted into the Not I/Xho I sites of pcDNA 3.1 (Invitrogen, Groningen, The Netherlands). Sequence analysis of the cloned DNA fragment revealed the identity with the published inhibiņ βA sequence (GenBank entry NM_002192). Transfection of Kelly and embryonic kidney cells was achieved using "Superfect Transfection Reagent" (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Briefly, 2 x 10^7 cells were incubated with "Superfect" reagent and 10 µg of vector DNA containing the human inhibiņ□□-cDNA. Plasmid uptake was selected for by adding G418 (10 µg / µl) to the media. Transfected cells were isolated by ring cloning.

RT-PCR

Inhibiņ βA- or GAPDH-cDNA were amplified as described previously (Breit et al., 2000). Primer sets were used alone or together in a duplex-RT-PCR reaction.

For semi quantitative analysis of neuroblastoma specimens, inhbin βA/ GAPDH duplex RT-PCR products of neuroblastoma RNA were separated on 1.5% agarose gels. Scanned images were analysed using "NIH image for MacIntosh". Inhibin βA expression was defined according to the inhibin βA/ GAPDH band intensity ratio as arbitrary density units (du). Lanes without detectable GAPDH signals were excluded. Primers used for amplification of genes were as described (Breit et al., 2000).

Western Blotting

Cells were extracted by exposure to RIPA lysis buffer (50 mM Tris-HCl containing NaCl (300 mM), NP-40 (1%), deoxycholate (0.5%), sodium dodecylsulfate (0.1%) and "complete" protease inhibitor (Roche Diagnostics, Mannheim, Germany) (pH 8.0)). Protein concentration was determined using DC Protein assays (Bio-Rad, Munich, Germany). Extracts (20 µg of protein / lane) were separated by 10% SDS-PAGE under reducing conditions and transferred to nitrocellulose membranes (Schleicher & Schüll, Dassel, Germany) previously exposed for 1 h at 20°C with non-fat dried milk (3%) in phosphate-buffered saline (PBS). Membranes were incubated overnight with a 1: 1000 dilution of monoclonal activin A-specific antibodies (Serotec, Düsseldorf, Germany), washed three times with PBS containing Tween20 (0.4%), and exposed to secondary antibodies coupled to horseradish peroxidase. Activin A-immunoreactivity was visualized using ECL chemiluminescence (Amersham Pharmacia).

Microarray analyses

Total RNA was prepared from neuroblastoma cells during logarithmic growth phase using TRIZOL (Life Technologies, Rockville, MA) according to standard protocols (Chomczynski, 1993). RNA was cleaned and DNA removed by using RNeasy columns (QIAGEN, Hilden, Germany) according to the manufacturer's suggestions. Labelling of cRNA and hybridization of labelled probes with the Human Cancer G110 Gene Chip® was done according to the manufacturer's recommendations (Affymetrix, Santa Clara, CA). Data readout and global scaling was essentially as described (Wai et al., 2002). Data analysis was performed with "Microarray Analysis Suite 5.0" and "Data Mining Tool 3.0" (Affymetrix, Santa Clara, CA). The criteria for a transcript to be called differentially expressed between Kelly and empty-vector control or activin A-expressing Kelly cells were: 1) signal > 400 and call = "P" (present) in at least one sample; 2) DiffCall !="NC" no change); 3) FC (fold change) > 2.5. Signals <100 were set to 100.

Cell culture and bioassays

All cells were cultured in RPMI 1640 containing 10 % foetal calf serum and penicillin and streptomycin/ (100 U/ml) (Fotsis et al., 1999; Fotsis et al., 1997; Rössler et al., 1999; Schweigerer et al., 1990). Transfected neuroblastoma cells received additionally G418 (10 µg / µl twice a week) for continuous selection. Cell proliferation was quantified as previously reported (Schweigerer et al., 1987).

Animal experiments

Experimental neuroblastomas were established as described (Schweigerer et al., 1990). Palpable masses were defined as tumors. Subsequently, tumor size, body weight, and general status of the animals were recorded every 2 or 3 days. Perpendicular tumor volume was calculated from the

formula: (longest diameter) x (shortest diameter)^2 x 0.5. Mice were sacrificed at times regulated by local institutional guidelines, i.e., once tumors had reached sizes of approximately 2.5 cm^3 or 10 % of the body weight.

Immunohistochemistry

Tumors generated from KT3, KT2 or native Kelly cells were excised from sacrificed animals, fixed in formalin (4 %) over night, embedded in paraffin, sectioned and mounted on glass slides. Sections were deparaffinized through graded xylene and alcohol washes and treated for 10 min with proteinase K (DAKO, Hamburg, Germany) prior to immunohistochemistry. For Ki67 or CD31 staining, we incubated sections with monoclonal mouse anti-human Ki67 antibodies (clone MiB-1, DAKO, Hamburg, Germany, 1: 100) for 1 h at room temperature or with monoclonal rat anti-mouse PECAM-1 antibodies (BD Pharmingen, San Jose, CA, 1: 50) overnight at 4^0 C. Glass slides were washed with PBS and antibody binding was determined with Fuchsin (DAKO, Hamburg, Germany) as a substrate. For tumor morphology, sections were also stained with hematoxylin-eosin. For analysis of human specimen, paraffin sections of human neuroblastomas (n=7) were deparaffinized with rotihistol (Carl Roth, Karlsruhe, Germany). Endogenous peroxidase was blocked with 0.3% hydrogen peroxide in methanol for 30 minutes. Sections were then incubated for 5 min. in PBS containing 0.05% Tween 20, washed 2 times in PBS and blocked with 1%BSA in PBS for 20 minutes. Primary antibody, mouse-anti-human inhibin βA (Serotec, Oxford, UK), was diluted 1:20 in PBS and incubated for 1 hour. The secondary antibody was peroxidase-conjugated goat-anti-mouse IgG, diluted 1:200 (Sigma, St. Luis, USA). Diaminobenzidine (Sigma) was used as chromogen. Sections were counter-stained with nuclear fast red.

Patients and specimens

The local ethics committee approved the study. Neuroblastoma specimens, clinical data and written informed consent of the patients' guardians were obtained within the treatment studies NB 85 and NB 90 of the German Society for Paediatric Oncology and Haematology (GPOH). From a bank of neuroblastoma specimens, we randomly chose 95 samples (collected between 1987 and 1998), and excluded post hoc those without sufficient mRNA quality. The remaining 78 patients (40 male, 33 female, 5 unspecified) were aged between 0 and 116 months (median, 19 months). According to the International Neuroblastoma Staging System (INSS (Brodeur et al., 1993), 17 patients had stage 1, 11 stage 2, 17 stage 3, 25 stage 4, and 8 stage 4S tumors, respectively. 17 neuroblastomas (22%) showed *MYCN* amplification. During follow-up time (minimum and maximum 1 and 96 months, respectively, median 27 months), 26 major events occurred, resulting in a 5 years event-free survival rate (EFS) of 57.7% ± 6.8% (standard error). Investigators were blind to all clinical data until mRNA analysis was completed.

Statistical analyses

For experimental animals, we calculated and compared tumor take (from inoculation to tumor formation) and survival times (from tumor inoculation to sacrifice). Microvessel densities and the percentage of Ki67-positive cells in animal tumors were tested using Student's t-test for independent groups. All statistical tests were two-sided, and a p-value of 0.05 was considered statistically significant.

Chromatography, mass fingerprint analysis and microsequencing

Concentration of conditioned medium (47 liters), acidification and SP-Sepharose Fast Flow (SSFF) chromatography were described earlier. Fractions containing SI.2 were purified through a second SSFF column (320 ml bed volume) using a salt gradient at lower pH (4.8) for increased resolution. The desalted and lyophilized active fractions were then dissolved in 0.25% trifluoroacetic acid (TFA)/dH2O and loaded onto a Bakerbond Wide-Pore C-18 reversed phase high performance liquid chromatography (HPLC) column (4.6x250 mm; Malinckrodt Baker, Philipsburg, NJ) equilibrated with 0.1% TFA. Bound material was eluted with linear gradients from 0-30% acetonitrile in 10 minutes and 30-60% acetonitrile in 45 minutes at a flow rate of 1 ml/min. Fractions of 1 ml were collected, lyophilized and resuspended in culture water. An aliquot (10 Bl) was tested for activity on BBCE cell proliferation. The active fractions were separated on 12.5% SDS-polyacrylamide gel under reducing conditions. Protein bands were excised from the Coomassie blue stained gel, destained, reduced and alkylated with iodoacetamide, and digested with trypsin. The resulting peptides were subjected to mass spectroscopy analysis using an MALDI-TOF instrument (Voyager DE-STR, PerSeptive Biosystems). Peptide samples were prepared using dihydroxybenzoic acid as matrix. From the calibrated MS peptide mass map, a peak table list was generated omitting signals observed in the chemical background spectrum. This peak table list was used as input to search the SWISSPROT and NCBI protein databases for sequence similarities. All searches were carried out using the MS-Fit software program (Clauser KR and Baker PR, UCSF). MS-Fit search results were checked regarding the MOWSE score, molecular mass, isoelectric point, species and percentage of matching masses.

Transfections and IL-6 promoter reporter assays

Full-length N-*myc* (pcDNA3-N-*myc*) was generated by PCR amplification using pN-*myc* as template (a gift from M. Schwab, German Cancer Research Center, Heidelberg, Germany) (Hiller, 1991). All deletion mutants of N-*myc* were generated from the above construct by ligation of PCR products. All constructs were sequenced. Transient transfections were performed, according to the manufacturer's instructions, using either effectene (Qiagen) or lipofectamine plus reagent (Invitrogen) for CAT and luciferase IL-6 promoter reporter constructs, respectively. A reporter CAT construct containing the -1200 to +15 promoter fragment of the human IL-6 (pIL6-CAT (-1200/+15)) was kindly provided by Dr. Scala (University Federico II, Naples, Italy). HeLa cells ($5x10^5$ per 60 mm dish) were transfected with 0.4 µg of the pIL6-CAT (-1200/+15) construct, alone or in combination with 0.4 µg of wild-type or mutated N-*myc* expression vectors, for 40 hours. In each transfection a constant total DNA concentration was used. Cells were collected and analysed for CAT activity using a CAT-ELISA kit according to the manufacturer's instructions (Roche Molecular Biochemicals). Luciferase reporter assays were carried out in SH-EP 007 cells using identical conditions except that three different luciferase constructs of the human IL-6 promoter (pIL-6-Luc) were used (-840, -417 and –140 to +12). Luciferase activity was analysed by a commercial kit (Promega) using a luminometer (EG&G junior, Berthold). In all transfections, 0.2 µg of β-galactosidase (β-gal) expression vector (CMV-β-gal) was constransfected as an internal control. The expression and nuclear localisation of all constructs were determined by Western blot using a mouse anti-human N-Myc antibody (Cymbus Biotech, Hants, NF).

The full-length human IL-6 cDNA, kindly donated by Dr Podacks (University of Miami, Florida) was subcloned into the Xho I site of a CMV promoter construct. WAC2 cells (70% confluent) were transfected with effectene using 1.8 µg of the IL-6 construct and 0.2 µg of a construct containing puromycin resistance gene (Clontech). Clones were selected in medium containing 0.7µg/ml puromycin and expression of IL-6 was evaluated by Western blot analysis of supernatants using a

goat anti-IL-6 antibody (R&D). Several independently derived clones were obtained for each construct. Control tranfectants were generated using the empty CMV promoter construct.

Phosphorylation of STAT3 protein in BBCE cells

BBCE cells were seeded in 12-well plates (400 000 cells/well), cultured for 2 days in DMEM containing 0.5 % NCS and STA3 and phospho-STAT3 were detected as described in a kit purchased from New England-Biolabs.

Preparation of adenoviruses expressing Stat3 and [^{3}H] thynidine incorporation assays

Recombinant adenoviruses harbouring wild-type (AD/WT) and dominant negative (Y705F) (AD/DN*) Stat3* cDNAs as well as a control adenovirus carrying only the vector (AD) were prepared as previously [Kunisada, 1998 #84]. Adenoviruses were amplified in 293 cells. The efficiency of infection and the expression of the proteins were monitored by immunofluorescence and Western blot analysis, respectively. BBCE cells grown to subconfluency in 24-well plates were infected with the adenoviruses at a multiplicity of infection of 3:1 (3 plaque-forming units (pfu)/cell) for 8 h in full medium. The medium was renewed and various concentrations of rhIL-6 together with 2.5ng/ml bFGF or 30 ng/ml VEGF were added for an additional 24h. Then, 1BCi/ml of [^{3}H] methyl-thymidine (ICN) was added to each of the wells for the last 4 h of the incubation. Culture medium was removed and the cells were fixed with ice-cold 10% trichloroacetic acid for 20 min at 4ΓC, washed three times with water and solubilised in 0.1 M NaOH overnight at 4^{O}C. The radioactivity was counted in a beta liquid scintillation counter. Translocation of STAT3 to the nucleus was studied after infection of the cells with 3 plaque-forming units (pfu)/cell for 8 h in full medium, allowing the expression for another 20h and adding 20 ng/ml rh-IL-6 for 15 min. Then, the cells were fixed and STAT3 and phospho-STAT3 were detected with a kit from New England-Biolabs. Leica TCS-SP confocal microscope was used.

RESULTS

Down-regulation of an angiogenesis inhibitor identified as activin A

We transfected this cell line with a vector containing a geneticin resistance gene and human inhibin βA cDNA so as to establish high activin A expression. In a parallel experiment, we transfected Kelly cells with the empty vector and used these and the native, untransfected cells as controls. Kelly cells transfected with the inhibiņ βĄ cDNA were selected in the presence of geneticin and gave rise to a cell line (termed KT3) which expressed inhibin βA mRNA and secreted immunoreactive (1 ng / µg total protein as determined by ELISA) and bioactive activin A into the medium. In contrast, the control cell line (named KT2) expressed neither inhibiņ βĄ nor immunoreactive activin A. To determine the character and quality of the potential biological consequences of enhanced activin A expression, we compared gene expression profiles of KT3, KT2 and native Kelly cells using Affymetrix "cancer gene" chips with oligonucleotides representing about 1600 cancer-related genes. Activin A modulated expression of various molecules. Down-regulation was most prominent for insulin-like growth factor-II (IGF-II), which has been implicated in neuroblastoma progression. Up-regulation was strongest in the case of vasoactive intestinal protein (VIP), BIGH3 and LMO4, which have been demonstrated to inhibit neuroblastoma growth. All data were confirmed by RT-PCR. These properties suggested that the activin A-mediated transcriptional program would eventually result in the inhibition of

neuroblastoma growth and / or progression. In fact, growth rates of activin A-expressing (KT3) cells were considerably attenuated when compared to those of KT2 or native Kelly cells and this effect could be antagonized by recombinant follistatin. Thus, activin A appeared to suppress the malignant potential of neuroblastoma cells. Since the ability to grow anchorage-independent at clonal density and in colonies is a feature of malignant cells, we compared the abilities of the neuroblastoma cells to grow in soft agar. Native Kelly cells and the control transfectants (KT2), but not the activin A-producing neuroblastoma cells (KT3) were able to form colonies in soft agar, indicating that activin A suppresses the malignant phenotype of human neuroblastoma cells. In order to investigate the consequences of enhanced activin A expression for neuroblastoma growth in vivo, we compared the abilities of the control cells with those of KT3 cells to grow in immune-deficient nude mice. Native Kelly cells or KT2 cells formed measurable tumors 14 (mean; 95% confidence interval 13 – 15 days) or 17 (14 – 20) days, respectively, after subcutaneous injection. In contrast, KT3 cells formed measurable tumors only with significant delay, i.e., 30 (23 – 37) days after tumor cell injection ($p < 0.001$). Mice carrying tumors derived from KT3 cells lived significantly longer (mean 44 days $\pm$ 3 days) ($p < 0.001$) from inoculation of tumor cells to the primary end point (i.e., when tumors had expanded to 10 % or more of the body weight) than mice carrying tumors derived from KT2 or native Kelly cells (mean 24 days $\pm$ 5 days or 27 days $\pm$ 4 days, respectively). In addition, KT3 cell-derived tumors appeared considerable paler than those derived from the control cells suggesting activin A-mediated suppression of tumor angiogenesis.

To determine whether activin A-mediated suppression of neuroblastoma growth *in vivo* resulted from the inhibition of neuroblastoma cell proliferation and / or tumor angiogenesis, we compared experimental neuroblastomas by immunohistochemistry using antibodies directed against the proliferation marker Ki67 or the blood vessel marker CD31, respectively. Tumors derived from control (native Kelly or KT2) cells were composed of 75.8 ± 3.3 or 71.0 ± 1.7 % Ki67-positive cells, whereas those derived from KT3 cells contained significantly less ($p < 0.001$), i.e., 53.8 $\pm$ 0.7%, Ki67-positive cells than the combined controls (n=5 for either group). Tumors derived from the controls (native Kelly or KT2 cells) contained 25.0 $\pm$ 4.5 or 26.1 $\pm$ 5.9 microvessels per visual field, respectively, whereas those derived from KT3 cells contained 18.6 $\pm$ 0.7 microvessels per field. Vessel densities in KT3-derived tumors were lower than in the combined controls (25.5 $\pm$ 3.4), but the difference did not reach statistical significance (p=0.214). Thus, activin A suppresses neuroblastoma growth and, apparently, neuroblastoma angiogenesis.

Our experimental data suggested that activin A could suppress neuroblastoma progression clinically. To evaluate this hypothesis, we first excluded expression of inhibin βB and inhibin α and subsequently determined expression of inhibiņ βA in 78 human neuroblastoma samples using semi-quantitative RT-PCR and inhibin βA specific primers. While inhibiņ A expression was low or undetectable in most samples, it was high in the remainders (range 0 – 1.36 density units (du); quartiles 0.01 – 0.05 – 0.14 du; 80th percentile 0.22 du). Inhibiņ A expression did not differ significantly in neuroblastomas with regard to sex, age or stage. In contrast, neuroblastomas with *MYCN* amplification showed a significantly lower inhibiņ βA expression (n=17; quartiles 0.00 – 0.03 – 0.06 du) than neuroblastomas without *MYCN* amplification (n=61; quartiles 0.01 – 0.05 – 0.21 du; p=0.042). When patients were categorized for high (group 1, n=15) or low (group 2, n=63) inhibiņ A expression, both groups included male and female patients of all ages and stages, with no significant differences detectable. Only one tumor (6.7%) in group 1 of patients contained *MYCN* amplification, as opposed to 16 tumors in group 2 of patients (25.4%). This difference was also not significant.

We investigated whether inhibiņ βA expression would predict the outcome of our patients. High inhibiņ βA expression predicted an excellent outcome (group 1, EFS 92.9% $\pm$ 6.9%) as compared with low inhibiņ βA expression (group 2, EFS 49.1% $\pm$ 7.7%, p=0.014). The favourable outcome of

group 1 could not be explained solely by the higher number of *MYCN*-amplified tumors in group 2. When the analysis was restricted to patients with non-amplified *MYCN* (n=61), the estimated outcome of patients with high inhibin βA expression (n=14, EFS 100%) still differed significantly from that of patients with low inhibin βA expression (n=47, EFS 55.1% ± 8.9%, p=0.012). Since we did neither detect inhibin βB nor inhibin α, our data suggest that inhibin βA expression results in production of activin A. These data are in line with preliminary results demonstrating elevated activin A levels in neuroblastoma patients, but not healthy children.

To further validate these data, we studied expression of activin A by immunohistochemistry using paraffin sections of human neuroblastomas and an antibody which detects both mature inhibin βA and its precursor protein but not related activin or inhibin molecules. In undifferentiated, cell-dense neuroblastomas we were unable to detect activin A. In contrast, activin A was readily detected in neuroblastomas with a differentiated phenotype including large perikarya, pale nuclei with nucleoli, polarity and axon formation. The activin A signal was preferentially observed in perikarya and nerve fibers. We also detected inhibin βA in the tunica intima and media of blood vessels suggesting a function for both neuroblastoma cells and blood vessels. Our results demonstrate that activin A suppresses neuroblastoma growth and potentially angiogenesis and they suggest that these mechanisms are of clinical benefit. Finally, inhibin βA expression may serve as an independent indicator to better define the prognosis of patients of class 2 neuroblastomas.

SI.2 is identical with IL-6

Purification of SI.2 from SH-EP007 supernatants resulted in a major peak of inhibitory activity eluted at a concentration of 52% acetonitrile from a C-18 reverse-phase HPLC column. In SDS-PAGE analysis, we observed some minor quantities of Mr 55 – 116 kDa proteins in addition to a major protein of Mr 24 kDa. Since previous gel filtration experiments had revealed a Mr of about 25-30 kDa for the activity, the 24 kDa band was excised from the gel and subjected to in-gel digestion followed by mass fingerprint analysis. Sequence analysis of the 9 peptides obtained unequivocally identified SI.2 as IL-6. The identity was fully confirmed by Western blot analysis where the intensities of IL-6 immuno-reactivity in the respective HPLC fractions correlated strongly with SI.2 activity. SI.2 activity could be abrogated by specific neutralizing antibodies against IL-6.

IL-6 expression is down-regulated by N-Myc

Detection and isolation of IL-6 in SH-EP007 cells (vs. WAC2) suggested that N-Myc down-regulated IL-6. Indeed, further correlation of N-Myc and IL-6 expression in 13 neuroblastoma cell lines, both at the mRNA and protein levels, showed inverse relationship (data not shown). To substantiate N-Myc-induced down-regulation of IL-6, we used promoter-reporter assays. Upon transfection of HeLa cells with a vector containing wild-type N-*myc* cDNA, IL-6 promoter activity was suppressed substantially. Transfection of N-*myc* mutants lacking the myc box domains MBI or MBII (mutants d1-134, d20-90 and d96-140) or the DNA-binding (mutant d381-395) or the helix/loop/helix leucine zipper regions (mutant d395-464) resulted in an unabated suppression of IL-6 promoter-CAT activity. In contrast, N-Myc mutants lacking both the basic region (BR) and helix/loop/helix leucine zipper regions (HLH-Zip) (mutant d350-464) had an impaired ability to suppress promoter activity and N-Myc mutants lacking the first 300 amino acids (including the MBI, MBII and EX2/EX3 regions) had completely lost this ability. Thus, N-Myc regions highly conserved among myc-box genes (MBI, MBII) are not necessary while a contiguous BR-HLH-Zip region is important with the central region of the N-Myc protein being decisive for its suppressive effects on the IL-6 promoter.

We have used HeLa cells because of low transfection efficiency of SH-EP 007 cells resulting in detection problems with the CAT reporter construct of the IL-6 promoter. Subsequent generation of IL-6 promoter constructs fused to the more sensitive luciferase reporter gene allowed confirmation of the suppressing effect of N-Myc on transcription of IL-6 promoter in SH-EP 007 cells and defined domains involved in this effect. These data indicate an N-Myc-specific role in inhibiting IL-6 promoter activity.

IL-6 inhibits endothelial cell proliferation via the STAT3 pathway

We had identified IL-6 due to its ability to inhibit an important partial step of angiogenesis, i.e., vascular endothelial cell proliferation. In agreement with this finding, in addition to bovine brain capillary ECs, recombinant human IL-6 (rhIL-6) inhibited the bFGF-stimulated proliferation of aorta (BAE) and human dermal microvascular ECs. At the concentrations used, rhIL-6 was not cytotoxic, as seen by microscopic evaluation and by the fact that cell densities never fell below those present at seeding. IL-6 can mediate its effects by binding to specific cell surface receptors with subsequent phosphorylation of the transcription factor STAT3 at Tyr705. When rhIL-6 was added to BBCE cells, it was, in fact, able to phosphorylate STAT3 in a time- and concentration-dependent manner. Simultaneous administration of angiogenic factors such as bFGF and VEGF did not alter the phosphorylation pattern of STAT3. We wished to determine whether or not the STAT3 pathway was necessary and crucial for the IL-6-induced inhibition of vascular endothelial cell proliferation. To that aim, we infected BBCE cells with recombinant adenoviruses containing various *stat3* forms and investigated the ability of rhIL-6 to inhibit proliferation of the infected cells. rhIL-6 was able to inhibit proliferation of BBCE cells infected with the empty vector controls (AD) or with the vector containing the wild-type *stat3* (AD/WT). In contrast, rhIL-6 was unable to substantially inhibit proliferation of BBCE cells transfected with the dominant-negative *stat3* mutant (AD/DN). These effects were equally observed in BBCE cells stimulated by bFGF or VEGF. In order to mediate its effects on cell proliferation, STAT3 must be translocated from the cytosol to the cell nucleus. Therefore, we examined the ability of rhIL-6 to induce nuclear STAT3 translocation in the BBCE infected cells by using confocal laser microscopy. In fact, IL-6 induced STAT3 translocation in the BBCE cells transfected with the control vector (AD) or the vector containing wild-type *stat3* (AD/WT), but not the dominant-negative *stat3* mutant (AD/DN). These data clearly demonstrate that IL-6 inhibits vascular endothelial cell proliferation induced by major angiogenesis stimulators and that inhibition is mediated via the STAT3 pathway.

IL-6 inhibits VEGF-induced neovascularisation in the rabbit cornea assay

To evaluate whether or not IL-6 inhibited angiogenesis *in vivo*, we used the rabbit cornea assay. Initial experiments, in which pellets containing 0.25 or 1.0 µg of rhIL-6 were implanted, revealed no macroscopic evidence of IL-6-induced inflammation. In 2 out of 7 animals receiving 1 µg rhIL-6 pellets, we observed evidence of a mild angiogenic response. However, whereas pellets containing 0.25 µg of rhIL-6 had a minor effect, pellets containing 1.0 µg of rhIL-6 inhibited VEGF-induced angiogenesis completely as compared to control pellets. Thus, IL-6 inhibits VEGF-induced angiogenesis.

IL-6 inhibits tumor angiogenesis and growth in xenograft tumorigenesis assays in mice. As demonstrated earlier, overexpression of N-myc oncogene down-regulates IL-6 expression. Though the effect of IL-6 on EC proliferation and corneal angiogenesis suggested an attenuating role of IL-6 on malignant transformation via inhibition of angiogenesis, the result did not exclude a direct autocrine inhibitory effect of IL-6 on neuroblastoma cell proliferation. Towards this end, rhIL-6 had no

effect on the proliferation of various neuroblastoma cell lines, including SH-EP007, WAC2, NBL-S, Kelly cells, even at very high concentrations. The same results were obtained when we transfected WAC2 neuroblastoma cells with a vector containing the human IL-6 cDNA under the control of a CMV promoter (not down-regulated by N-Myc) as compared to cells transfected with the empty vector. IL-6-expressing (c3, c11 and c21) and control (cve-1 and cve-2) WAC2 clones had an unabated ability for rapid proliferation, which was very similar to that of the parental WAC2 cells and clearly distinct from that of the low N-*myc* expressing SH-EP007 cells. Thus, forced overexpressiom of IL-6 in WAC2 cells did not inhibit their *in vitro* proliferation. Yet, the fast growing IL-6 expressing WAC 2 clones (c3, c11, c21) did grow slower as mouse xenografts (compared to control cve-1 and cve-2 WAC2 clones) exhibiting reduced vascularization. Indeed, 7 days following intradermal implantation in the flanks of immune-deficient mice, the weight of tumors derived from the IL-6 expressing WAC2 cells (c3, c11, and c21) was decreased in a statistically significant manner compared to both control clones (cve-1 and cve-2). Similarly, the tumor volumes were significantly smaller for clones c3 and c21 on day 7 compared to vector controls. At the same time point (7 days), the number of capillaries was significantly lower in the IL-6 clones compared to that of all the control clones. The slow growth of the IL-6 expressing WAC2 clones was still visible in a set of mice sacrificed at 4 weeks.

DISCUSSION

MYCN expression down-regulates activin A

We have demonstrated that enhanced *MYCN* expression down-regulates activin A and that it inhibits angiogenesis (Breit et al., 2000), but we did not evaluate the direct consequences of activin A on neuroblastoma growth and angiogenesis. The next study was designed to address these questions. We selected a human neuroblastoma cell line (Kelly) with amplified *MYCN* and transfected the cells with a vector containing human inhibiņ βA cDNA or with the empty vector as a control. Using this cellular model, we sought to define the biological and clinical consequences of enhanced activin A expression in human neuroblastomas.

Activin A is already present in the early embryo where it participates, in mesoderm induction (Altaba and Melton, 1989). During later development, it contributes to various inductive events generally leading to differentiation and a concomitant loss of proliferation (Mather et al., 1997). Here, we have demonstrated that activin A inhibits proliferation of human neuroblastoma cells. Our findings appear to be in contrast to those of other groups who have demonstrated that activin A supports proliferation and inhibits differentiation of non-transformed neuronal cells (Iwahori et al., 1997; Wu et al., 1999). These apparent discrepancies may be explained by the different embryonic origin of the cells and their differentiation status. That activin A inhibits neuroblastoma proliferation has been confirmed recently by another group (Cinatl et al., 2002). It is possible that this occurs by suppression of an autocrine IGF-II-involving pathway. Alternatively, activin A may mediate these effects indirectly through molecules implicated in the modulation of cell proliferation and differentiation. This possibility is supported by activin A-induced up-regulation of the homeobox or homeobox-related molecules Hox5.4 or LMO4. Both molecules are implicated in cell and tissue differentiation (Giusti et al., 2000; Hermanson et al., 1999; Visvader et al., 2001). For example, LMO4 participates in the differentiation of progenitor cells of motoneurons, of the cranial neural crest and of Schwann cells and induces regression of mammary tissue (Hermanson et al., 1999; Visvader et al., 2001). That activin A suppresses neuroblastoma growth and angiogenesis is supported by the clinical data presented. Here, we have demonstrated for the first time that high expression of the activin A-encoding gene inhibin βA is associated with favourable outcome of neuroblastoma patients and vice versa. This behaviour is found in, but not exclusive to, *MYCN*-amplified neuroblastomas

suggesting that factors other than MYCN regulate inhibin βA and / or activin A expression. In fact, this assumption is supported by various recent studies (Andreasson and Worley, 1995; Cinatl et al., 2002; Hübner & Werner, 1996; Tuuri et al., 1996; Woodruff et al., 1997). Clinical and biological data support the existence of three groups of patients with neuroblastomas (see Introduction and reference (Brodeur, 2003). Whereas the 5-year survival rates of patients classified into groups 1 and 3 can be accurately estimated, those of stage 2 patients vary widely, i.e., between 25 and 50%. The data presented here demonstrate that inhibin βA expression may serve as a novel molecular marker, which can accurately specify survival probabilities within group 2 patients. Thereby, inhibin βA determination may be helpful for therapeutic decisions.

N-*myc* Oncogene Overexpression Down-Regulates IL-6

We have previously shown that neuroblastoma cell supernatants contained three EC proliferation inhibitors (SI.1, SI.2 and SI.3), the expression of which was dramatically down-regulated upon N-Myc overexpression. In the present study, SI.2 activity was identified as IL-6 on the basis of mass fingerprinting analysis of tryptic peptides. Moreover, using reporter assays, we conclusively show, for the first time, that N-Myc overexpression dramatically down-regulates IL-6 promoter transcription. Though several genes have been shown to be regulated by the *myc* gene family members using various assays including crosslinking and cDNA microarrays, the IL-6 gene has never been included in these lists. Initial transfection experiments with deletion mutants of the Il-6 promoter revealed that the smallest construct (-140/+12) was still inhibited by N-Myc. This construct contained several characterized binding sites such as AP-1, NF-κB, RCE and GRE, however, it is not known whether one or more of these elements or additional DNA sequences are invloved in IL-6 promoter repression by N-Myc. Indirect repression of IL-6 gene via some of the known N-Myc/Max regulated genes is excluded, as neither the HLH-Zip nor the BR deletion released the transcriptional repression by N-Myc. Interestingly, simultaneous deletion of both BR and HLH-Zip domains abolished IL-6 promoter suppression. Perhaps, a contiguous BR-HLH-Zip domain is the binding site of regulatory proteins such as Ying Yang-1 (YY-1), and the presence of either BR or HLH-Zip domains alone is sufficient for such binding. Alternatively, the BR and HLH-Zip domains are required as part of two independent complexes that are both necessary for the repressive effect of N-Myc on IL-6 promoter.

Direct effect of N-Myc on the Inr element of the IL-6 promoter is unlikely as combined deletions of the MBI, MBII and HLH domains appeared to only minimally release the N-Myc repression. However, a significant contribution was assigned to the central area of N-Myc between amino acids 134-300 which encompasses the interaction domain with Nmi (aa 177 to 298). Nmi is a predominantly cytoplasmic protein that can be translocated to the nucleus where it augments STAT-dependent transcription in response to cytokines by interacting with STATs (except STAT 2) and enhancing association of CBP/p300 to STATs. It is tempting to speculate that recruitment of N-Myc via Nmi to STAT or other transcriptional complexes or competition of N-Myc for Nmi might influence transcription. The JAK-STAT pathway, activated by the IL-6R/gp130 receptors, plays a central role in IL-6 signalling. Concerning cell proliferation, activation of STAT3 can either induce cell proliferation via cyclin D1 overexpression inducing cell proliferation or cause growth arrest by enhancing expression of p21 or p27 cell cycle inhibitors. Indeed, we have also observed that proliferation of adrenal cortex (ACE) ECs were not affected by rhIL-6. In all cases, dominant negative mutants of STAT3 reverse the effects on proliferatio, indicating that the STAT3 pathway is the target of extensive crosstalk with other pathways. In the present study, infection of BBCE cells with adenoviruses expressing negative mutant STAT3 (Y705F) effectively reversed the inhibitory effect of IL-6 on bFGF and VEGF-induced proliferation. This result indicates an important role for STAT3 in modulating angiogenic responses of ECs. Some recent studies have shown that both bFGF and

VEGF can induce STAT3 phosphorylation and this is an excellent point for crosstalk. However, we were unable to observe phosphorylation of STAT3 from either angiogenic factor. Perhaps, cyclical, recurrent or delayed phosphorylation as previously reported for bFGF phosphorylation of STAT3 might have been the reason.

Inhibition of VEGF-induced neovascularization in the rabbit corneal assay reserved a truly *in vivo* antiangiogenic function for this cytokine. Previous studies have suggested a stimulatory role of IL-6 on angiogenesis and vasculogenesis judging from the expression pattern of the cytokine in ovarian follicles, maternal decidua and murine brain. Further xenograft tumorigenesis assays in mice, confirmed a specific antiangiogenic effect of IL-6 that was actually translated into reduced tumor growth rates. Indeed, though *in vitro* proliferation of IL-6-expressing WAC2 clones was not substantially different from that of the parental and control WAC2 clones, their xenograft tumor growth rate was decreased and correlated with a statistically significant reduction in the number of new capillaries. Previous overexpression of IL-6 cDNA in Lewis lung carcinoma cells did not alter the growth rate of syngeneic tumors suggesting that, indeed, IL-6 might play an important role in the balance of angiomodulators in the vicinity of N-*myc* amplified neuroblastomas. Taken together our results indicate that i) IL-6 is an angiogenesis inhibitor and ii) amplification of N-*myc* in neuroblastomas, in addition to effecting cell proliferation, could potentially enhance malignant phenotype progression by activating the angiogenic switch via down-regulation of IL-6 expression. Thus, contrary to other cancers, where STAT3 needs to be constitutively activated for transformation and blockage of apoptosis, in N-*myc* amplified neuroblastomas, silencing of STAT3 (via down-regulation of IL-6) might be required for activation of angiogenesis defining, perhaps, a novel, tumor-selective angiogenesis pathway. Alteration of the balance of stimulators and inhibitors of angiogenesis seem to participate also in the malignant transformation repertoire of other members of the Myc family. Indeed, c-Myc has been shown to down-regulate the promoter of thrombospondin-1, a strong angiogenesis inhibitor**.** These results await confirmation in primary neuroblastoma tumors.

REFERENCES

Altaba ARI, Melton DA: Interaction between peptide growth factors and homoeobox genes in the establishment of antero-posterior polarity in frog embryos. *Nature* 341: 33-38, 1989.

Andreasson K, Worley PF: Induction of beta-A activin expression by synaptic activity and during neocortical development. *Neuroscience* 69: 781-796, 1995.

Breit S, Ashman K, Wilting J, Rössler J, Hatzi E, Fotsis T, Schweigerer L: The N-myc oncogene in human neuroblastoma cells: down-regulation of an angiogenesis inhibitor identified as activin A. *Cancer Res.* 60: 4596-4601, 2000.

Breit S, Rössler J, Fotsis T, Schweigerer J: N-myc down-regulates activin A. *Biochem. Biophys. Res. Commun*. 274: 405-409, 2000.

Brodeur GM: Neuroblastoma: biological insights into a clinical enigma. *Nat Rev Cancer* 3: 203-16, 2003.

Brodeur GM, Pritchard J, Berthold F, Carlsen NLT, Castel V, Castleberry RP, De Bernardi B, Evans AE, Favrot M, Hedborg F, Kaneko M, Kemshead J, Lampert F, Lee REJ, Look AT, Pearson ADJ, Philip T, Roald B, Sawada T, Seeger RC, Tsuchida Y, Voute PA: Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment *J.Clin.Oncol.* 11: 1466-1477, 1993.

Cantero R, Torres AJ, Maestro M, Fernandez C, Hernando F, del Barco V, Sanz T, Balibrea JL: Use of possible synergistic expression of p53 and p185 as a prognostic tool for stage I non-small-cell lung cancer. *World J Surg* 23: 1294-9; discussion 1299-300, 1999.

Chen YG, Lui HM, Lin SL, Lee JM, Ying SY: Regulation of cell proliferation, apoptosis, and carcinogenesis by activin. *Exp Biol Med (Maywood)* 227: 75-87, 2002.

Chomczynski P: A reagent for the single-step simultaneous isolation of RNA, DNA and proteins from cell and tissue samples. *Biotechniques* 15: 532-4, 536-7, 1993.

Cinatl J Jr, Kotchetkov R, Blaheta R, Driever PH, Vogel JU, Cinatl J: Induction of differentiation and suppression of malignant phenotype of human neuroblastoma BE(2)-C cells by valproic acid: enhancement by combination with interferon-alpha. *Int J Oncol* 20: 97-106, 2002.

Fotsis T, Breit S, Lutz W, Rössler J, Hatzi E, Schwab M, Schweigerer L: Down-regulation of endothelial cell growth inhibitors by enhanced MYCN oncogene expression in human neuroblastoma cells. *Eur.J.Biochem.* 263: 757-764, 1999.

Fotsis T, Pepper MS, Aktas E, Breit S, Rasku S, Adlercreutz H, Wähälä K, Montesano R, Schweigerer L: Flavonoids, dietary-derived inhibitors of cell proliferation and in vitro angiogenesis. *Cancer Res.* 57: 2916-2921, 1997.

Giusti AF, Hinman VF, Degnan SM, Degnan BM, Morse DE: Expression of a Scr/Hox5 gene in the larval central nervous system of the gastropod Haliotis, a non-segmented spiralian lophotrochozoan. *Evol Dev* 2: 294-302, 2000.

Gong H, Pöttgen C, Stüben G, Havers W, Stuschke M, Schweigerer L: Arginine deiminase and other antiangiogenic agents inhibit unfavourable neuroblastoma growth: potentiation by irradiation. *Int. J. Cancer* 106: 391-394, 2003.

Hatzi E, Murphy C, Zoephel A, Rasmussen H, Morbidelli L, Ahorn H, Kunisada K, Tontsch U, Klenk M, Yamauchi-Takihara K, Ziche M, Rofstad EK, Schweigerer L*, Fotsis T*: N-myc oncogene overexpression down-regulates IL-6; evidence that IL-6 inhibits angiogenesis and suppresses neuroblastoma tumor growth. *Oncogene* 21: 3552-3561, 2002 (*equal contribution).

Hatzi E, Murphy C, Zoephel A, Ahorn H Tontsch U, Bamberger AM, Yamauchi-Takihara K, Schweigerer L, Fotsis T: N-myc overexpression down-regulates leukemia inhibitory factor in neuroblastoma. *Eur. J. Biochem.* 269: 3732-3741, 2002.

Hermanson O, Sugihara TM, Andersen B: Expression of LMO-4 in the central nervous system of the embryonic and adult mouse. *Cell Mol Biol (Noisy-le-grand)* 45: 677-86, 1999.

Hübner G, Werner S: Serum growth factors and proinflammatory cytokines are potent inducers of activin expression in cultured fibroblasts and keratinocytes. *Exp.Cell Res.* 228: 106-113, 1996.

Iwahori Y, Saito H, Torii K, Nishiyama N: Activin exerts a neurotrophic effect on cultured hippocampal neurons. *Brain Res* 760: 52-8, 1997.

Mather J.P, Moore A, Li RH: Activins, inhibins, and follistatins: further thoughts on a growing family of regulators. *Proc.Soc.Exp.Biol.Med.* 215: 209-222, 1997.

Pavlakovic H, Havers W, Schweigerer L: Multiple angiogenesis stimulators in a single malignancy. *Angiogenesis* 4: 259-262, 2001.

Rössler J, Breit S, Havers W, Schweigerer L: Vascular endothelial growth factor expression in human neuroblastoma: up-regulation by hypoxia. *Int.J.Cancer* 81: 113-117, 1999.

Schweigerer L, Breit S, Wenzel A, Tsunamoto K, Ludwig R, Schwab M: Augmented MYCN expression advances the malignant phenotype of human neuroblastoma cells: evidence for induction of autocrine growth factor activity. *Cancer Res.* 50: 4411-4416, 1990.

Schweigerer L, Neufeld G, Friedman J, Abraham J.A, Fiddes JC, Gospodarowicz D: Capillary endothelial cells express basic fibroblast growth factor, a mitogen that promotes their own growth. *Nature* 325: 257-259, 1987.

Tuuri T, Erämaa M, Van Schaik RHN, Ritvos O: Differential regulation of inhibin/activin alpha- and beta A-subunit and follistin mRNAs by cyclic AMP and phorbol ester in cultured human granulosa-luteal cells. *Mol.Cell.Endocrinol.* 121: 1-10, 1996.

Visvader JE, Venter D, Hahm K, Santamaria M, Sum EY, O'Reilly L, White D, Williams R, Armes J, Lindeman GJ: The LIM domain gene LMO4 inhibits differentiation of mammary epithelial cells in vitro and is overexpressed in breast cancer. *Proc Natl Acad Sci U S A* 98: 14452-7, 2001.

Wai DH, Schaefer KL, Schramm A, Korsching E, Van Valen F, Ozaki T, Boecker W, Schweigerer L, Dockhorn-Dworniczak B, Poremba C: Expression analysis of pediatric solid tumor cell lines using oligonucleotide microarrays. *Int J Oncol* 20: 441-51, 2002.

Westermann F, Schwab M: Genetic parameters of neuroblastomas. *Cancer Lett* 184: 127-47, 2002.

Woodruff TK, Sluss P, Wang E, Janssen I, Mersol-Barg MS: Activin A and follistatin are dynamically regulated during human pregnancy. *J.Endocrinol.* 152: 167-174, 1997.

Wu DD, Lai M, Hughes PE, Sirimanne E, Gluckman PD, Williams CE: Expression of the activin axis and neuronal rescue effects of recombinant activin A following hypoxic-ischemic brain injury in the infant rat. *Brain Res*. 835: 369-78, 1999.

Vascular differentiation

Endothelial cell differentiation and integrity of cell-cell-contact complexes during angiogenesis

Dr. Birgit Kräling
Dept. of Cell Biology
Deutsches Krebsforschungszentrum
Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Dr. Hjalmar Kurzen
Dept. of Dermatology
University Clinic Mannheim
University of Heidelberg
Theodor-Kutzer-Ufer 1-3, 68167 Mannheim, Germany

Member of the SPP 1069 from 1999 until 2001

This project was funded for only one funding period. The final report has been submitted to the DFG after conclusion of the project.

Modulation of Choroidal Neovascularization by Age-Related End Products (AGE) and Angiogenesis-Related Molecules

Prof. Dr. Peter Wiedemann

Dept. of Ophthalmology
University of Leipzig
Liebigstr. 10-14, 04103 Leipzig, Germany

Member of the SPP from 1999 until 2001

SUMMARY

Advanced glycation endproducts (AGE) have been implicated in the pathogenesis of choroidal neovascularization (CNV), a key process in exsudative age-related macular degeneration (AMD). We have exposed CNV-related cells, i.e., choroidal endothelial cells (CEC) and retinal pigment epithelial (RPE) cells to AGE. These experiments demonstrated that AGE upregulate vascular endothelial growth factor (VEGF) expression in CEC. Concomitantly, AGE increased proliferation, and invasion of CEC, and promote matrix metalloproteinase- (MMP-) 2 expression in CEC. Moreover, AGE were found to bind to RPE cells, thereby stimulating VEGF secretion and MMP-2 expression. However, AGE elicited only weak effects on RPE cellular proliferation and migration. We have also evaluated the pattern of ocular cell types that produce pigment epithelium-derived factor (PEDF), a potent angiostatic molecule. We have shown for the first time that retinal glial (Müller) cells are a major source of PEDF in the eye. These results suggest that AGE may play an important role as a stimulatory factor in CNV even though their effects may be counterbalanced by natural anti-angiogenic factors such as PEDF.

Furthermore, we have demonstrated the impact of VEGF on CEC-related nitric oxide (NO) production. Whereas unstimulated CEC continuously produced low levels of NO, VEGF stimulated increased NO generation. This effect may be important in the control of angiogenesis, and appropriate blockers of NO signaling may be implicated in efforts to reduce neovascularization in AMD.

In other studies we have determined the effects of therapeutically interesting compounds such as triamcinolone acetonide (TA) and endostatin on CNV-related cells *in vitro*. We could demonstrate that TA inhibits proliferation, migration and tube formation of basic fibroblast growth factor (bFGF)-stimulated CEC. Furthermore, the expression of activated MMP-2 on CEC was down-regulated. These data suggest that TA is a promising drug for the treatment of CNV. Other experiments demonstrated that that endostatin inhibits significantly bFGF-induced migration of CEC. Moreover, bFGF-induced MMP-2 mRNA expression in CEC and MMP-2 protein secretion by CEC were suppressed by endostatin.

We have also evaluated the efficacy of a cyclic RGD (Arg-Gly-Asp) peptide in a rat model of laser-induced CNV. Cyclic RGD inhibited adhesion of CEC in a dose-dependent manner without changing cell viability. In eyes treated with two injections of 100 or 200 µg of cyclic RGD peptide, development of CNV was significantly inhibited in the area of leakage as determined by fluorescein angiography. These experiments demonstrated that cyclic RGD peptides may effectively inhibit CNV progression suggesting that α_v-integrin antagonists may be beneficial in the treatment of CNV.

We have also developed a novel model of AMD in rabbits which involves drusen formation and choroidal neovascularization (CNV). Subretinal administration of crosslinked microspheres, which

had been produced through glycoxidation, resulted in age-related alterations such as accumulation of lipofuscin. The results obtained raise the possibility that choroidal vessels play a role in the clearance of deposits possibly in active communication with RPE, a process that may be associated with CNV formation.

INTRODUCTION

Age related macular degeneration (AMD) is the leading cause of irreversible blindness in patients aged 65 years or over. In our aging population this disease becomes more and more important (Ferris, 1983). AMD can be divided into two types, the non-exsudative and the exsudative form. The exsudative form, which is characterized by CNV, is less common, but its effects on vision are devastating. CNV denotes vessel formation under the macula, i.e., new vessels originating from the choriocapillaris invade the subretinal space under the macula and lie between the choroid and the RPE or between the RPE and the sensory retina (Amin *et al.*, 1994; Kliffen *et al.*, 1997). New vascular membranes are generated by the interaction of choroidal endothelial cells (CEC), RPE cells, macrophages, fibroblasts, and Müller cells (Ishibashi *et al.*, 1997). However, the exact pathogenic mechanisms leading to the induction of CNV, especially through secretion of proangiogenic cytokines and the activity of matrix metalloproteinases, are not known. Advanced glycation end products (AGE) were proposed to contribute to the pathogenesis of CNV, since they accumulate during aging, largely in the choriocapillaris and Bruch's membrane (Handa 1998). AGE are highly expressed in CNV membranes (Hammes *et al.*, 1991; Ishibashi et al, 1991). Notably, AGE were known to possess angiogenesis-promoting abilities in diabetic retinal neovascularization (Ishibashi 2000). AGE promote neovascularization in diabetes mellitus and they bind to two cell surface proteins, i.e., the receptor for AGE (RAGE) or to lactoferrin-like peptide. Binding to these receptors results in the upregulation of angiogenic cytokines modulating cellular proliferation, enhanced extracellular matrix accumulation, and hemodynamic permeability (Schmidt *et al.*, 1994a; Schmidt *et al.*, 1994b).

Prior work has demonstrated that exposure of CNV-related cells to AGE may induce the expression of cytokines, for example, TNF-α (Vlassara *et al.*, 1988) and PDGF-BB (Handa, 1998). AGE are also able to induce upregulation of extracellular matrix molecules in RPE cells (Doi, 1992). The cytokines, VEGF, PDGF, aFGF, bFGF and TGF-β, are expressed in CNV membranes (Kliffen *et al.*, 1997; Amin *et al.,* 1994). Immunohistochemical studies showed an increased expression of VEGF, TGF-β, aFGF and bFGF in RPE cells of maculae with AMD (Ishibashi *et al.,* 1997; Amin *et al.,* 1994). CEC located close to the RPE layer in choroidal neovascular membranes are immunopositive for bFGF and the FGF receptor (Amin *et al.*, 1994). Müller cells, located in the outer nuclear layer express VEGF and PDGF (Ishibashi *et al.,* 1997; Kliffen *et al.,* 1997). The contribution of Müller cells to the pathogenesis of CNV had been neglected in the past. However, the enhanced expression of VEGF and PDGF in CNV membranes forces the assumption that Müller cells are of importance for progression of neovascular AMD. The mechanisms leading to increased expression of the angiogenic cytokines, VEGF, PDGF-BB, bFGF, TGF-β, TNF-α and HGF, as well as modulate the activity of matrix metalloproteinases in context with neovascular AMD are not defined. In this study the possibility was considered that AGE have a significant impact on the process of CNV by upregulating pro-angiogenic cytokines and by modulating the activity of matrix metalloproteinases.

METHODS

Isolation and culture of ocular microvascvular cells

Microscopic techniques (confocal microscope, immunohistochemistry)

Analyses of endothelial cell proliferation, migration, and tube formation

ELISA techniques

Expression analyses: Real-time PCR, Western Blotting

Transfection

Amperometric NO measurement

Analysis of MMP activity by zymography

Animal model of laser-induced CNV

RESULTS

Advanced glycation end products induce choroidal endothelial cell proliferation, matrix metalloproteinase-2 and VEGF upregulation *in vitro* (Hoffmann *et al.*, 2002)

To determine whether AGE have an impact on endothelial cell growth, CEC were cultured in the presence of AGE (concentration range 50-100 µg/ml). These experiments revealed that AGE may increase the proliferation of CEC significantly when compared to the non-glycated BSA control (Fig. 1a). We observed increasing cellular proliferation of 46.1% ($P<0.05$) by using 100 µg/ml AGE. After application of 50 µg/ml AGE, a 41% ($P<0.05$) increase in CEC proliferation was observed. Otherwise, CEC stimulation by the cytokines, VEGF (10 ng/ml) and HGF (10 ng/ml), revealed stronger effects on CEC proliferation than stimulation with non-glycated BSA and AGE. VEGF and HGF each induced the strongest increase in CEC proliferation (56%, $P<0.05$).

We have also investigated the effect of AGE on RPE and Müller cells. In a concentration range covering 10 µg/ml to 100 µg/ml AGE, there was no effect on the proliferative response of RPE cells. The cytokine, bFGF (10 ng/ml), used as a positive control, revealed stronger effects on RPE cell proliferation than AGE (Fig. 1b). Moreover, no proliferative response of Müller cells was seen after AGE exposure in the indicated concentration range (Fig. 1c).

We have performed quantitative RT-PCR analyses to determine whether AGE (10, 50, 100 µg/ml) stimulate VEGF mRNA expression in CEC. These experiments demonstrated that AGE may upregulate VEGF expression in CEC. AGE induced the strongest VEGF expression at a concentration of 50 µg/ml. In addition, 100 µg/ml AGE was able to upregulate VEGF, but not as strong as by using 50 µg/ml AGE (Fig. 2).

In further experiments (*results not shown*) zymography was used to determine the expression level of the 72-kDa matrix metalloproteinase, MMP-2, in AGE-stimulated CEC. These experiments demonstrated a dose-dependent MMP-2 increase after AGE exposure to CEC in a concentration range covering 20 to 100 µg/ml. Comparison of these samples with 4-aminophenylmercuric acetate-preincubated samples revealed the expression of MMP-2 as an inactive proenzyme. Densitometry of the zymograms disclosed that AGE at a concentration of 100 µg/ml induce a 237% increase of MMP-2, 50 µg/ml an increase of 187% and 20 µg/ml an increase of 133%. In addition, quantitative PCR confirmed the upregulation of MMP-2 in CEC and confirmed the zymography results on mRNA level. These results suggest that AGE may play an important role as a stimulatory factor in CNV.

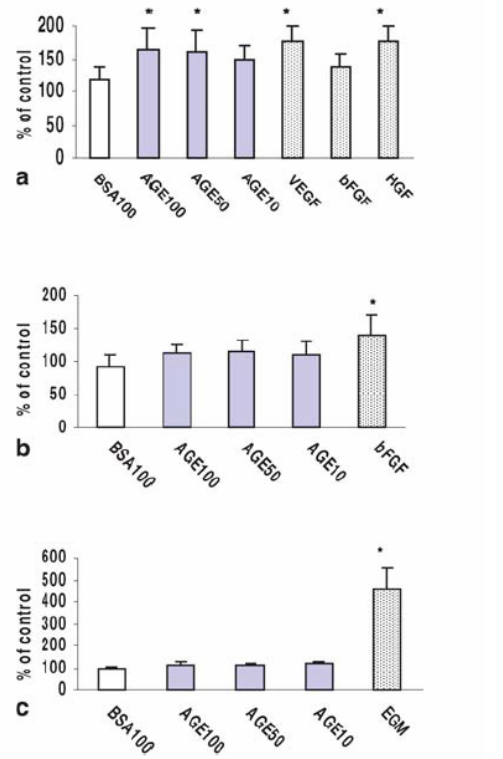


Fig. 1: Proliferative responses of (**a)** CEC, (**b)** RPE cells and (**c)** Müller cells after exposure to AGE (10-100 µg/ml). Proliferation was determined by a MMT assay (* P<0.05). Results were demonstrated as proliferation in percent of control. Medium without AGE was used as a control. In the proliferation assay, the cytokines bFGF, VEGF and HGF (10 ng/ml) were used as positive controls for CEC, bFGF (10 ng/ml) as a positive control for RPE cells and EGM medium with 20% FBS as a positive control for Müller cells.

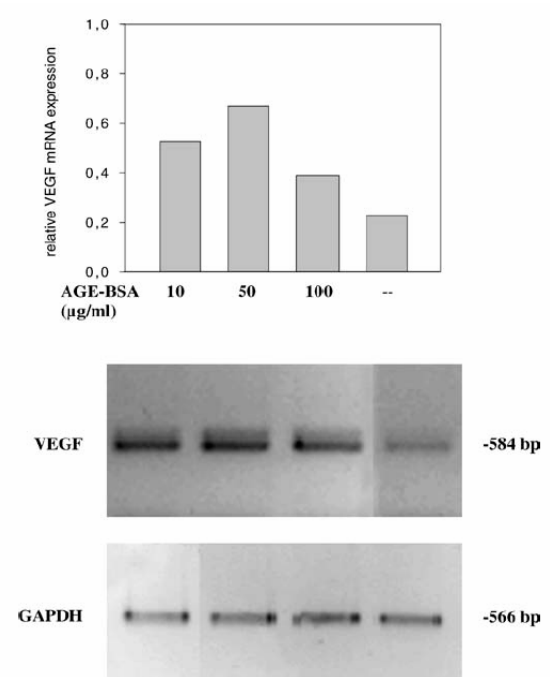


Fig. 2: CEC were incubated with BSA (-) or stimulated with AGE (10, 50 and 100 µg/ml) for 36 h. After this time period VEGF mRNA expression was determined by RT-PCR. Representative data out of three experiments with similar results are shown. The cDNA samples were adjusted to equal GAPDH inputs before use.

Müller cells express and secrete PEDF (Eichler *et al.*, 2004)

To explore the possibility that retinal glial (Müller) cells express PEDF, immunofluorescence studies were performed. Two-color stained cryosections of retinae revealed that the majority of vimentin-positive cells express PEDF (Figs. 3A and B). Moreover, Müller cells freshly isolated from human retinal tissue (Fig. 3C), a Müller cell line (Fig. 3D), or guinea pig Müller cells (Fig. 3E) were also found to express PEDF. PEDF expression in Müller cells was additionally demonstrated on the mRNA level (data not shown). To demonstrate release of PEDF from retinal cells, PEDF was identified in supernatants of cultured Müller cells using Western Blotting (Fig. 3F).

VEGF induces nitric oxide (NO) production by CEC (Uhlmann *et al.*, 2002)

To investigate whether AGE exposure modulates NO production in CEC, an amperometric NO sensor was used. We demonstrated that VEGF (1, 10, and 100 ng/ml) causes a dose-dependent NO production by CEC (Fig. 4). CEC were also preincubated with the competitive inhibitor of NO synthase, N_G-nitro-L-arginine methyl ester (L-NAME; 1 mM) for 2 h before VEGF (10 ng/ml) stimulation. This caused a significant reduction in peak NO responses.

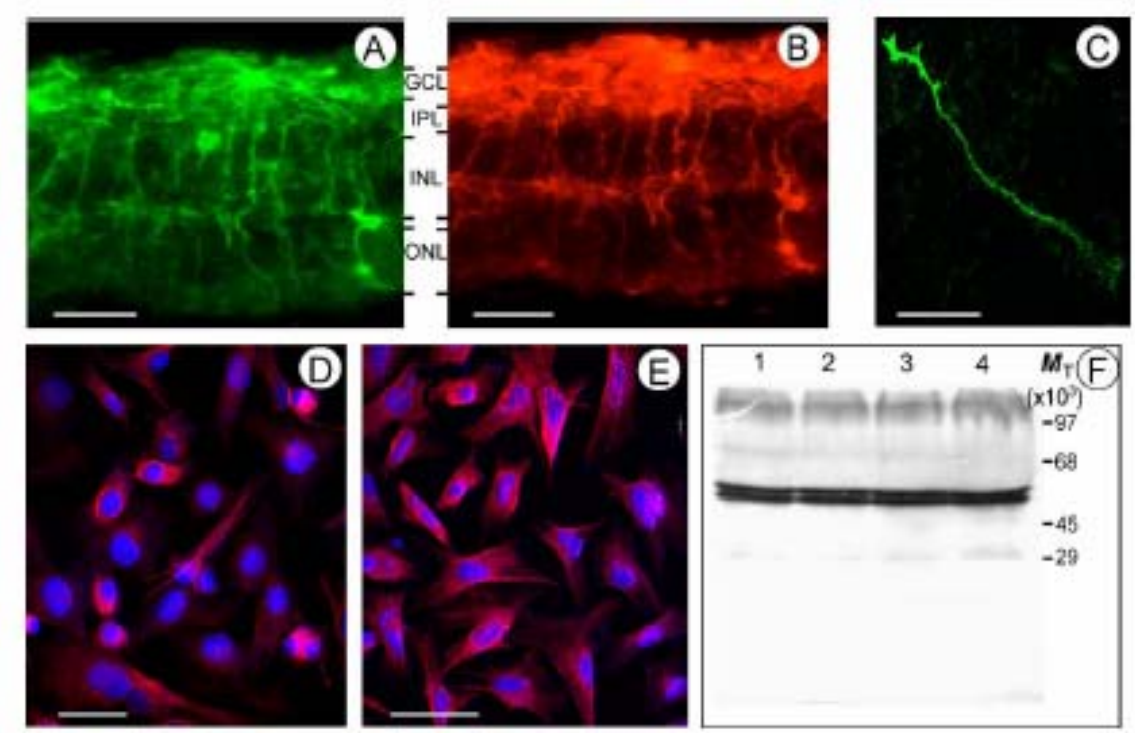


Fig. 3: Demonstration of PEDF expression in Müller cells using immunofluores- cence labeling of retinae and isolated Müller cells (A–E) or Western blotting (F). Immunofluorescence double-labeling of human retinae revealed co-localization of PEDF (A, green) and vimentin (B, red). GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; ONL, outer nuclear layer. PEDF was also detected in freshly dissociated human Müller cells (C, green staining) and in cultured human MIO-M1 (D, red staining) and guinea pig Müller cells (E, red staining). In Western blot experiments (F), the release of PEDF into the culture media of guinea pig Müller cells (lane 1) and a Müller cell line (MIO-M1) (lane 2) was detected. For reference, human vitreous (lane 3) and RPE-cell-conditioned media (lane 4) were used as a positive standard control. The molecular weight of SDS-PAGE standards is indicated on the right. Scale bars: 25 µm (A and B), 50 µm (C–E).

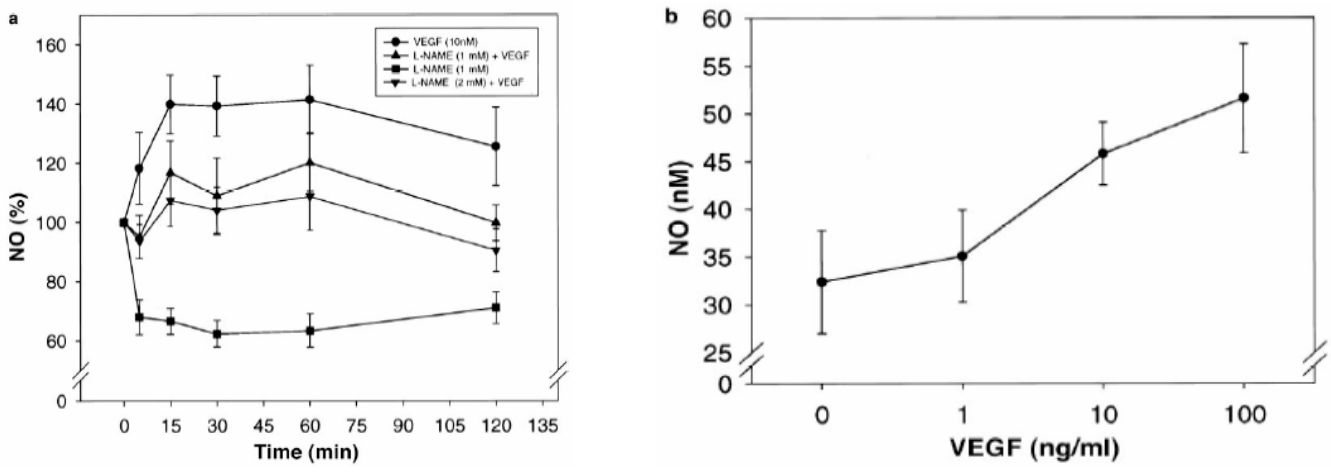


Fig. 4: (a) VEGF (10 ng/ml) causes a significant increase in NO release after 60 min of stimulation of CEC. Preincubation of CEC with L-NAME (1 and 2 mM) causes a reduction in peak NO responses to VEGF. **(b)** Dose dependence of the effect of different concentrations of VEGF on NO production after 30 min of stimulation of CEC.

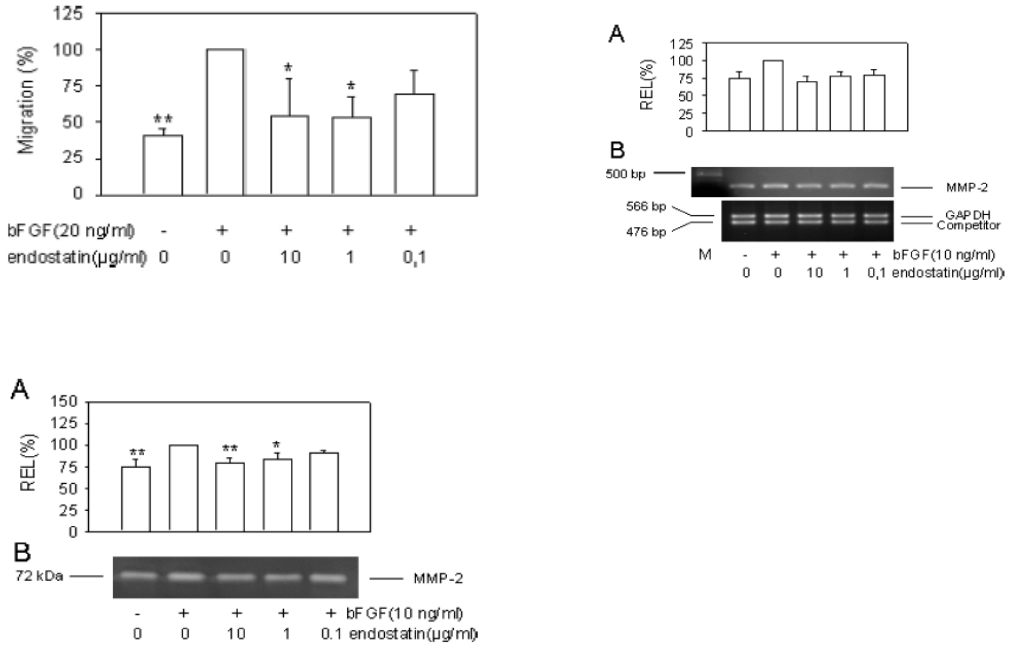
Endostatin inhibits bFGF-induced migration of CEC and suppresses MMP-2 expression in CEC (Wang et al., 2002)

To investigate the potential role of endostatin, an endogenous angiogenesis inhibitor, in the prevention of choroidal angiogenesis-related disorders we treated CEC with basic fibroblast growth factor (bFGF) alone or combined with endostatin. Stimulation of CEC with 20 ng/ml bFGF resulted in a significant ($p<0.01$) increase in CEC migration activity compared to basal levels. However, bFGF-induced migration was attenuated by preincubation of CEC for 20 min with endostatin at 1 and 10 μg/ml ($p < 0.05$) (Fig. 5, upper left panel).

To evaluate the effect of endostatin on MMP-2 expression, total RNA was isolated from CEC and semi-quantitative RT-PCR was performed. The expression of MMP-2 mRNA was obviously enhanced by 10 ng/ml bFGF, and this increase was suppressed by endostatin at concentrations ranging from 0.1 to 10 μg/ml (Fig. 5, lower left panel). Gelatin zymography of the CEC-conditioned medium was performed to evaluate protein levels of MMP-2. These experiments revealed a 72-kDa pro-MMP-2 band in basal conditions (Fig. 5, right panel). The intensity of the MMP-2 band was significantly increased by 10 ng/ml bFGF, and this increase was dose-dependently inhibited by endostatin at concentrations ranging from 0.1 to 10 μg/ml. Conditioned media were further subjected to Western blot analysis. As shown in Figure 8, the 72-kDa MMP-2 band in the bFGF+/endostatin-free group was strong, while it became faint in the endostatin-treated groups.

Inhibition of Experimental Choroidal Neovascularization in Rats by an α_v-Integrin Antagonist (Yasukawa *et al.,* 2004)

Since the integrin $\alpha_v\beta_3$ is predominantly expressed on activated endothelial cells in CNV we have evaluated the efficacy of cyclic RGD (Arg-Gly-Asp) peptide, a putative α_v-integrin antagonist, in a rat model of laser-induced CNV. By determining fluorescein leakage from the CNV lesions by fluorescein angiography, we demonstrated that neovascularization was reduced in a significant dose-dependent manner in eyes treated with 100 and 200 µg of cyclic RGD; in the eyes treated with vehicle, the leakage tended to remain unchanged or to increase in area ($P<0.01$) (Fig. 6, *left panel*).



Fíg. 5: Upper left panel: Endostatin inhibits bFGF-induced migration of CEC. Values are mean ± standard deviation of four independent experiments performed in triplicate, and shown as the percentage (%) of the bFGF+/endostatin-free group. **Right panel:** Effect of endostatin on expression of MMP-2 mRNA in CEC: RT-PCR analysis of dose effects. (A) Relative expression levels (REL) of MMP-2 mRNA in CEC treated for 3 days with 10 ng/ml bFGF alone or combined with various concentrations of endostatin are shown. Values are mean ± standard deviation of three amplification reactions performed on a single cDNA sample and shown as the percentage (%) of the bFGF+/endostatin-free group. (B) Representative semiquantitative RT-PCR of MMP-2. Expression of GAPDH housekeeping gene and its competitor were used to verify adjustment of samples to equal cDNA amounts. **Lower left panel:** Effect of endostatin on MMP-2 secretion from CEC. Gelatin zymography analysis of dose effect. (A) Relative expression levels (REL) of MMP-2 protein in CEC-conditioned media are shown. CEC were treated for 3 days with 10 ng/ml bFGF alone or combined with various concentrations of endostatin. Values are mean ± standard deviation of three independent experiments and shown as the percentage (%) of the bFGF+/endostatin-free group. (B) Representative gelatin zymogram of MMP-2. The bands at 72 kDa correspond to pro-MMP-2.

Moreover, the eyes were also observed histologically. The neovascular membranes in the control eyes were in the subretinal space and did not extend into the retina and included numerous new vessels. The lesions in the eyes treated with cyclic RGD showed a statistically significant reduction in thickness (P<0.01) (Fig. 6, *right panel*).

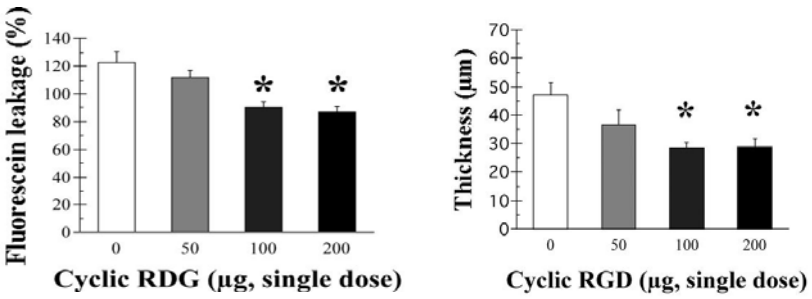


Fig. 6: Left panel: Cyclic RGD significantly reduced the area of fluorescein leakage from CNV compared with the group not treated with cyclic RGD. Change of fluorescein leakage areas after treatment compared to that before treatment. Values are means ± SEM (ANOVA**, ***P*<0.01). **Right panel:** Effect of cyclic RGD on the thickness of the CNV lesions 2 weeks after treatment. Each column shows means ± SEM of measurements. The lesions in eyes treated with cyclic RGD are significantly thinner than those in control eyes (ANOVA, P<0.01).

DISCUSSION

So far the pathophysiology of CNV formation in exsudative AMD is not understood. However, it is well established that pro-angiogenic cytokines and MMPs contribute significantly to the process of CNV formation. We do not know in detail what leads to upregulation of these factors, to CNV-associated cell proliferation, migration and, consequently, to CNV progression. In the present study we have demonstrated that AGE may induce VEGF upregulation and MMP-2 secretion by CEC and enhance CEC proliferation *in vitro*. Hence, AGE formation may be an important mechanism playing a role in CNV progression in exsudative AMD *in vivo*, by enhancement of substeps of angiogenesis. The stimulation of CEC proliferation in our study may be explained by direct effects of AGE on cell signaling of CEC and/or by upregulation of angiogenic cytokines. Thus upregulation of VEGF secretion resulting from exposure to AGE may explain the proliferative response of CEC.

It should be stressed that anti-angiogenic molecules play a major role in regulating VEGF-induced migration and growth of ocular endothelial cells (Eichler *et al.*, 2001; Bouck, 2002; Duh *et al.*, 2002). A major anti-angiogenic candidate molecule is PEDF. This protein occurs natively in the eye where it counteracts the stimulatory activity of inducers of angiogenesis, thus preventing ocular neovascularization under normal conditions (Bouck, 2002). PEDF levels in the vitreous were repeatedly found to be decreased in angiogenic eye diseases (Duh *et al.*, 2002; Gao *et al.*, 2001; Holekamp *et al.*, 2002). We have demonstrate for the first time that Müller cells produce and secrete significant amounts of PEDF. We have further shown that the decreased PEDF release from Müller cells under hypoxia is associated with downregulated PEDF mRNA levels, suggesting a hypoxia-dependent regulation of PEDF expression at the level of transcription and/or mRNA stability. Thus, a precise balance between stimulators and inhibitors of angiogenesis, such as VEGF and PEDF, respectively, is essential for angiogenic homeostasis in ocular tissues.

To our knowledge, our data describe for the first time enhanced MMP-2 secretion of CEC after AGE exposure. MMPs are involved in cell migration, in disruption of the Bruch' s membrane in exsudative AMD, and in tube formation. In this context MMP-2 is of special importance (Kvanta *et al.*, 2000). VEGF upregulates MMP-2 expression in endothelial cells (Chae *et al.*, 2000) and is therefore one feasible candidate to explain AGE-induced MMP-2 upregulation by CEC.

Since AGE promote CEC proliferation, the upregulation of VEGF and MMP-2 in vitro, accumulating glycated biomolecules may be of special importance for initiation and progression of CNV in exsudative AMD. Additional *in-vivo* studies are necessary to explore the significance of AGE for initiation and progression of exsudative AMD and to develop new therapeutic strategies that may inhibit CNV.

REFERENCES

Amin R, Puklin JE, Frank RN: Growth factor localization in choroidal neovascular membranes of age-related macular degeneration. *Invest Ophthalmol Vis Sci.* 35: 3178–3188, 1994.

Bouck, N: PEDF: anti-angiogenic guardian of ocular function. *Trends Mol Med.* 8: 330–334, 2002.

Chae JK, Kim I, Lim ST, Chung MJ, Kim WH, Kim HG, Ko JK, Koh GY: Coadministration of angiopoietin-1 and vascular endothelial growth factor enhances collateral vascularization. *Arterioscler Thromb Vasc Biol* 20: 2573-2578, 2000.

Doi T, Vlassara H, Kirstein M, Yamada Y, Striker GE, Striker LJ: Receptor-specific increase in extracellular matrix production in mouse mesangial cells by advanced glycosylation end products is mediated via platelet-derived growth factor. *Proc Natl Acad Sci USA* 89: 2873-2877, 1992.

Duh EJ, Yang, HS, Suzuma I, Miyagi M, Youngman E, Katai K, Katai M, Yan L, Suzuma K, West K, Davarya S, Tong P, Gehlbach P, Pearlman J, Crabb JW, Aiello LP, Zack PA, Zack DJ: Pigment epithelium-derived factor

suppresses ischemia-induced retinal neovascularization and VEGF-induced migration and growth, *Invest Ophthalmol Vis Sci* 43: 821–829, 2002.

Duh EJ, Yang HS, Haller JA, De Juan E, Humayun MS, Gehlbach P, Melia M, Pieramici D, Harlan JB, Campochiaro PA, Zack DJ: Vitreous levels of pigment epithelium-derived factor and vascular endothelial growth factor: implications for ocular angiogenesis. *Am J Ophthalmol* 137: 668–674, 2004.

Eichler W, Friedrichs U, Thies A, Tratz C, Wiedemann P: Modulation of matrix metalloproteinase and TIMP-1 expression by cytokines in human RPE cells. *Invest Ophthalmol Vis Sci* 43: 2767-2773, 2002

Eichler W, Yafai Y, Kuhrt H, Gräter R, Hoffmann S, Wiedemann P, Reichenbach A: Hypoxia: modulation of endothelial cell proliferation by soluble factors released by retinal cells, *Neuroreport* 12: 4103–4108, 2001.

Eichler W, Kuhrt H, Hoffmann S, Wiedemann P, Reichenbach A: VEGF release by retinal glia depends on both oxygen and glucose supply. *Neuroreport* 11: 3533-357, 2000.

Eichler W, Yafai Y, Keller T, Wiedemann P, Reichenbach A: PEDF derived from glial Müller cells: a possible regulator of retinal angiogenesis. *Exp Cell Res* 299: 68-78, 2004.

Ferris FLD: Senile macular degeneration: review of epidemiologic features. *Am J Epidemiol* 118: 132–151, 1983.

Gao G, Li Y, Zhang D, Gee S, Crosson C, Ma J: Unbalanced expression of VEGF and PEDF in ischemia-induced retinal neovascularization, *FEBS Lett* 489: 270–276, 2001.

Holekamp NM, Bouck NP, Volpert OV: Pigment epithelium-derived factor is deficient in the vitreous of patients with choroidal neovascularization due to age-related macular degeneration. *Am J Ophthalmol* 134: 220–227, 2002.

Hammes HP, Hoerauf H, Alt A, Schleicher E, Clausen JT, Bretzel RG, Laqua H: N(epsilon)(carboxymethyl) lysin and the AGE receptor RAGE colocalize in age-related macular degeneration. *Invest Ophthalmol Vis Sci* 40: 1855–1859, 1999.

Handa JT: Immunohistochemical evidence for deposition of advanced glycation endproducts (AGEs) in Bruch's membrane and choroid with age. *Invest Ophthalmol Vis Sci* 39: 1725–B1606, 1998.

Hoffmann S, He S, Wiedemann P: Carboxyamido-triazole inhibits substeps of choroidal neovascularization on retinal pigment epithelial cells and choroidal endothelial cells in vitro *Ophthalmologe* 101: 993-997, 2004.

Hoffmann S, He S, Jin ML, Masiero L, Wiedemann P, Ryan SJ, Kohn EC: Carboxyamido-triazole modulates retinal pigment epithelial and choroidal endothelial cell attachment, migration, proliferation, and MMP-2 secretion of choroidal endothelial cells. *Curr Eye Res* 30: 103-113, 2005.

Hoffmann S, Friedrichs U, Eichler W, Rosenthal A, Wiedemann P: Advanced glycation end products induce choroidal endothelial cell proliferation, matrix metalloproteinase-2 and VEGF upregulation in vitro. *Graefe`s Arch Clin Exp Ophthalmol* 240: 996-1002, 2002.

Ishibashi T, Hata Y, Yoshikawa H, Nakagawa K, Sueishi K, Inomata H: Expression of vascular endothelial growth factor in experimental choroidal neovascularization. *Graefe`s Arch Clin Exp Ophthalmol* 235: 159–167, 1997.

Ishibashi T, Murata T, Hangai M, Nagai R, Horiuchi S, Lopez PF, Hinton DR, Ryan SJ: Advanced glycation end products in age-related macular degeneration. *Arch Ophthalmol* 116: 1629–1632, 1998.

Ishibashi T: Cell biology of intraocular vascular diseases. *Jpn J Ophthalmol* 44: 323–324, 2000.

Kliffen M, Sharma HS, Mooy CM, Kerkvliet S, de Jong PT: Increased expression of angiogenic growth factors in age-related maculopathy. *Br J Ophthalmol* 81: 154–162, 1997.

Kvanta A, Shen WY, Sarman S, Seregard S, Steen B, Rakoczy E: Matrix metalloproteinase (MMP) expression in experimental choroidal neovascularization. *Curr Eye Res* 21: 684–690, 2000.

Schmidt AM, Hasu M, Popov D, Zhang JH, Chen J, Yan SD, Brett J, Cao R, Kuwabara K, Costache G: Receptor for advanced glycation end products (AGEs) has a central role in vessel wall interactions and gene activation in response to circulating AGE proteins. *Proc Natl Acad Sci USA* 91: 8807–8811, 1994a.

Schmidt AM, Mora R, Cao R, Yan SD, Brett J, Ramakrishnan R, Tsang TC, Simionescu M, Stern D: The endothelial cell binding site for advanced glycation end products consists of a complex: an integral membrane protein and a lactoferrinlike polypeptide. *J Biol Chem* 269: 9882–9888, 1994b.

Uhlmann S, Friedrichs U, Eichler W, Hoffmann S, Wiedemann P: Direct measurement of VEGF-induced nitric oxide production by choroidal endothelial cells. *Microvasc Res* 62: 179-89, 2001.

Uhlmann S, Rezzoug K, Friedrichs U, Hoffmann S, Wiedemann P: Advanced glycation end products quench nitric oxide in vitro. *Graefe`s Arch Clin Exp Ophthalmol* 240: 860-866, 2002.

Vlassara H, Brownlee M, Cerami A: Specific macrophage receptor activity for advanced glycosylation end products inversely correlates with insulin levels in vivo. *Diabetes* 37: 456-61, 1988.

Wang YS, Eichler W, Friedrichs U, Yafai Y, Hoffmann S, Yasukawa T, Hui YN, Wiedemann P: Impact of endostatin on bFGF-induced proliferation, migration, and matrix metalloproteinase-2 expression/secretion of bovine choroidal endothelial cells. *Curr Eye Res* 30: 479-489, 2005.

Yafai Y, Iandiev I, Wiedemann P, Reichenbach A, Eichler W: Retinal endothelial angiogenic activity: effects of hypoxia and glial (Müller) cells. *Microcirculation* 11: 577-586, 2004.

Yasukawa T, Hoffmann S, Eichler W, Friedrichs U, Wang YS, Wiedemann P: Inhibition of experimental choroidal neovascularization in rats by an alpha(v)-integrin antagonist. *Curr Eye Res* 28: 359-366, 2004.

Cardiac angiogenesis and arteriogenesis

Coronary Angiogenesis Induced by Retroinfusion of Angiogenic Growth Factors and Liposomal cDNA

Prof. Dr. med. Peter Boekstegers
PD Dr. med. Christian Kupatt

Medical Clinic I
University Clinic Munich-Großhadern
University of Munich
Marchioninstr. 15, 81377 Munich

Members of the SPP 1069 from 1999 until 2006

SUMMARY

Although therapeutic neovascularization is an attractive option in patients who suffer from chronic ischemic cardiomyopathy and are not eligible for surgical or interventional treatment, recent clinical studies failed to translate this concept to the clinical scenario. Besides the search for new therapeutic agonists and enhanced biological activity (e.g. by use of longlasting gene vectors), effective regional application to the chronic ischemic myocardium is a predominant issue in the cardiovascular field. Recently, we demonstrated the feasibility of retroinfusion as regional approach to treatment of ischemic myocardium. The projects embedded in SPP 1069 aimed at retroinfusion of pro-angiogenic agents / vectors for efficient induction of therapeutic neovascularization.

At first, we established a percutaneous model of chronic ischemia in the heart. For this purpose, a reduction stent was developed by controlled ligation of a PTFE-membrane coated stent. 4 weeks after stent placement, hibernating myocardium was demonstrated by $^{13}NH_3$-PET and ^{18}FDG-PET. Then, retroinfusion of basic fibroblast growth factor at d7 was successfully applied, inducing functionally relevant neovascularization at d28. After extensive testing of other proangiogenic agents, mainly targeting the PI3K-AKT-eNOS pathway in a rabbit hindlimb ischemia model, we next applied liposomal cDNA transfer of eNOS S1177D, to the hibernating myocardium. To avoid potential overlapping treatment of acute ischemia, when retroinfusion was applied at d7, we next postponed treatment till d28, using liposomal eNOS cDNA transfection via retroinfusion. Endothelial NOS S1177D overexpression increased endothelial cell proliferation as well as capillary and collateral growth at d49. Concomitantly, regional myocardial perfusion and regional functional reserve were improved .

Future perspective: The establishment of a percutaneous chronic ischemic model and the successful application of two proangiongenic agonists (bFGF, eNOS) have been demonstrated during the SPP 1069 funding period. Further studies are dedicated to an inducible long-acting expression system (adeno-associated virus) which, if successful, is an attractive candidate for application in clinical studies.

INTRODUCTION

Access for reperfusion of chronically ischemic myocardium may be limited despite current techniques of bypass grafting or percutaneous approaches such as PTCA and stenting. Thus, a subgroup of patients suffering from angina pectoris or heart insufficiency due to totally occluded arteries or diffuse coronary artery disease is not suitable for current revascularization strategies. However, chronically ischemic but viable myocardium which has ceased myocardial function (= hibernating myocardium), may recover to normal function if adequate perfusion is reestablished. Therefore, therapeutic angiogenesis (Khan et al. 2003b) and arteriogenesis (Schaper et al. 2003) have been suggested as new options for the treatment of patients who suffer from myocardial ischemia despite optimal medication and who are no more candidates for percutaneous interventions or coronary artery bypass surgery.

Various strategies for the induction for therapeutic angiogenesis and arteriogenesis have been evaluated in experimental models and part of them have then been introduced into clinical pilot studies so far. With this respect, growth factors like acidic FGF, basic FGF, VEGF in experimental models (Lopez et al. 1998), cDNAs encoding for angiogenic factors or adenoviral vectors leading to overexpression of angiogenic factors or transcription factors (for review cf. (Khan et al. 2003a). More recently, the concept of cell mediated induction of growth factor release and subsequent induction of angiogenesis has been reported for adult bone marrow (Kocher et al. 2001) or endothelial progenitor cells (Kawamoto et al. 2001), an approach with therapeutic potential in the heart (Strauer et al. 2002), (Assmus et al. 2002).

Severe stenosis of a coronary artery induces hypoxia of the dependent myocardium, which activates transcription e.g. via through hypoxia-inducible factors HIF 1 and 2, results in increased expression of a variety of proangiogenic ligands such as VEGF proteins and their receptors. Auto- and paracrine stimulation subsequently triggers endothelial proliferation and migration, tube formation, thus leading to the occurence of new capillary vessels (Waltenberger 1997). Intracellular signal transduction after angiogenic growth factor stimulation includes activation of the phosphoinositol-3-kinase, which in turn phosphorylates protein kinase B (AKT), inducing phosphorylation of endothelial nitric oxide synthase (eNOS), a mediator of VEGF-induced angiogenesis (Papapetropoulos et al. 1997). This intracellular signal transduction pathway is also utilized by statins (Kureishi et al. 2000). Ligand-induced AKT-based eNOS activation is biochemically mimicked by overexpression of essential elements of the eNOS phosphorylation multicomplex such as heat shock protein 90 (Brouet et al. 2001) or active AKT or a constitutively active eNOS mutant such as eNOS S1177D (Dimmeler et al. 1999), (Fulton et al. 1999). Arteriogenesis, on the other hand, is preferentially induced by growth factors like bFGF (after receptor upregulation, (Deindl et al. 2003) and – to a smaller extent – by PlGF-1, a selective VEGF-R1 ligand (flt-1, (Pipp et al. 2003). MCP-1, a monocyte chemoattractant protein, has been demonstrated to induce profound arteriogenesis, implying monocyte adhesion and subsequent release of growth factors and chemokines as potent mechanism of arteriogenesis induction (Schaper et al. 2003). To what extent this effect is mediated by resident macrophages or circulating monocytes is controversially discussed (Khmelewski et al. 2004), (Heil et al. 2002).

A conceptional dissection of arteriogenesis and angiogenesis appears intriguing, in order to generate a more comprehensive understanding of the therapeutic potential of either level of vessel neoformation. To test this concept, local MCP-1 application might appear attractive as an inducer of arteriogenesis. However, this approach is ambiguous in the context of coronary artery disease for two main reasons: first, targeted local application of MCP-1 around the collateral vessels (in the nonischemic tissue) is difficult to achieve in the heart, since no in situ landmarks for these collaterals exist. Secondly, its capability to recruit monocytes at the wall of injured vessels might result in

accelerated atherosclerosis (de Lemos et al. 2003), (Egashira 2003). A stimulus avoiding overlap of arteriogenesis and atherosclerosis is physical shear stress, induced by e.g. shunt formation. Chronicially increased FFS has been shown to induce arterial remodeling of the affected artery, which in part is also observed in growing collaterals (Schaper et al. 2003). This mechanism might be used in comparison with capillary and venular eNOS S1177D overexpression to compare arteriogenesis and angiogenesis induction in our pig model of chronic ischemia and hibernating myocardium.

METHODS

Application of a proangiogenic protein, cDNA or cell population requires particular care, since arterial injection into coronary arteries suffers from limited access to hibernating myocardium by the arterial route as well as limited efficacy due the short passage time (Banai et al. 1994), (Henry et al. 2003) (Sato et al. 2001). Therefore, among others (Haga et al. 1994), our group established the use of coronary veins as an alternative access to ischemic myocardium (Boekstegers et al. 1990), (Boekstegers et al. 1994), (Boekstegers et al. 1998), (Boekstegers et al. 2000). Selective catheterization and retroinfusion of coronary veins provides a unique approach to deliver therapeutic factors to ischemic myocardium which cannot be reached through the coronary arteries (Boekstegers et al. 2000). As capillary sprouting originates mainly from venules and capillaries, retrograde administration into the vein may also target angiogenic growth factors and vectors to these vessel segments probably most receptive and responsive to them (Folkman et al. 1992), (Battegay 1995). The results obtained in the current project support this concept of increasing the efficacy of therapeutic angiogenesis by prolonging and enhancing tissue binding of growth factors using selective pressure-regulated retroinfusion of the coronary veins (von Degenfeld et al. 2003).

The surgically implanted ameroid constrictor model has been used in the majority of experimental studies designed to study therapeutic angiogenesis. The placement of an ameroid constrictor at the circumflex artery, however, was not suitable for studying retrograde delivery of angiogenic growth factors in the pig. The anatomy of the veins draining the myocardium supplied by the circumflex artery is highly variable in the pig whereas the anterior cardiac vein is very consistent. As a consequence, selective retroinfusion of the anterior cardiac vein is mandatory to achieve reproducible results of regional drug or gene delivery. Placement of an ameroid constrictor around the LAD, however, was associated with a high incidence of ventricular fibrillation and significant infarctions of the LAD territory. Furthermore surgical placement of an ameroid constrictor around the LAD might be associated with compression of the adjacent anterior cardiac vein which hampers or inhibits access through the vein in a reproducible and predictable manner.

During the first period of the project, the percutaneous reduction stent graft model was established and during the second period it was characterized in more detail (von Degenfeld et al. 2003) . Unlike the ameroid constrictor model on the circumflex artery, the percutaneous placement of the reduction stent graft into the LAD resulted in a significant decrease of resting blood flow in the LAD territory after 28 days. As the decrease in perfusion was associated with a significant decrease in regional myocardial function, hibernating myocardium was present 28 days after implantation of the reduction stent (von Degenfeld 2003). In principle, hibernating myocardium is capable to regain up to normal myocardial function if perfusion is restored completely e.g. by coroanry artery bypass grafting. Whether therapeutic angiogenesis or arteriogenesis are able to recover hibernating myocardium, has not been addressed in preclinical studies so far which was mainly due to the limitations of the ameroid constrictor model of the circumflex artery pointed out above. Other pig models which have been used to establish chronically hibernating myocardium in the LAD territory

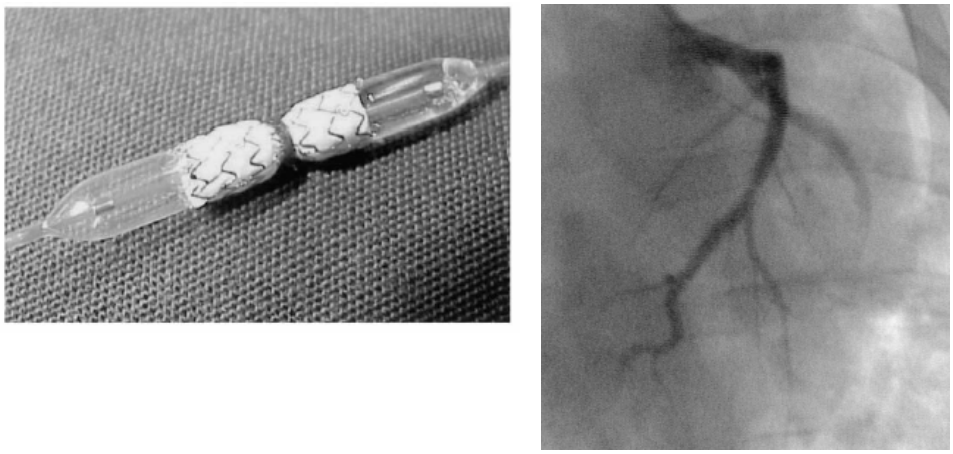


Fig. 1: Left panel: Example of a reduction stent prepared for percutaneous implantation. **Right panel:** Angiogramm of the LAD after placement of a reduction stent (white arrow) providing a 75% stenosis, which typically progresses to 100% at d28 after placement.

needed a complex surgical procedure and a much longer follow-up period (3-6 months) than neccessary in the reduction stent model (3-4 weeks).

Confirmation of induction of hibernating myocardium was obtained by radionuclide tracer experiments at d28 after percutaneous reduction stent implantation. Comparing the metabolic rate of myocardium detected by ^{18}FDG-uptake mapping and the myocardial perfusion obtained by ^{13}Ammonium-PET, induction of hibernating myocardium was demonstrated (collaboration Prof. Schwaiger, TU Munich).

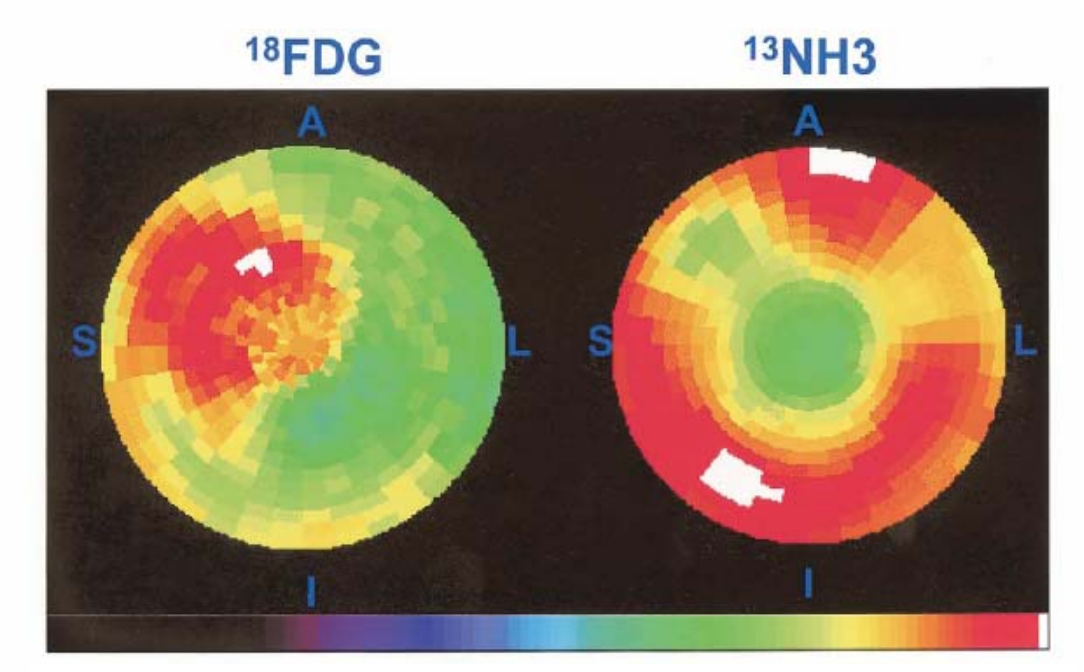


Fig. 2: Polar map display of relative myocardial uptake of ^{18}FDG and ^{13}N-labeld ammonia. S = Septum, I = inferior, L = lateral, A = anterior. Sectors with brighter colors represent higher uptake values. The perfusion deficit (right panel, anteroseptal) corresponds to an area with increased ^{18}FDG-uptake.

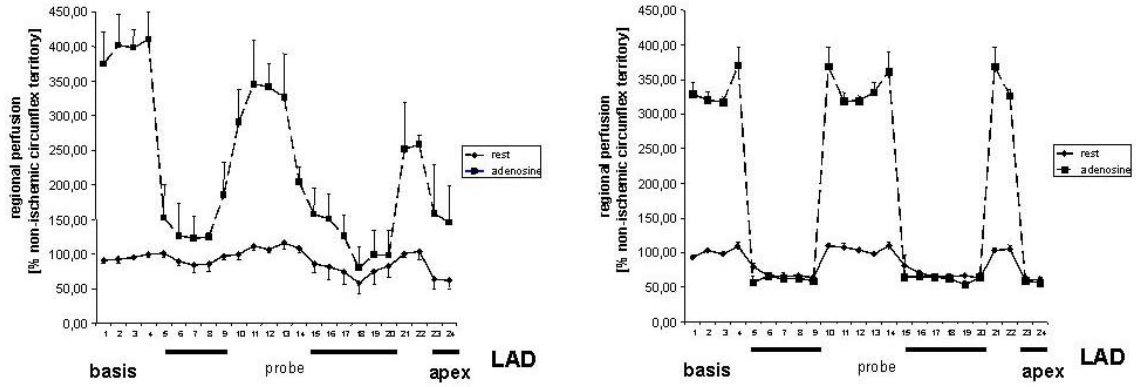


Fig. 3: Regional perfusion map of the left ventricle after slicing from the basis to the apex. Fig. 1A: 7 days after implantation (n=7); Fig. 1B: 28 days after implantation (n=7). Probe= number of transmural probe. Rest= regional myocardial perfusion at rest; Adenosine= regional myocardial perfusion determined after intracoronary adenosine.

The impact of percutaneous reduction stent implantation on myocardial perfusion was characterized by microsphere distribution methodology (cf. (von Degenfeld et al. 2003). An important prerequisite for the reduction stent model is to assure that the extent of transmural myocardial infarction is very limited (<5%) and does not influence the assessment of angiogenic or arteriogenic treatments. Therefore, repeated angiograms were used to determine the progression of the LAD stenosis in the reduction stent model. At day 7, resting blood flow was not yet significantly reduced, whereas at day 28 hibernating myocardium with significantly reduced resting blood flow developed (Fig. 3). In between day 7 and day 28, complete occlusion of the LAD occurred in all pigs but the exact time point of total coronary artery occlusion is unknown in the reduction stent model which is similar to the ameroid constrictor model.

RESULTS

During the first period of the project, the percutaneous reduction stent graft model was established, during the second period it was characterized in more detail and used for basic fibroblast protein application(v Degenfeld et al. 2003). During the third period, the model was extended to a 2 month experimental period and used for eNOS S1177D gene transfer to the chronic ischemic myocardium. During all periods, a chronic hindlimb model was used in parallel to investigate novel proangiogenic signaling pathways with respect to potential therapeutic targeting and application of candidate genes in the pig model.

1. The impact of proangiogenic protein application

Two powerful candidates for angiogenesis induction had emerged from *in vitro* and small animal *in vivo* experiments at the onset of the project, vascular endothelial growth factor ($VEGF_{165}$) and basic fibroblast growth factor (bFGF). Since therapeutic dosage showed considerable variability and (at higher doses), $VEGF_{165}$ as a protein displayed serious hemodynamic side effects such as hypotension and edema formation, we first tested the feasibility of (high dose) bFGF application with or without VEGF-coapplication in a rabbit chronic ischemic hindlimb model (art. Femoralis excision, (Lebherz et al. 2003). Here we first investigated the uptake behaviour of retrograde versus antegrade applied ^{99}technetium colloids. As displayed in Figure 4, the recruitment of radioactively labelled nanocolloids was significantly increased in the retrogradely treated experiments. Since in this model,

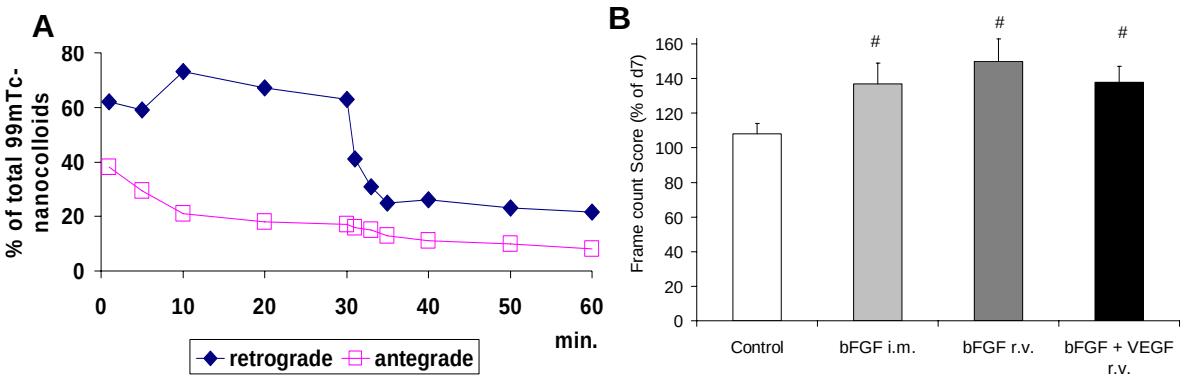


Fig. 4: A Quantitative analysis of the distribution of 99mTC-nanocolloids revealed a 5.8fold increase of specific radioactivity immediately after retrograde as compared to antegrade delivery. 30min later, the increase was still 2.8 fold (data are given in % of total detectable radioactivity = whole body region of interest). **B** Ratio of frame count score d35 / d7 (cf. Methods) displays a similar increase in blood flow after i.m. (= intramuscular), r.v. (= retrograde intravenous) and bFGF+VEGF r.v. treatment. (n=6, #=p<0.05 vs. saline retroinfused controls)

micro- and macrovessel density remained stable from d7 to d28 after excision of the femoral artery, we choose d7 as treatment timepoint.

Moreover, in this study the equipotency of retrograde bFGF application (20µg/kg) with or without $VEGF_{165}$ (20µg/kg) was found, indicating that the bFGF effect was not enhanced by a low dose, side-effect-free VEGF-coapplication (Lebherz et al. 2003).

Applying the bFGF-protein-retroinfusion approach to the pig model, we compared retrograde vs. antegrade infusion of 150µg bFGF with respect to perfusion and function of the chronic ischemic myocardium (cf. Fig.2) at d28. In this preclinical model we were able to demonstrate that 1) retroinfusion doubled the acute binding of radioactively labebelled bFGF in the ischemic region, 2) retrograde but not antegrade application increased myocardial blood flow significantly and 3) that regional myocardial function of the LAD-region benefited in the retrogradely bFGF-treated group only (von Degenfeld, 2003).

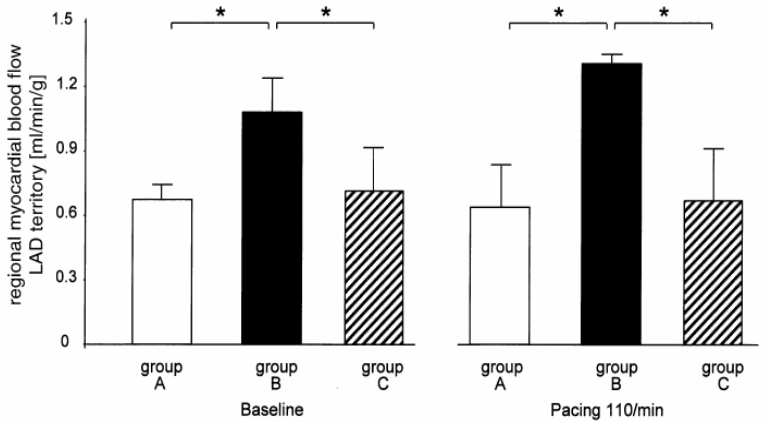


Fig. 5: Regional myocardial blood flow (microspheres) at d28 in the LAD territory (left: at rest, right: during pacing (110/min). **A**: controls, **B**: retroinfusion of bfGF, **C**= antegrade delivery (*=p<0.05)

Over time, two shortcomings of protein treatment for therapeutic neovascularization became increasingly obvious: first, treatment of ischemia early in the course of chronic ischemia development aims at both, acute and chronic ischemic events, obscuring the effect of the given treatment in chronic stable ischemia alone. Secondly, one-time protein application, although effective in experimental settings of otherwise healthy animals, does not necessarily induce a similar effect in systemically arteriosclerotic organisms.

2. Novel signaling pathways

Since the relevance of the PI3K-AKT-eNOS pathway for the proangiogenic pathways of VEGF, insulin-like growth factors, hepatocyte growth factor as well as statins has become increasingly clear, we attempted to target the downstream elements of this pathway, in particular the activation of eNOS phosphorylation step downstream of receptor tyrosine kinase activation. With this respect, we studied the effect of heatshock protein 90, a chaperone instrumental for the assembly of the AKT-eNOS complex which provides serine 1177 phosphorylation.

Since hps90 overexpression itself induced eNOS phosphorylation, and induction of an angiogenic phenotype in endothelial cells *in vitro* (Brouet et al. 2001) Therefore, we assessed the efficacy of hsp 90 cDNA transfection in the chronic ischemic hindlimb model (rabbit). Here, we detected an initial capillary growth response upon transfection, followed by collateral growth only days later (Pfosser et al. 2005). Geldanamycin, a specific hsp90 protein-protein interaction inhibitor, blocked the hsp90-induced neovascularization. L-NAME, an unselective nitric oxide blocker, also inhibited the sequence of capillary growth and collateral growth *in vivo*. Notably, selective blockade of collateral growth was achieved by delaying L-NAME application for 3 days, a time point where capillary growth has already occurred (see Fig. 7). A similar angiogenic and arteriogenic potency was found when eNOS S1177D was applied (data not shown).

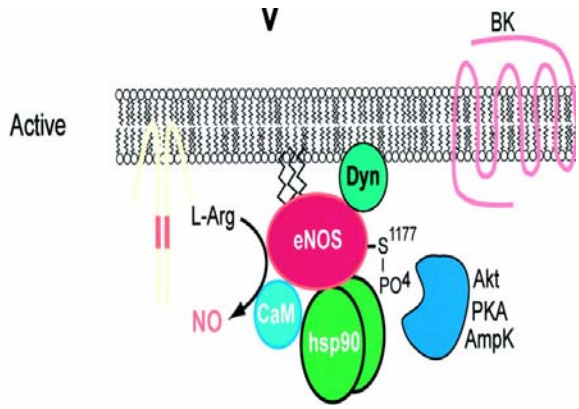


Fig. 6: Activation of membrane bound eNOS by AKT (protein kinase B) critically requires hsp90-mediation. BK = bradykinin, Dyn = dynamin, hsp90 = heat shock protein 90, CaM = Calmodulin.

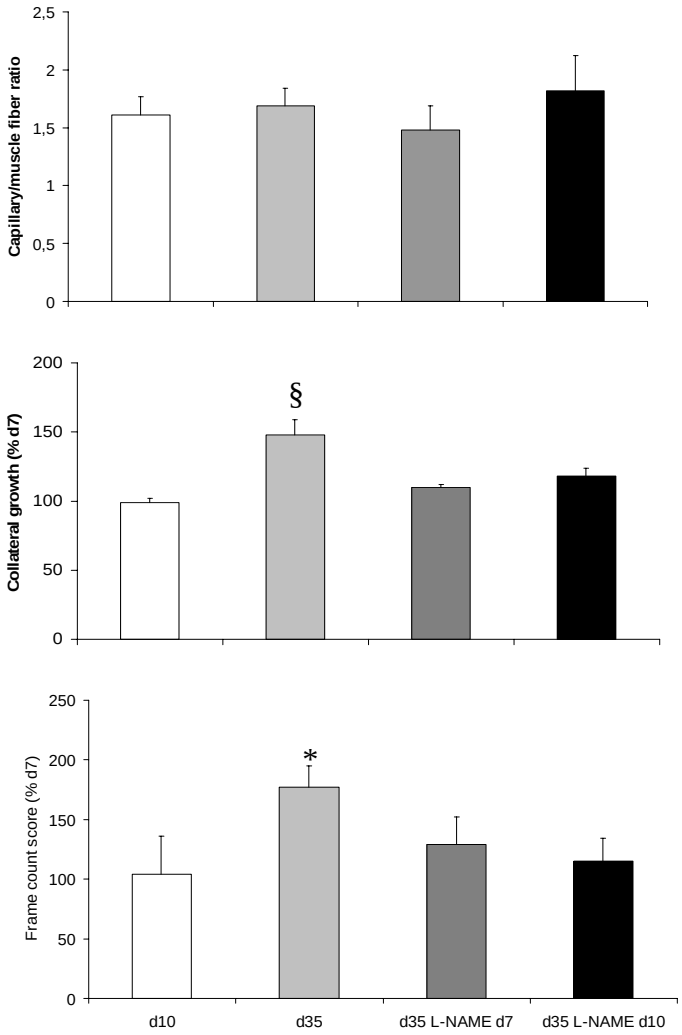


Fig. 7: A Capillary/muscle fiber (C/MF) ratio of mock- or Hsp90-transfected hindlimbs (Hsp90) explanted at d10 (n=4) or d35 without or with L-NAME minipump insertion at d7 (n=5) or d10 (n=4). B collateral growth and C perfusion score of the same experimental groups. *=p<0.05 vs.d10 and L-NAME d10, §=p<0.05 vs. all other groups.

Having assessed the potency of nitric oxide formed after Hsp90-facilitated eNOS activation, we used the preclinical pig model to study the effect of a constitutively active eNOS mutant (eNOS S1177D) on neovascularization of the heart. In this study, we modified our protocol to confine the treatment selectively to the hibernating myocardium (see Fig. 8).

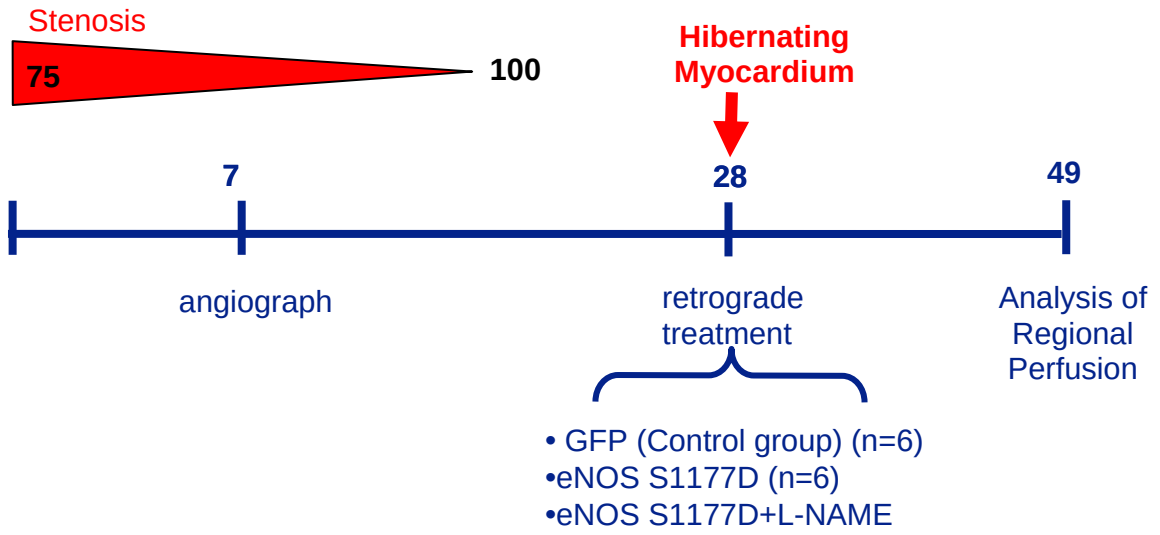


Fig. 8: Protocol of the eNOS S1177D cDNA retroinfusion study in the preclinical pig model.

In this refined model, the prediction that nitric oxide provides an adequate stimulus for therapeutic neovascularization proved correct: Capillary growth and collateral growth were obtained 3 weeks after transfection, improving perfusion of the hibernating myocardium (LAD-region) as well as regional myocardial function. As a mechanism, we could observe an increase in proliferating (Ki67+) cells in the treated vs. the untreated region. Moreover, co-staining revealed the majority of the Ki67+ cells as endothelial cells.

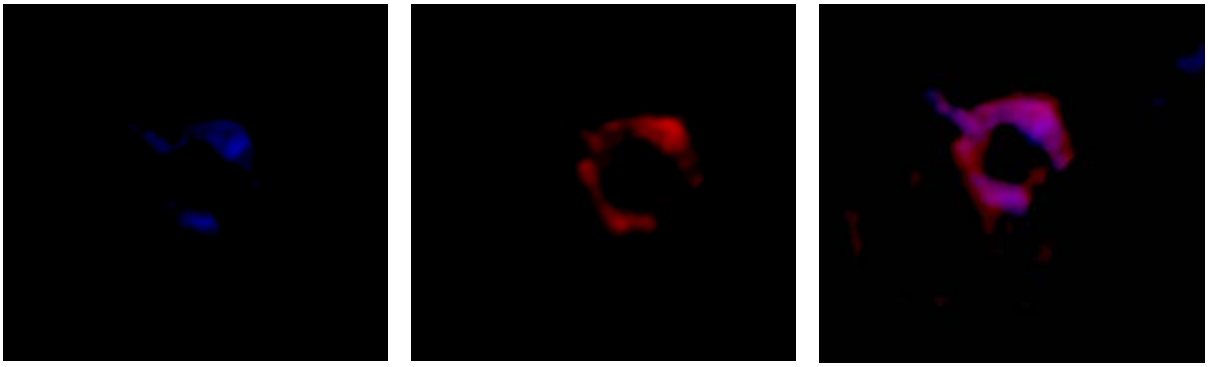


Fig. 9: Histochemical evaluation proliferation (Ki67 positivity) in control (A) and ischemic (B) tissue. Red bar = 50 µm. C. Quantitative analysis revealed an increased proliferation in eNOS S1177D transfected tissue. D. Fluorescence microscopy of Ki67 (left panel), endothelial marker PECAM-1 (middle) and overlay (right panel), red bar = 10µm.

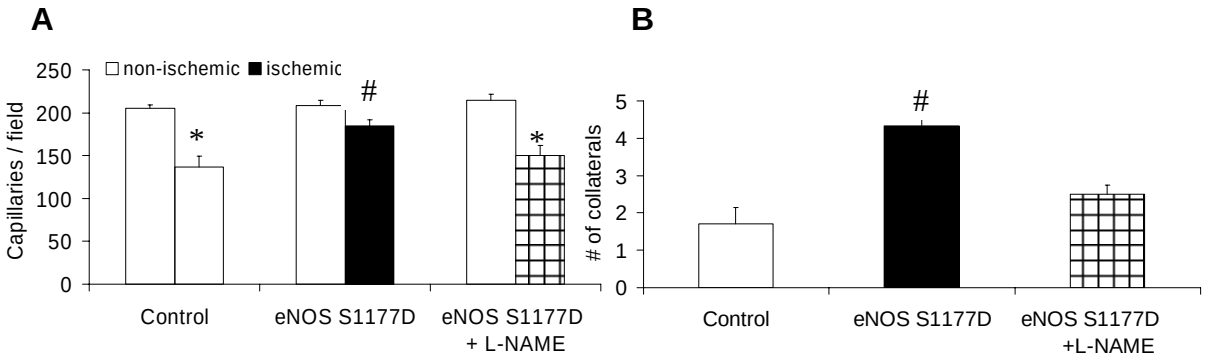


Fig. 10: A. The ischemic area of control animals displayed rarification of capillaries in the ischemic area (*=p<0.05 vs. non-ischemic area), which was attenuated by eNOS S1177D-transfection (#=p<0.05 vs. control ischemic area), unless L-NAME was present. **B.** The number of collaterals at d49 (post mortem angiogram) was concomitantly increased after eNOS transfection.

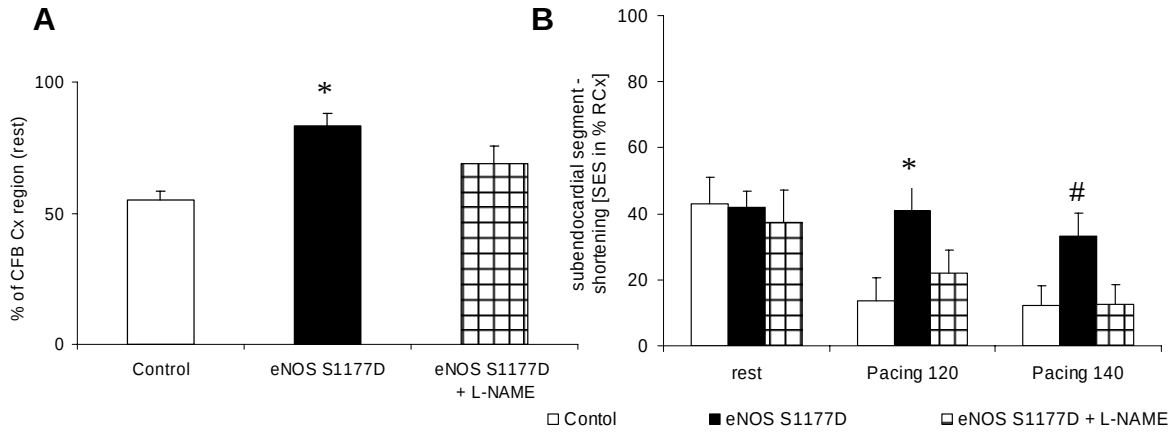


Fig. 11: A At d49, myocardial perfusion of the ischemic area was improved in the eNOS S1177D treated hearts, as compared to control or L-NAME cotreated animals. B Regional myocardial function was assessed by subendocardial segment shortening (cf. methods) at d49 in the proximal (left panel) or distal LAD region (right panel) at rest or after atrial pacing (120 and 140/min). *=p<0.05 vs. control group, #=p<0.05 vs. both other groups.

3. Pro-angiogenic cell factory

Since the advent of endothelial cell therapy, numerous experimental and clinical trials have been conducted using adult endothelial progenitor cells. Since we had the privilege to cooperate with A. Hatzopoulos in the SPP framework, we analyzed the paracrine effects exerted by the embryonic endothelial progenitor cells developed in his lab (Hatzopoulos et al. 1998) for their pro-angiogenic and pro-arteriogenic potential.

For this purpose, we used eEPCs in the rabbit hindlimb model (Kupatt et al. 2005). In this subproject, we were able to show that a clonal embryonic mouse cell line expressing a variety of chemokines, cytokines, insulin-like growth factors, wnt- and bmp-proteins is capable of inducing therapeutic neovascularization *in vivo*, even though the xenotypic nature of the experiment had the eEPC population disappeared at d14 after application.

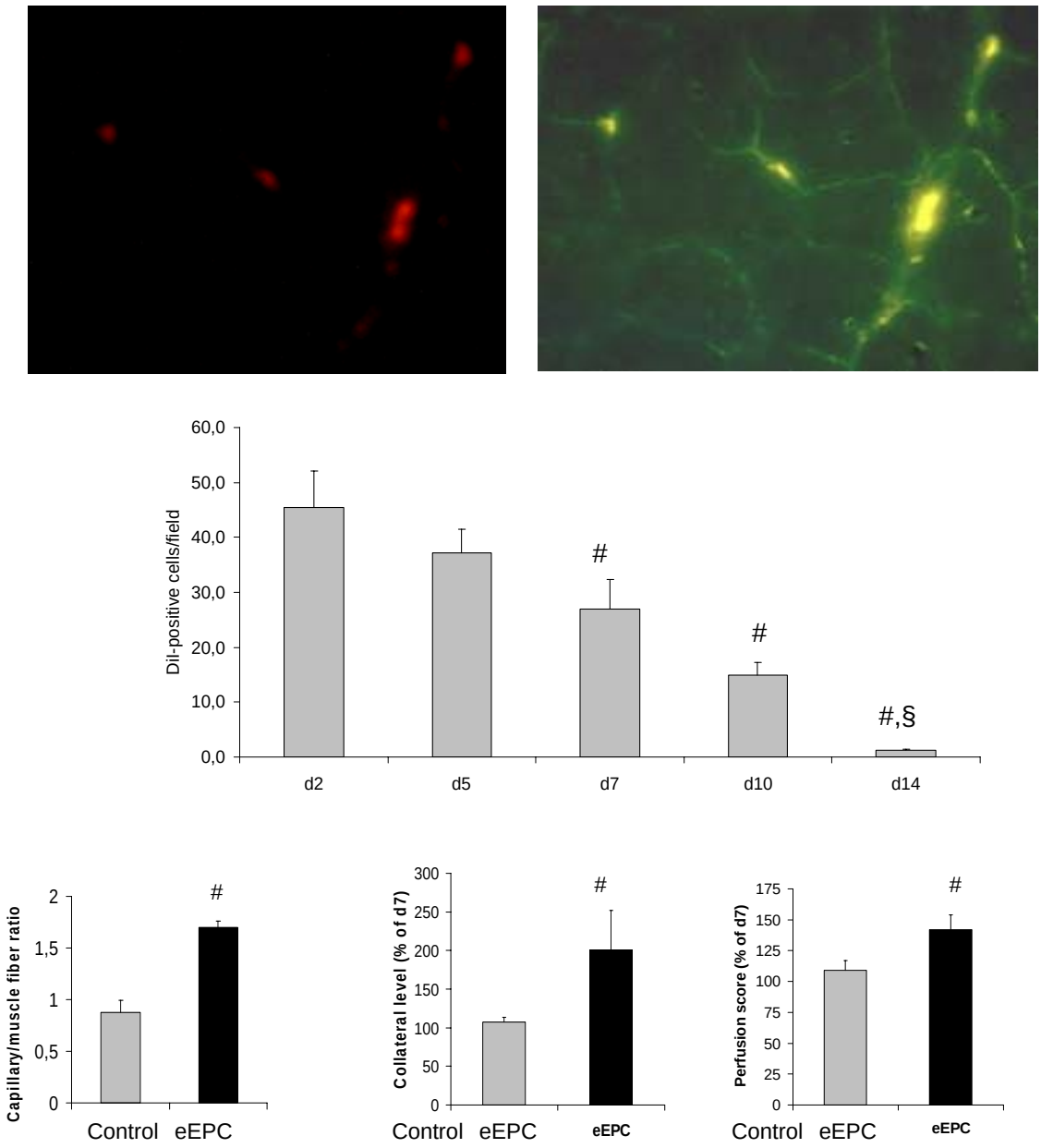


Fig. 12: Embryonic EPCs labelled with Di-I (upper left panel) and eGFP (upper right panel) harbour in the ischemic, but not the non-ischemic limb. After gradual decrease, eEPCs disappear at d14 (mid-panel). However, their presence sufficed to induce capillary and collateral growth, resulting in significant improvement of perfusion (lower panels). #=p<0.05 vs. saline treated control group.

In summary, during the six year period of SPP 1069 participation, we developed a novel model of percutaneous induction of hibernating myocardium and a regional percutaneous treatment approach via pressure regulated retroinfusion of pro-angiogenic factors (protein and cDNA). We have successfully improved function of the chronic ischemic myocardium with bFGF retroinfusion (28d model), and eNOS S1177D transfection (49d model). Furthermore, we have been investigating the pro-angiogenic potential of embryonic endothelial progenitor cells, an approach also proving effective in a chronic ischemic hindlimb model, and developing vectors for future long-acting gene transfer (Raake, Müller, Boekstegers, unpublished results).

REFERENCES

Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S, Zeiher AM: Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation* 106: 3009-3017, 2002.

Banai S, Shweiki D, Pinson A, Chandra M, Lazarovici G, Keshet E: Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesi. *Cardiovasc Res* 28: 1176-1179, 1994.

Battegay EJ: Angiogenesis: mechanistic insights, neovascular diseases, and therapeutic prospect. *J Mol Med* 73: 333-346, 1995.

Boekstegers P, Diebold J, Weiss C: Selective ECG synchronised suction and retroinfusion of coroanry veins: first results of studies in acute myocardial ischemia in dog. *Cardiovasc Res* 24: 456-464, 1990.

Boekstegers P, Giehrl W, von Degenfeld G, Steinbeck G: Selective suction and pressure-regulated retroinfusion: an effective and safe approach to retrograde protection against myocardial ischemia in patients undergoing normal and high risk percutaneous transluminal angioplast. *J Am Coll Cardiol* 31: 1525-1533, 1998.

Boekstegers P, Peter W, von Degenfeld G, Nienaber CA, Abend M, Rehders TC, Habazettl H, Kapsner T, von Lüdinghausen M, Werdan K: Preservation of regional myocardial function and myocardial oxygen tension during acute ischemia in pigs: Comparison of selective synchronized suction and retroinfusion of coronary veins to synchronized coronary venous retroperfusio. *J Am Coll Cardiol* 23: 459-469, 1994.

Boekstegers P, von Degenfeld G, Giehrl W, Heinrich D, Hullin R, Kupatt C, Steinbeck G, Baretton G, Middeler G, Katus H, Franz WM: Myocardial gene transfer by selective pressure-regulated retroinfusion of coronary vein. *Gene Ther* 7: 232-240, 2000.

Brouet A, Sonveaux P, Dessy C, Moniotte S, Balligand JL, Feron O: Hsp90 and caveolin are key targets for the proangiogenic nitric oxide-mediated effects of statin. *Circ Res* 89: 866-873, 2001.

de Lemos JA, Morrow DA, Sabatine MS, Murphy SA, Gibson CM, Antman EM, McCabe CH, Cannon CP, Braunwald E: Association between plasma levels of monocyte chemoattractant protein-1 and long-term clinical outcomes in patients with acute coronary syndrome. *Circulation* 107: 690-695, 2003.

Deindl E, Hoefer IE, Fernandez B, Barancik M, Heil M, Strniskova M, Schaper W: Involvement of the fibroblast growth factor system in adaptive and chemokine-induced arteriogenesi. *Circ Res* 92: 561-568, 2003.

Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM: Activation of nitric oxide synthase in endothelial cells by Akt- dependent phosphorylatio. *Nature* 399: 601-605, 1999.

Egashira K: Molecular mechanisms mediating inflammation in vascular disease: special reference to monocyte chemoattractant protein-. *Hypertension* 41: 834-841, 2003.

Folkman J, Shing Y: Angiogenesis. *J Biol Chem* 267: 10931-10934, 1992.

Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC: Regulation of endothelium-derived nitric oxide production by the protein kinase Ak. *Nature* 399: 597-601, 1999.

Haga Y, Uriuda Y, Bjorkman JA, Hatori N, Johansson L, Nordlander R, Sjoquist PO, Ryden L: Ischemic and nonischemic tissue concentrations of felodipine after coronary venous retroinfusion during myocardial ischemia and reperfusion: an experimental study in pig. *J Cardiovasc Pharmacol* 24: 298-302, 1994.

Hatzopoulos AK, Folkman J, Vasile E, Eiselen GK, Rosenberg RD: Isolation and characterization of endothelial progenitor cells from mouse embryo. *Development* 125: 1457-1468, 1998.

Heil M, Ziegelhoeffer T, Pipp F, Kostin S, Martin S, Clauss M, Schaper W: Blood monocyte concentration is critical for enhancement of collateral artery growt. *Am J Physiol Heart Circ Physiol* 283: H2411-H2419, 2002.

Henry TD, Annex BH, McKendall GR, Azrin MA, Lopez JJ, Giordano FJ, Shah PK, Willerson JT, Benza RL, Berman DS, Gibson CM, Bajamonde A, Rundle AC, Fine J, McCluskey ER: The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesi. *Circulation* 107: 1359-1365, 2003.

Kawamoto A, Gwon HC, Iwaguro H, Yamaguchi M, Uchida S, Masuda H, Silver M, Ma H, Kearney M, Isner JM, Asahara T: Therapeutic potential of ex vivo expanded endothelial progenitor cells for myocardial ischemi. *Circulation* 103: 634-637, 2001.

Khan TA, Sellke FW, Laham RJ: Gene therapy progress and prospects: therapeutic angiogenesis for limb and myocardial ischemi. *Gene Ther* 10: 285-291, 2003a.

Khan TA, Sellke FW, Laham RJ: Therapeutic angiogenesis: protein-based therapy for coronary artery diseas. *Expert Opin Pharmacother* 4: 219-226, 2003b.

Khmelewski E, Becker A, Meinertz T, Ito WD: Tissue Resident Cells Play a Dominant Role in Arteriogenesis and Concomitant Macrophage Accumulatio. *Circ Res* 95: e56-e64, 2004.

Kocher AA, Schuster MD, Szabolcs MJ, Takuma S, Burkhoff D, Wang J, Homma S, Edwards NM, Itescu S: Neovascularization of ischemic myocardium by human bone-marrow-derived angioblasts prevents cardiomyocyte apoptosis, reduces remodeling and improves cardiac functio. *Nat Med* 7: 430-436, 2001.

Koczulla R, von Degenfeld G, Kupatt C, Krötz F, Zahler S, Gloe T, Issbrücker K, Unterberger T, Zaiou M, Lebherz C, Karl A, Raake P, Pfosser A, Boekstegers P, Welsch U, Hiemstra PS, Vogelmeier C, Gallo RL, Clauss M, Bals R: An angiogenic role for the human peptide antibiotic LL-37/hCAP-18. *J Clin Invest* 111: 1665-1672, 2003.

Kupatt C, Horstkotte J, Vlastos GA, Pfosser A, Lebherz C, Semisch M, Thalgott M, Buttner K, Browarzyk C, Mages J, Hoffmann R, Deten A, Lamparter M, Muller F, Beck H, Buning H, Boekstegers P, Hatzopoulos AK: Embryonic endothelial progenitor cells expressing a broad range of proangiogenic and remodeling factors enhance vascularization and tissue recovery in acute and chronic ischemia. *FASEB J* 19: 1576-8, 2005.

Kureishi Y, Luo Z, Shiojima I, Bialik A, Fulton D, Lefer DJ, Sessa WC, Walsh K: The HMG-CoA reductase inhibitor simvastatin activates the protein kinase Akt and promotes angiogenesis in normocholesterolemic animal. *Nat Med* 6: 1004-1010, 2000.

Lebherz C, von Degenfeld G, Karl A, Pfosser A, Raake P, Pachmayr F, Scholz D, Kupatt C, Boekstegers P: Therapeutic angiogenesis/arteriogenesis in the chronic ischemic rabbit hindlimb: effect of venous basic fibroblast growth factor retroinfusio. *Endothelium* 10: 257-265, 2003.

Lopez JJ, Laham RJ, Stamler A, Pearlman JD, Bunting S, Kaplan A, Carrozza JP, Sellke FW, Simons M: VEGF administration in chronic myocardial ischemia in pig. *Cardiovasc Res* 40: 272-281, 1998.

Papapetropoulos A, Garcia-Cardena G, Madri JA, Sessa WC: Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cell. *J Clin Invest* 100: 3131-3139, 1997.

Pfosser A, Thalgott M, Büttner K, Brouet A, Feron O, Boekstegers P, Kupatt C: Liposomal Hsp90 cDNA induces neovascularization via nitric oxide in chronic ischemi. *Cardiovasc Res* 65: 728-736, 2005.

Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, van den Martin S, Heuvel J, Weich H, Fernandez B, Golomb G, Carmeliet P, Schaper W, Clauss M: VEGFR-1-selective VEGF homologue PlGF is arteriogenic: evidence for a monocyte-mediated mechanis. *Circ Res* 92: 378-385, 2003.

Raake P; von Degenfeld G; Hinkel R, Vachenauer-R; Sandner-T; Beller S; Andrees M; Kupatt C; Schuler G; Boekstegers P: Myocardial gene transfer by selective pressure-regulated retroinfusion of coronary veins – comparison to surgical and percutaneous intramyocardial gene delivery. *J Am Coll Cardiol* 44: 1124-1129, 2004.

Raake P, Hinkel R, Kupatt C, von Bruhl ML, Beller S, Andrees M, Vicol C, Boekstegers P: Percutaneous approach to a stent-based ventricle to coronary vein bypass (venous VPASSTM): comparison to catheter-based selective pressure-regulated retro-infusion of the coronary vein. *Eur Heart J* 12:1228-34, 2005.

Sato K, Wu T, Laham RJ, Johnson RB, Douglas P, Li J, Sellke FW, Bunting S, Simons M, Post MJ: Efficacy of intracoronary or intravenous VEGF165 in a pig model of chronic myocardial ischemi. *J Am Coll Cardiol* 37: 616-623, 2001.

Schaper W, Scholz D: Factors regulating arteriogenesi. *Arterioscler Thromb Vasc Biol* 23: 1143-1151, 2003.

Strauer BE, Brehm M, Zeus T, Kostering M, Hernandez A, Sorg RV, Kogler G, Wernet P: Repair of infarcted myocardium by autologous intracoronary mononuclear bone marrow cell transplantation in human. *Circulation* 106: 1913-1918, 2002.

von Degenfeld G, Raake P, Kupatt C, Lebherz C, Hinkel R, Gildehaus FJ, Munzing W, Kranz A, Waltenberger J, Simoes M, Schwaiger M, Thein E, Boekstegers P: Selective pressure-regulated retroinfusion of fibroblast growth factor-2 into the coronary vein enhances regional myocardial blood flow and function in pigs with chronic myocardial ischemi. *J Am Coll Cardiol* 42: 1120-1128, 2003.

Waltenberger J: Modulation of growth factor action: implications for the treatment of cardiovascular disease. *Circulation* 96: 4083-4094, 1997.

Interaction of Hemodynamic Changes, Chemoatractants, Cell Adhesion Molecules and Macrophage Recruitment during Collateral Growth

PD Dr. Wulf D. Ito

Head of research in Angiology
Medical Department II, Campus Lübeck
University Medical Centre Schleswig-Holstein
Ratzeburger Allee 160, 23538 Lübeck, Germany

Member of the SPP 1069 from 1999 until 2006

SUMMARY

Several observations regarding the growth of collateral arteries had prompted us to develop the arteriogenesis hypothesis. According to this hypothesis the attenuation of the main arterial supply leads to drastic hemodynamic changes in preexisting arteriolar anastomoses. These hemodynamic changes stimulate homing mechanisms for monocyte/macrophages which accumulate around growing collateral vessels supplying growth factors but also metalloproteinases and thus triggering outward remodeling. Goal of our research proposals was to test the hypothesis and to further elucidate the mechanisms of arteriogenesis with particular emphasis on the interaction of hemodynamic changes, chemoatractants, cell adhesion molecules and macrophage recruitment. We first developed a model that allowed us to follow the process of collateral formation in a single preexisting arteriolar shunt, the remnant arteria ischiadica of the rat. We demonstrated that collateral growth can be divided into an early proliferative phase characterized by proliferation of endothelial- and smooth muscle cells, macrophage recruitment and vessel lumen enlargement and a remodeling phase characterized by declining proliferation, disappearance of macrophages, “corkscrew-like” deformation and increased wall to lumen ratios. In an attempt to determine the contribution of monocytes we detected that neither macrophage recruitment nor collateral proliferation was attenuated by the depletion of circulating monocytes and that fluorescently labelled cells did not incorporate significantly into growing vessels. The arteriogenic impact of chemoatractants like MCP-1 was also not affected. We concluded that local proliferation and differentiation of macrophages and vascular cells constitute the rate limiting step in arteriogenesis. In order to elucidate the pattern of hemodynamic changes in collateral arteries we determined the time course of expression of NO-related enzymes, known indicators of shear force levels. eNOS-, iNOS-, PKG-I-, p-VASP and p-Akt expression was significantly downregulated 12 h post occlusion indicating reduced shear force levels. Not until 24 h post occlusion expression of NO-donating enzymes had risen significantly beyond normal levels. We concluded that shear force is initially downregulated in collateral vessels allowing the recruitment of circulating progenitor cells that divide and differentiate locally during the following days. Within 24 h shear force rises due to ischemia triggered peripheral vasodilatation inhibiting further recruitment of circulating cells. Further support for this sequence of events came from investigations showing that CD 133 positive progenitor cells are responsive to MCP-1 but rapidly downregulate the receptor CCR2 during proliferation and differentiation. Our findings focused the attention onto local changes at the single vessel level. In contrast to our initial concepts the cross talk of circulating cells and the endothelium appears to play a minor role. Hemodynamic changes, however, still appear to play a key role in the initiation and modulation of collateral growth. Two proteins the intermediate filament Vimentin and the cell adhesion molecule CEACAM-1 were recently

identified as possible candidates mediating mechanotransduction during collateral growth by our group within the SPP 1069 project. We are currently developing methods to determine hemodynamic changes within collateral vessels more precisely. Based upon these findings we will investigate the mechanisms of mechanotransduction with particular emphasis on CEACAM-1, Vimentin and cellular proliferation, migration and differentiation in cell and organ culture models currently under development.

INTRODUCTION

The physiology of developing collateral vessels

Collateral growth has been studied for more than two centuries. John Hunter, a Scottish surgeon, used the capability of higher organisms to develop bypassing arterial circuits therapeutically at the end of the 18th century to treat patients with femoral artery aneurysms (Kobler, 1960). Observations made during the past decades and supported by experimental evidence in the late 1990ies indicated that the growth of collateral arteries is not directly related to angiogenesis, the sprouting of capillaries (Fulton, 1965). Fulton already observed that collateral arteries in the human heart largely grow outside the ischemic area at risk (Fulton, 1965). Hollenberg demonstrated that collateral artery growth proceeds beyond compensation of tissue oxygenation models (Hollenberg, 1993). In our own experiments on collateral growth in the rabbit hindlimb we were able to confirm that collateral arteries grow largely outside the ischemic territory (Deindl, et al., 2001, Ito, et al., 1997a). Secondly, growth of collateral arteries is a very rapid adaptive process once a critical stenosis or occlusion persists. In a systematic study on collateral development after myocardial infarction Rentrop et al were able to show that 30% of all patients maintain an existing collateral circulation at the time of myocardial infarction, 60% develop a functional collateral circulation within 10 days after coronary artery occlusion and only 10% do not develop a collateral circulation within 2 weeks (Rentrop, et al., 1989). We demonstrated that the main rise in collateral conductance already occurred within the first week after occlusion (Ito, et al., 1997a). These findings were supported by the fact that BrdU incorporation appeared to be maximal during the first 3 days after occlusion (Arras, et al., 1998, Ito, et al., 1997a). Proliferation appears to be confined to certain vessels within a region supporting the notion that the primary stimulus does not come from the surrounding tissue. Thirdly, collateral growth does not appear to be guided by tissue need. This was already suggested by the experiments of Hollenberg et al mentioned above (Hollenberg, 1993). Furthermore, it is a common observation that despite collateral vessels patients experience severe chest pains especially when multiple vessels are involved which can be explained by the fact that each collateral vessel only compensates maximal blood flow by about 60% (Ito, et al., 1997a, Schaper, et al., 1999). In chronic ischemic patients the situation is characterized by several collateral vessels that grow in series and originate from stenotic vessels which leads to a consecutive diminishment of flow and only minimal compensation in the most distal territory. The reduced compensatory capacity of collateral vessels can also be deduced from their morphological appearance. The typical “corkscrew” appearance of collateral vessels is hemodynamically unfavorable. On the ultrastructural level we observed formation of asymmetrical neointima and inward remodeling resembling diseased vessels in patients suffering from arteriosclerosis, restenosis or hypertension (Scholz, et al., 2000).

The arteriogenesis concept

In order to explain the rapid proliferation of collateral arteries, the confinement of collateral proliferation to only certain vessels within a largely non-ischemic region, the hemodynamically unfavorable corkscrew formation and the obvious independence of collateral growth from tissue need

(either beyond or below compensation, depending on the situation) we developed the following concept of “arteriogenesis” (Ito, et al., 1997a, Schaper, et al., 1999):

After constraint of the main supplying arterial vessel blood flow is diverted through preexisting bypassing arteriolar anastomoses. This leads to a dramatic increase of blood flow velocities and thus of shear forces in these small preexisting shunts. In order to compensate for altered hemodynamic forces the vessel remodels in order to achieve baseline shear force levels. This remodeling process involves outward remodeling processes, like vascular distension via proliferation of endothelial and smooth muscle cells, as well inward remodeling processes, like neo-intima formation. The compensatory mechanisms depend on local hemodynamics that are likely to vary along the already curved and bent preexisting shunt and continue to vary as the growth process proceeds. The whole process comes to a standstill as soon as the preset shear force level is reached in a particular part of the vessel independently from the accomplished perfusion level in the collateral dependent region.

The concept of arteriogenesis has important clinical implications. It explains why the development of collateral arteries often is insufficient especially in “no-option” patients with multple stenotic vessels and interventions, where stenotic vessels feed collateral arteries to occluded vessels and they again feed collateral vessels to other occluded arteries. In order to achieve a lasting therapeutical effect without constant stimulation it might be necessary to mold the remodeling process into the direction of outward remodeling rather than enhancing the whole process of collateral growth.

Although the concept of arteriogenesis appeared to be plausible and promised to render answers to very important questions concerning the therapeutic enhancement of collateral growth, the mechanisms at the cellular and subcellular level were largely unknown at the beginning of the SPP 1069.

Macrophage accumulation is a hallmark of arteriogenesis

A clue towards the resolution of the cellular mechanisms appeared to reside in the accumulation of macrophages around growing collateral vessels. Previous studies demonstrated that collateral growth is associated with the accumulation of macrophages around growing vessels and that activation of monocytes and macrophages enhanced vascular growth (Arras, et al., 1998, Heil, et al., 2002, Ito, et al., 1997b, Polverini, et al., 1977). Schaper et al. were able to show in 1976 that monocytes/macrophage accumulation was associated with collateral growth in the dog heart (Schaper, et al., 1976). In analogy to many other human pathologies in which macrophage accumulation plays an important role it was assumed that these macrophages originate from circulating monocytes that home to the activated collateral vessel (Schaper and Ito, 1996, Schaper, et al., 1999). In fact we were able to demonstrate that local infusion of monocyte chemoatractant protein-1 (MCP-1) into the collateral vasculature enhanced significantly arteriogenesis (Ito, et al., 1997b). Heil et al. were able to demonstrate a dramatic augmentation of collateral conductance in the rebound phase after 5 FU treatment in rabbits and mice when monocyte concentrations rose more than 10-fold the normal level (Heil, et al., 2002). These studies indeed indicated that circulating inflammatory cells are sufficient for the induction of vascular growth, in particular arteriogenesis. Based upon these findings the hypothesis was conceived that after occlusion of the main blood supplying vessel high shear forces in preexisting arteriolar shunts lead to the upregulation of cell adhesion molecules and thus to increased homing of monocytes into growing vessels (Schaper, et al., 1999). The question, however, whether monocyte migration and macrophage accumulation were required for collateral growth and how they contribute to collateral growth remained unanswered.

The “arteriogenesis paradox”

The cardinal problem of the arteriogenesis hypothesis was that most data available about shear force, cell adhesion molecule and chemokine regulation as well as monocyte adhesion rather suggested that monocyte accumulation primarily occurred under low rather than high shear force conditions (Ando, et al., 1994, Luscinskas, et al., 1996, Patrick and McIntire, 1995, Resnick and Gimbrone, 1995, Sampath, et al., 1995, Springer, 1994, Tsao, et al., 1996, Tsuboi, et al., 1995, Walpola, et al., 1993). According to available data either shear force is down- and not upregulated in collateral vessels or the observed massive accumulation of macrophages around collateral vessels is not due to an increase of monocyte homing. There was no available data on shear force regulation in collateral vessels and the existing data did not prove that increased monocyte homing is responsible for macrophage accumulation around collateral vessels.

Cell adhesion molecules and cytoskeletal proteins as possible mechanotransducer

On the molecular level, one of the key questions of the arteriogenesis concept was how mechanical hemodynamic forces are translated into biochemical signals and how these biochemical signals are again translated into mechanical actions like migration and cytokinesis (Helisch and Schaper, 2003 Jan). Despite reports about “shear stress response elements” in the promotor regions of growth factor genes, the most appealing model of mechanotransduction is the “tensegrity” model pioneered by Donald Ingber (Ingber, 2003). According to this model mechanical forces lead to an integrated alteration of the energy status of the different cytoskeletal components and thus biochemical reactions at certain key structures of the cytoskeletal network in particular at focal adhesion sites. These focal adhesion sites contain apart from integrins and cell adhesion molecules a number of key proteins involved in several signaling cascades. Biochemical reactions at these focal adhesion sites change in turn the energy status of the cyoskeletal network eliciting mechanical activities like migration. We therefore focused our attention on cytoskeletal proteins and cell adhesion molecules when looking for the molecular players of arteriogenesis. Furthermore we needed a molecular explanation for the marked macrophage accumulation of macrophages during maximal collateral proliferation. Thus chemoatractants became a second focus of our attention when trying to reveal the molecular mechanisms underlying arteriogenesis.

METHODS

Within the course of the SPP 1069 program and our projects we established a number of different methods in order to elucidate the mechanisms of arteriogenesis with particular regard to the role of macrophages, chemoatractants and cell adhesion molecules. The core of our laboratory remains the physiological model of collateral growth in the rat hindlimb after femoral artery occlusion. The ability to isolate a single collateral vessel at any time during collateral formation enables us to perform targeted searches for the molecular systems involved in collateral growth (Herzog, et al., 2002). A thorough description of the cellular processes during the different phases of collateral formation was a prerequisite for aligning certain molecular mechanisms to different changes on the cellular and organ level. For this thorough description of morphological changes we primarily used immunohistochemical stainings and confocal microscopy, proliferation assays after BrdU infusions and stereoscopical post mortem micro-angiographies. These morphological studies were complemented with blood flow and conductance measurements using fluorescent microspheres as well as ultrasonic flow probes and pressure recordings with microcatheters. The same model is suitable for functional studies using direct infusions of test substances into the collateral circulation via osmotic minipumps. We recently extended our portfolio of in vivo models towards the mouse

model of peripheral ischemia allowing us to study certain molecular systems in transgenic- and knock-out models, which already lead to a publication, now in press in the Journal of Clinical Investigation (Horst, et al., 2006). In our search for molecular systems involved in the different phases of collateral growth we applied different methods ranging from antibody generation, biochemical purification, 2-D gel analysis and mass spectrometry, bio-panning of single-chain antibody phage expression libraries to laser dissections and real-time PCR (Obermeyer, et al., 2003). In particular collateral proteomics including biochemical purifications, 2-D gel analysis and mass spectrometric identification of molecular target systems became part of the core expertise of our laboratory. Our in-vivo models are complemented by in vitro analysis of certain molecular systems in cell culture systems of endothelial cells, smooth muscle cells, macrophages and CD 133 positive endothelial progenitor cells. We use a spontaneously immortalized rat heart endothelial cell line as well as primary cells from human origin and cells isolated via magnetic bead sorting from peripheral blood (CD 133) and tissue (rat macrophages). Phenotypic analysis is performed via flow cytometry as well as via fluorescent staining of cytospins, where cellular yield is not sufficient for flow cytometric analysis. Functional assays include proliferation assays after BrdU incorporation, migration assays (Videomicroscopic analysis as well as Boyden Chamber), sprouting assays as well cell adhesion assays. Molecular pathways are primarily studied on the protein level using immunoprecipitations and western blotting as well as different activity assays. For the investigation of the functional relevance of certain molecular targets we use functional antibodies as well as si-RNA, a method that has been established in our laboratory 2 years ago. Currently we are seeking to establish methods for stable si-RNA transfections that enables us to study long term effects in vitro and also the functionality of certain molecular systems in vivo.

RESULTS

Morphological and cellular changes in a preexisting arteriolar shunt becoming a collateral artery in the rat hindlimb

As mentioned above we proposed in our concept of arteriogenesis that the growth of collateral vessels is a local phenomenon and that the main molecular mechanisms occur at the single vessel level. In order to elucidate the molecular mechanisms of arteriogenesis we therefore needed to develop and describe a model that allowed the investigation of collateral growth in a single vessel from the very beginning. We succeeded to identify a certain preexisting arteriolar shunt in the rat hindlimb, the remnant of the arteria ischiadica, still one of the main blood supplies of the hindlimb in birds that connects the internal iliac artery and the arteria poplitea (Herzog, et al., 2002). Upon femoral artery occlusion this preesxisting shunt remodels in order to form the major collateral vessel. As seen in Figure 1 Corrosion casts of both lower extremities of Sprague Dawley rats revealed a preexisting arteriolar shunt connecting the internal iliac artery to the popliteal artery with a defined stem, midzone and reentry region. The anatomy was reproducible in 5 experiments. The vessel had only a slight tortuous appearance. Midzone diameter was 140 μm. After 2 weeks and 2 months of femoral artery occlusion this vessel had become the most prominent collateral artery with an extremely tortuous course and a midzone diameter of 300 μm. Localization and anatomy was the same in all casts examined and was identical for both the dormant anastomose as well as for the fully developed collateral vessels indicating that indeed the main collateral artery grows from this preexisting arteriolar anastomose. We were able to reproduce the anatomic localization of the preexisting arteriolar shunt on stereoscopic post-mortem angiographies obtained before as well as 1 week and 3 weeks post femoral artery occlusion for analysis of collateral anatomy and development using computerized imaging systems. Again the preexisting anastomose was clearly visible at a

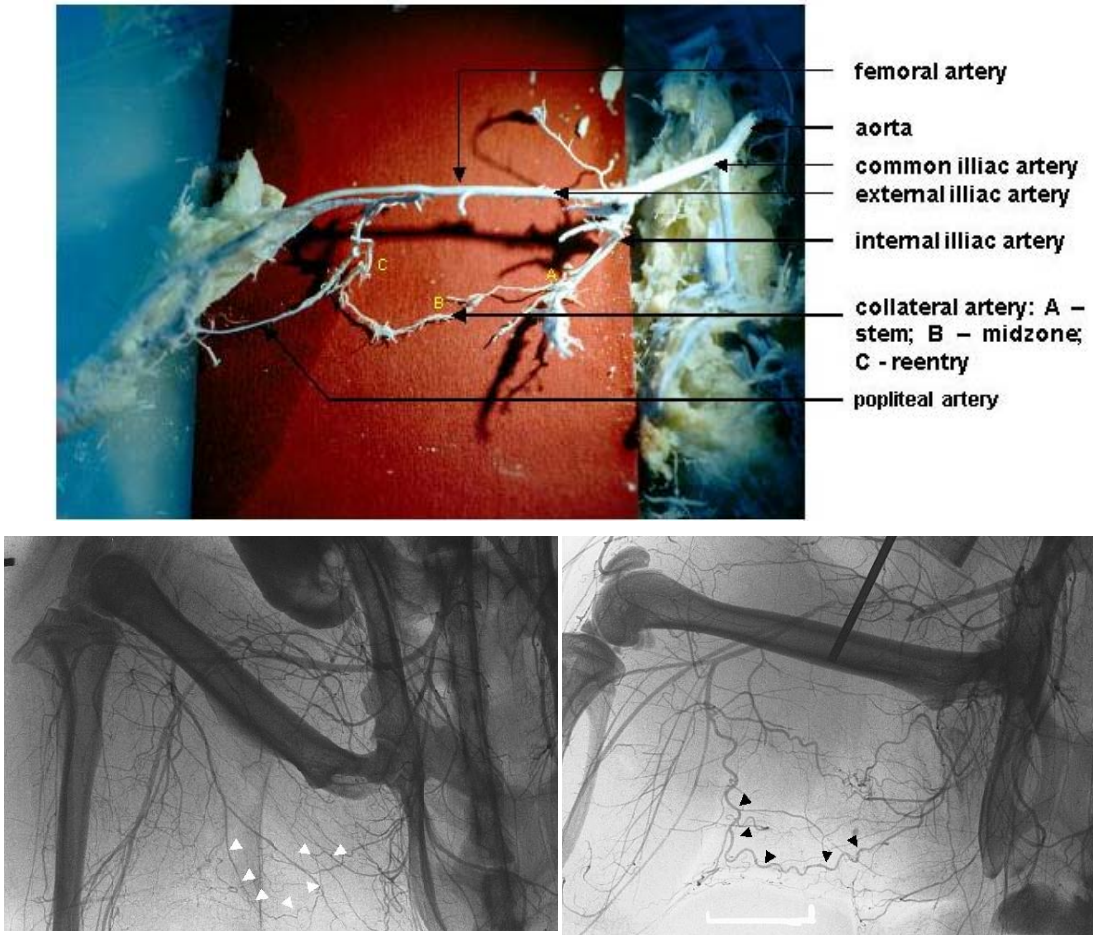


Fig. 1: Persistent arteria ischiadica remodeling into main collateral vessel. Top panel: corrosion cast of rat hindlimb vasculature without occlusion shows preexisting collateral vessel (arteria ischiadica) connecting internal iliac artery and popliteal artery. The same vessel (left lower panel, white arrows) remodels in order to give rise to the main collateral vessel (lower right panel, black arrows.

reproducible anatomical site in all animals studied and identified as the vessel becoming the main collateral artery (Fig. 1)

Midzone index (midzone diameter to diameter of femoral artery distal to the ligature) of the main collateral vessel increased form 0.17±0.05 before occlusion to 0.46±0.07 after 7 days of occlusion ($P<0.001$; Fig. 3B). Interestingly midzone index dropped again to 0.31±0.04 after 21 days of occlusion indicating regression of the collateral vessel after 7 days of occlusion ($P<0.02$). We observed not only an enlargement in diameter of the collateral vessel but also a distension of the length due to an increase in vessel tortuousity. Total length of the collateral artery increased by 21 % within 7 days after occlusion and significantly by 39% within 21 days after occlusion ($P<0.02$). Staining of the collateral vessel and surrounding structures for BrdU after continuous subcutaneous infusion of the thymidine analog during the first weak after femoral artery occlusion revealed that proliferation was restricted to preexisting arteriolar anastomoses (Fig. 2). No proliferation was seen in directly neighboring vessel of similar size and vessel architecture that did not connect the ischemic to the non-ischemic territory as revealed by post mortem angiography before obtaining the tissue sections. Proliferation kinetics of the collateral artery were obtained after continuous BrdU infusion for 1 day, 3 days, 7 days and 21 days. Positively stained nuclei of endothelial and smooth muscle cells represented the total amount of cells proliferating within the observed period and were related to the total amount of nuclei of the vascular tissue. This allowed the calculation of accumulative proliferation

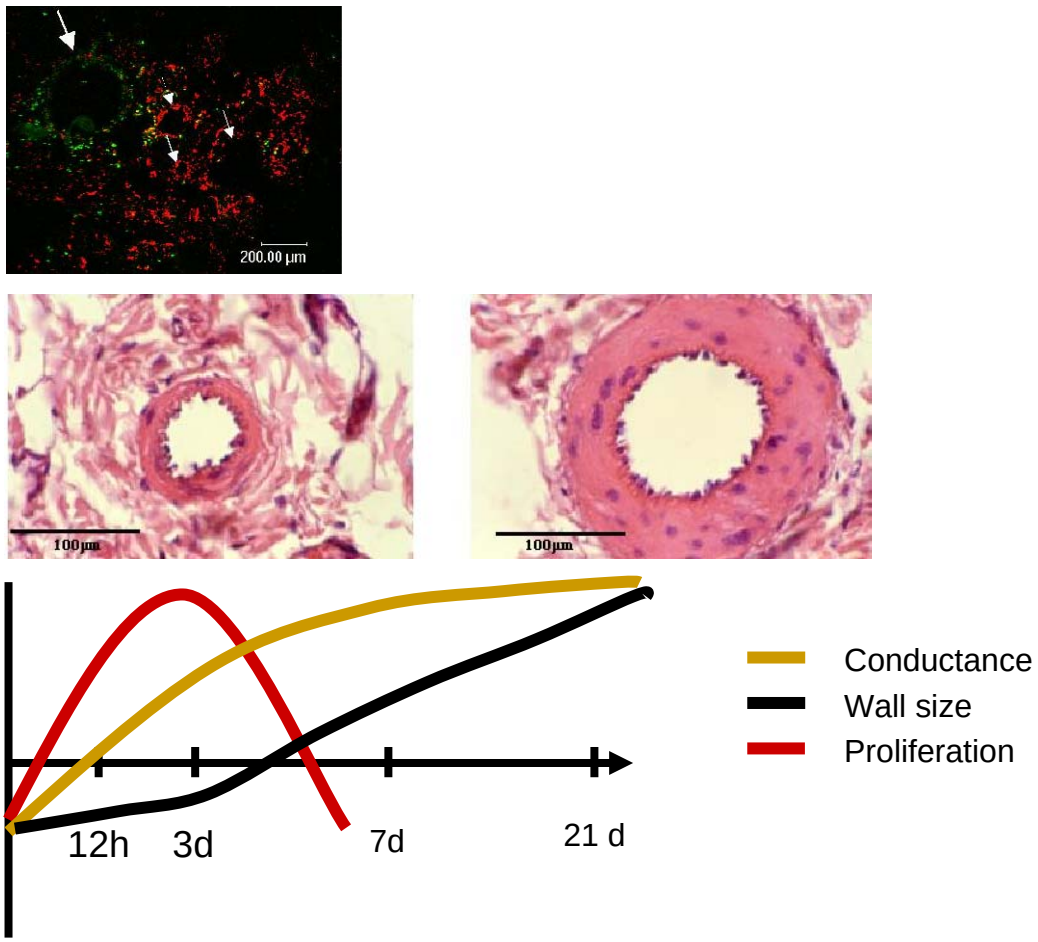


Fig. 2: Time course of development of collateral conductance, proliferation and wall size. Proliferation begins within 12h after femoral artery occlusion in the rat hindlimb, is maximal during the first 3 days and is restricted to the collateral artery (Top left panel; green fluorescent nuclei, showing BrdU incorporation). The main rise in collateral conductance is observed during the first week after femoral artery occlusion. After 7 days proliferation and rise in conductance slows down considerable. Instead, remodeling continues leading to a profound increase in wall to lumen ratio (Top middle panel: collateral vessel before occlusion; top right panel collateral vessel 4 month after femoral artery occlusion).

indices. The first BrdU positive nuclei were seen 3 days after occlusion indicating that proliferation started between 24 h and 3 days after occlusion. The main rise of proliferative index occurred between day 1 and 3 after occlusion reaching 35% at day 3. At day 7 proliferative index had risen to 59 %. From the onset of the second week after occlusion there was no further measurable proliferation. Proliferative Index at day 21 after occlusion was even slightly lower than at day 7 after occlusion in keeping with our angiographic data (24h vs. 3 days: $P<0.01$; 3 days vs. 7 days: $P<0.01$). Hematoxillin eosin staining of perfusion fixed tissue revealed pronounced remodeling processes beginning 7 days after occlusion (Fig. 2). Remodeling was particularly pronounced between day 7 and day 21 after femoral artery occlusion. Within this time an asymmetrical neo-intima was formed including several layers of smooth muscle cells and several laminae elasticae internae. Within 4 months after occlusion, vessel wall thickness increased several fold whereas lumen diameter of the main collateral vessel in the rat only doubled.

As schematically shown in Figure 2 we concluded that collateral growth is a biphasic process. It begins with a very rapid onset of massive proliferation leading to significant outward remodeling within a week after occlusion. As shown in previous studies on the rabbit hindlimb in our group and studies on the rat hindlimb in other groups this early proliferative phase is associated with the main

rise in collateral conductance. Proliferation slows down considerably or even comes to a standstill after a week of occlusion and is succeeded by a phase of intense inward remodeling that leads to neo-intima formation and pronounced increase of vessel wall diameter. In the remodeling phase we even may encounter regression of collateral lumen diameter as seen in our model.

Although reasoning based upon these findings indicated that not a general chemical factor such as ischemia but local acting hemodynamic forces was responsible for collateral growth we were not able to deliver direct evidence for the hypothesis that shear force is the primary stimulus for arteriogenesis. The ability, however, to identify a collateral artery at any stage after induction as presented in this paper was the prerequisite for modeling of flow patterns along a preexisting arteriolar shunt and their evolvement during collateral growth. Interventional studies in this model aimed at candidate genes and proteins help to identify mechanisms involved in proliferation and remodeling of collateral vessels at different stages of their development.

Collateral growth involves primarily local proliferation, migration and differentiation of tissue resident precursor cells possibly originating from the bone marrow

In an attempt to further identify the cellular mechanisms of collateral growth we took a closer look at the accumulation of circulating cells in particular macrophages in collateral vessels. We quantitatively correlated macrophage accumulation to collateral proliferation and then investigated whether a pronounced reduction of circulating cells including monocytes has an effect on collateral proliferation. We also investigated whether a significant number of circulating cells incorporate in the proliferating vessel (Khmelewski, et al., 2004).

Local macrophage accumulation around collateral vessels paralleled the time course of collateral proliferation after femoral artery occlusion. Macrophage accumulation was maximal at day 3 after femoral artery occlusion increasing three- to sixfold in comparison to control vessels (Fig. 3). The increase involved both immature macrophages (ED 3/TRPM 3 positive) and mature tissue macrophages (ED 2/KiM2R positive). Between day 3 and day 7 the number of macrophages accumulating around collateral vessels decreased again significantly by more than a third. Proliferative index was maximal during the first 3 days after occlusion and decreased towards day 7 paralleling the decline in the number of macrophages. Fluorescently labeled blood cells injected directly after femoral artery occlusion into the bed of the collateral circulation were clearly detectable 3 days after occlusion in the circulating blood. In particular large numbers of cells resembling monocytes and lymphocytes as well as thrombocytes were labeled. Fluorescent lymphocytes accumulated as a rim of cells around the pulpa of the spleen. Within the operation wound we detected cylindric fluorescent blood clots as well as fluorescent leukocytes. In contrast to spleen and wound tissue, not a single fluorescent cell was visible in tissue surrounding collateral vessels or in the collateral vessel itself indicating that homing of blood cells to collateral vessels does not occur during maximal collateral proliferation and macrophage accumulation. Monocyte depletion did not affect collateral proliferation and concomitant macrophage accumulation. Cyclophosphamide treatment led to a prounounced pancytopenia (Pre-treatment:10.5±1.4 x 10^9/l; time of occlusion:0.6±0.4 x 10^9/l ; 3 days post occlusion: 0.1±0.07 x 10^9/l) (Fig. 4). Differential counts revealed that the reduction of leukocytes involved all subpopulations (Data only shown for monocytes in Fig. 3a). Monocytes were reduced to 1% of their original population (Pre-treatment: 9.4±1.1% respectively 0.991 x 10^9/l ; time of occlusion: 10.6±2.7% respectively 0.067 x 10^9/l; 3 days post occlusion: 7±2.8% respectively 0.012 x 10^9/l)(Fig. 4). Despite the pronounced pancytopenia, we observed the same increase in proliferative index in depleted as in non-depleted animals after femoral artery occlusion. (Fig. 4). Furthermore, we observed the same increase in all macrophage subpopulations in monocyte depleted animals as in non-depleted animals (Fig. 4). After staining of

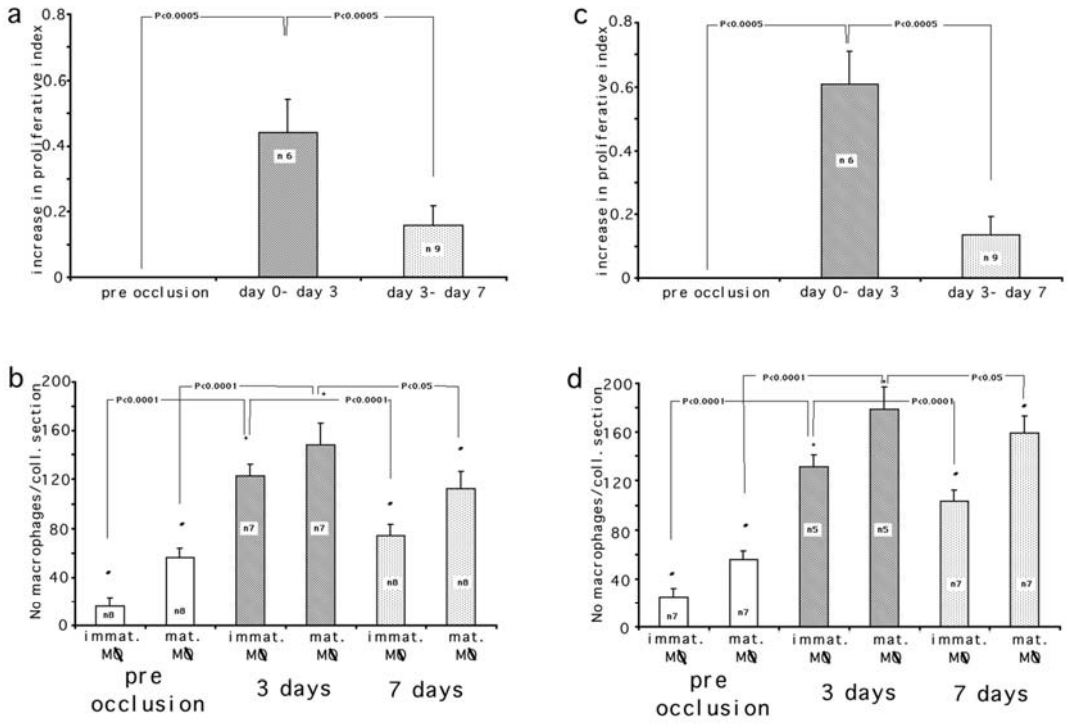


Fig. 3: Time course of macrophage accumulation in relation to collatera proliferation. The accumulation of immature (immat.) and mature (mat.) macrophages (panel b) parallels development of collateral proliferation (panel a). Treatment with the chemokine MCP-1 enhances both macrophage accumulation (panel d) and proliferation (panel c). Both proliferation and macrophage accumulation peaks at day 3 and then wanes off.

collateral sections and bone marrow with antibodies against the stem cell markers c-Kit, SSEA 1 and Thy 1 we detected not a single positively stained cell around the collateral vessel whereas numerous cells were positive for these stem cell markers in the bone marrow. Double staining with ED 2 and a BrdU antibody revealed that all macrophages accumulating around collateral vessels had divided within the first 3 days after femoral artery occlusion. Proliferation also involved endothelial and smooth muscle cell staining for VE-Cadherin and alpha smooth muscle actin. This proliferative response of macrophages and vascular cells was observed in depleted as well as in non-depleted animals. MCP-1 infusion increased both parameters maximally during the first three days after femoral artery occlusion (Fig. 4) and lead to the same rise in proliferative index and enhancement of macrophage accumulation in monocyte depleted animals as in monocyte competent animals (Fig.4). Thus, the increase of mature as well as immature macrophages is not dependent upon monocyte homing. In vitro, we were able to exclude a direct effect of MCP-1 on vascular cells .

In conclusion, we were able to demonstrate that indeed macrophage accumulation parallels maximal collateral proliferation. As also shown by other groups we were not able to demonstrate the acute integration of a significant number of circulating cells in the collateral vessels and we did not detect significant numbers of cells carrying markers of bone marrow derived stem cells in the vicinity of collateral vessels. To our own surprise depletion of circulating monocytes did not have an effect on collateral proliferation and to an even greater surprise this also didn't have an effect on the pronounced macrophage accumulation of collateral vessels. We therefore concluded that during the initial proliferative phase of collateral growth some to date unknown resident progenitor cells proliferate and transdifferentiate into vascular cells as well as into macrophages. This hypothesis was actually confirmed in a follow-up study conducted in collaboration with Süleyman Ergün in which we

were able to demonstrate in aortic ring assays that arterial vessels contain a regenerative zone situated in the adventicial space. This regenerative zone contains CD 34 positive progenitor cells that give rise to endothelial cells, smooth muscel cells and CD 68 positive monocytes/macrophages in absence of circulating cells (Zengin, et al., 2006).

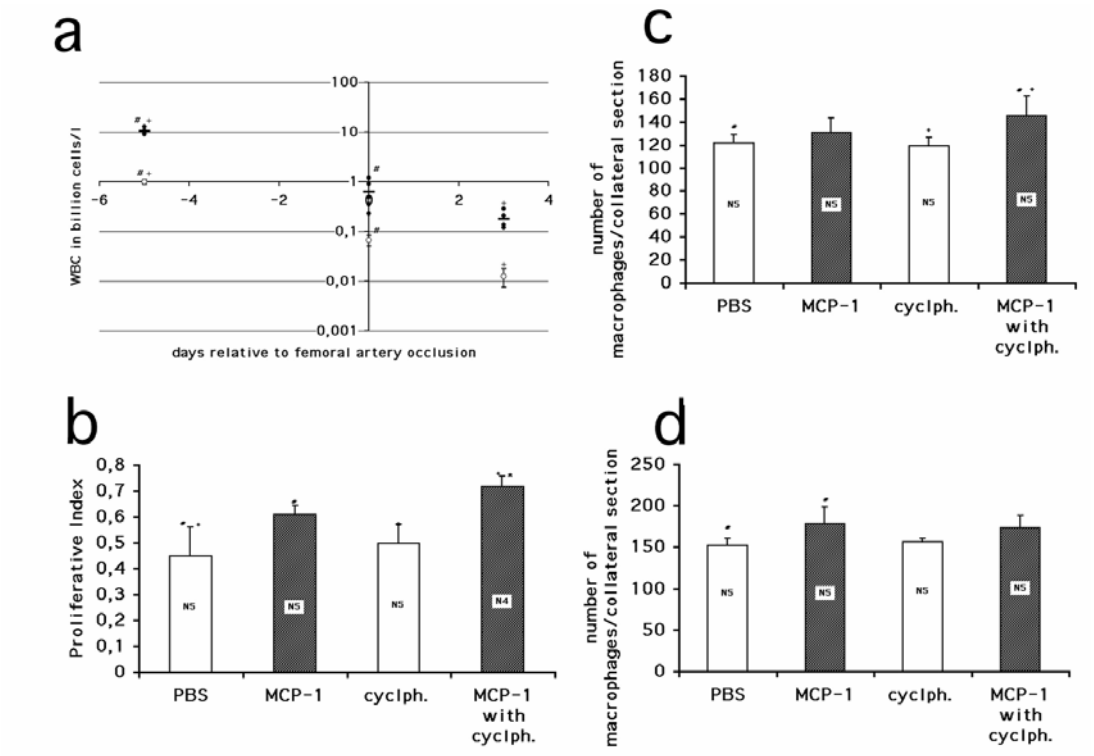


Fig. 4: No influence of reduction of circulating monocytic cells on collateral proliferation and macrophage accumulation. Panel a: Pre-treatment with cyclophosphamid leads to a reduction of circulating monocytic cells to less than 1% of the original population at day 3 after femoral artery occlusion. Panel b: No effect of monocyte depletion on collateral proliferation and its enhancement via MCP-1 treatment. Panel c: No influence of monocyte depletion on the accumulation of immature macrophages 3 days after femoral artery occlusion and the enhancement via MCP-1. Panel d: No influence of monocyte depletion on the accumulation of mature macrophages 3 days after femoral artery occlusion and the enhancement via MCP-1.

Time course of NO-donating enzyme expression during collateral growth suggests presence of only a very small time window that allows homing of circulating cells

Another line of evidence that most of cellular proliferation and differentiation during collateral growth occurs locally came from studies investigating expression levels of NO-donating enzymes and their role during collateral growth (Sager, et al., 2006). Arteriogenesis and its enhancement via VEGF-A, FGF 2 or exercise is NO dependent (Lloyd, et al., 2001). NO, however, counteracts pro-arteriogenic macrophage recruitment (Tsao, et al., 1995 Dec 15). In order to resolve this paradox we investigated eNOS-, iNOS-, PKG-I-, p-VASP-, sGC- and p-Akt protein expression in growing rat collateral vessels 12h, 24h, and 72h after femoral artery occlusion and correlated expression patterns to macrophage recruitment and shear forces calculated from blood flow in the collateral stem region (transonic flow probe). Interdependency of macrophage recruitment and NO production was investigated via a combination of local MCP-1 infusion (osmotic minipump) and NO synthesis blockade (oral L-NAME). 7 days post occlusion we assessed collateral vessel number (post mortem angiographies),

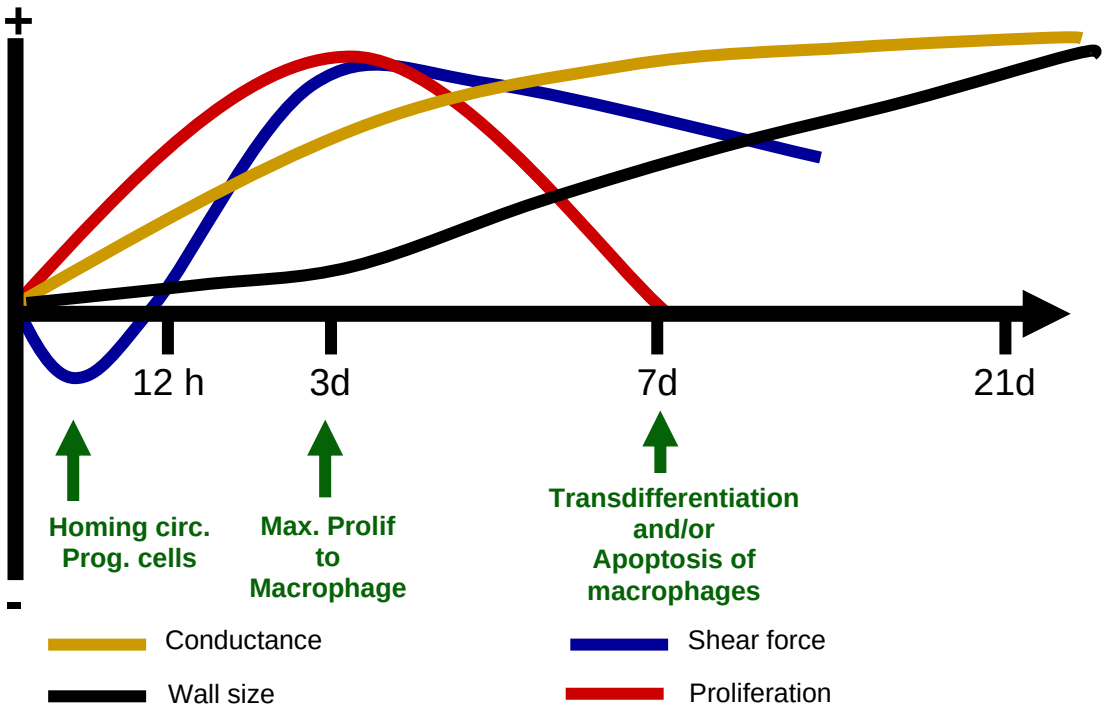


Fig. 5: Presumed time course of shear force, conductance, wall size and proliferation as well as homing, proliferation and differentiation of circulating cells in collateral vessels after femoral artery occlusion. Upon occlusion shear force initially is downregulated allowing homing of circulating progenitor cells. Within 24 h shear force is upregulated due to peripheral vasodilatation, further homing is impeded and originally homed cells start to proliferate and transdifferentiate locally. At this time there is a maximal proliferation of collateral vessels paralleling the main increase in collateral conductance. After 7 days this first proliferative phase is followed by a remodeling phase. Conductance- development and proliferation slows down and macrophages transdifferentiate or die an apoptotic dead. Instead an increase in wall to lumen ratio, neo-intima formation and increased cork-screw deformations are observed.

proliferative index (PI: BrdU incorporation) and amount of perivascular macrophages (ED2 staining). eNOS-, iNOS-, PKG-I-, p-VASP and p-Akt expression was significantly downregulated 12 h post occlusion correlating with reduced shear force and initial macrophage recruitment. 24 h post occlusion expression of NO-donating enzymes and shear force had risen significantly beyond normal levels. L-NAME administration lead to a reduction of visible collateral vessels but not of PI in vehicle treated animals despite a significant increase in macrophage levels and had no impact on the arteriogenic effect of MCP-1. These findings indicate that NO-concentrations and shear forces are initially downregulated in collateral vessels allowing the recruitment of circulating cells that divide and differentiate locally during the following days. Within 24 h shear force and NO levels rise due to ischemia triggered peripheral vasodilatation inhibiting further recruitment of circulating cells. This proposed sequence of events is outlined in Figure 5.

MCP1 receptor CCR2, a novel homing receptor for endothelial precursor cells, is rapidly downregulated in proliferating and differentiating cells

In our search for molecules possibly involved in the initial recruitment of precursor cells into collateral arteries we detected that CCR2 the receptor for MCP-1, a chemokine that had a pronounced proarteriogenic effect, was expressed on CD 133 positive endothelial progenitor cells directly after isolation (Ito, et al., 2006a). We demonstrated in this study that $CD133^{+}$ cells from G-CSF mobilized blood, which comprise precursors with hematopoietic, endothelial and hemangioblastic potential,

express CCR2 the main functional receptor for MCP-1. Given the fact that the population of CD 133 positive cells is very small in peripheral blood an pronounced local proliferation leads to the dilution of any fluorescent marker it is conceivable that we were not able to detect any bone marrow derived fluorescent cells in the growing collateral vessel even if at the beginning a small number of progenitor cells were homing to the collateral vessel. Stimulation with the chemokine MCP-1 resulted in migration but not in proliferation of the progenitor cells.

Diabetes mellitus II and arterial hypertension affect collateral growth in different phases

Further indication of the limitation of macrophage involvement to the early proliferative phase of collateral development and the physiological importance of the late remodeling phase came from experiments investigating the influence of different risk factors, namely the metabolic syndrom and arterial hypertension on collateral growth (Ito, et al., 2006b). We investigated at which level two prominent risk factors, diabetes mellitus type II and arterial hypertension, impair collateral formation and assessed proliferative index (PI; BrdU infusion), macrophage accumulation (MØ; ED 2 staining), collateral score (post-mortem angiography), collateral conductance (CC= collateral flow/pressure gradient; under maximal vasodilatation) and effect of MCP-1 treatment one week after femoral artery occlusion in normotensive Zucker Fatty Diabetic (ZDF) rats and control animals (ZDL rats). Results were compared with those of ZDF and ZDL rats rendered hypertensive via the Goldblatt method. While diabetic animals showed reduced collateral proliferation and macrophage accumulation (PI: ZDF vs. ZDL: 2.62±1.85% vs. 22.25±3.33%; p=0.001; MØ: 39.83±18.06 vs. 74.00±5.44; p<0.01) hypertensive animals had reduced collateral conductances without altered macrophage accumulation and a smaller reduction in collateral proliferation (CC: Hypertensive vs. normotensive: 0.747±0.427ml/min/100mmHg vs. 2.76±1.914 ml/min/100mmHg; p=0.011; MØ: 70.25±7.63 vs. 74.00±5.44; NS; PI: 12±2.71% vs. 22.25±3.33%; p<0.01). MCP-1 treatment only had significant pro-arteriogenic effects in diabetic but not in hypertensive animals. We concluded that diabetes mellitus impairs collateral proliferation via impaired monocyte/macrophage recruitment whereas arterial hypertension influences the later remodeling phase of collateral growth which is unresponsive to increased monocyte recruitment. Only the enforcement of the remodeling phase in arterial hypertension but not the impairment of collateral proliferation and macrophage recruitment in diabetes mellitus had a profound influence on collateral conductance suggesting that the remodeling phase constitutes the rate limiting step with regard to collateral functionality.

Identification and characterization of Vimentin as important regulator of endothelial migration and proliferation and possible mechanotransducer during collateral growth

Another important question regarding the molecular mechanisms of arteriogenesis apart from macrophage recruitment concerned the transduction of mechanical forces into biochemical signals and vice versa. Mechanotransduction in endothelial cells is not only a key to the understanding of the influence of local hamodynamic forces on vascular plasticity but also to the understanding of principle cellular activities like migration and proliferation, which are essential to any form of vascular formation. In pilot experiments using 2-D gel electrophoresis and mass spectrometry to identify molecules involved in arteriogenesis we discovered that vimentin was upregulated in collateral vessels. In a parallel proteomic analysis of migrating and non-migrating endothelial cells we also identified vimentin as a major protein discriminating migrating from non-migrating endothelial cells. We subsequently performed siRNA assays, in which vimentin expression in migrating endothelial cells was suppressed to examine whether the intermediate filament has functional importance in cell migration (Obermeyer, et al., 2006). Transfection conditions were optimized for our cell line using

fluorescencelabeled siRNA. Using Lipofectamine (Invitrogen) about 99 % of the treated cells were succesfully transfected without cytotoxic effects. However, siRNA uptake may not reflect succesful suppression of the target gene and gives no information about functional effects of the target protein. Therefore, we examined vimentin expression in transfected cells in Western blots and performed migration and proliferation assays. The vimentin suppression in vimentin-siRNA transfected cells is illustrated in a Western blot. Untreated cells and cells transfected with scrambled siRNA oligo´s were used as controls. An antibody against PI3-kinase subunit p85 served as a loading control. In the si vimentin sample the vimentin expression is barely detectable, pointing to a clear suppression of the target protein.

Following the successful protein suppression, we examined potential functional concequences in endothelial cells. To this purpose, we performed migration assays with time lapse videomicroscopy. The migration velocity of vimentin-suppressed endothelial cells is significantly reduced compared to si-control transfected cells. The vimentin-siRNA transfected cells retained with 0.05 (± 0.008) µm/min only half the migration velocity of si-control transfected cells (0.1 (± 0.01) µm/min). The enormous reduction in migration speed of vimentin suppressed cells could also be verified in the Boyden chamber - a more conservative migration assay. Results comparable to the videomicroscopic analysis. In an overnight Boyden chamber assay, only 130.4 (± 20) vimentin-suppressed cells passed the filter, whereas 226 (± 33) si-control transfected cells migrated towards the lower chamber. This signifies a reduction of migration capacity of about 42,5 %. Thus, we could demonstrate a significant function of the intermediate filament vimentin in endothelial cell migration. However, the cytoskeleton plays a decisive role in many other cellular functions, including cell proliferation. Hence, we investigated if the suppression of vimentin expression also affects the proliferative activity of endothelial cells. After a 5.5 hour BrdU-incubation, 37 % of the si-control transfected cells stained BrdU positive. However, only 21 % of the si-vimentin transfected cells stained positive for BrdU. Hence, the diminished vimentin expression caused a reduction in proliferative activity of about 43 %.

These results clearly indicate, that vimentin exerts a regulatory role in endothelial cell migration and proliferation. Given that the vasodilator-stimulating phosphoprotein (VASP) is a known adaptorprotein linking the system to signal transduction pathways, we stripped a Western blot after the detection of vimentin and analyzed the VASP phosphorylation state (Bear, et al., 2000, Harbeck, et al., 2000). The suppression of vimentin expression goes hand in hand with a decrease of pSer239-VASP, whereas it has no influence on total VASP expression (not shown). Since the phosphorylation state of VASP regulates actin filament dynamics, we wanted to ensure that there are no side effects of vimentin suppression on actin expression which could be related to pSer239- VASP (Galler, et al., 2005, Harbeck, et al., 2000). Therefore, we determined actin expression in transfected cells. The suppression of vimentin does not alter actin expression. This excludes actin-dependent changes of pSer239-VASP expression as a possible side effect of vimentin suppression. Consequently, we show for the first time, that a decreased expression of pSer239-VASP can also be directly related to vimentin expression without affecting actin expression.

With regard to endothelial cell migration, our results suggest, that both proteins, Vimentin and VASP are dependently involved in this process. The migration of a cell is accomplished by lamellipodia protrusion via actin polymerization and attachment to the extracellular matrix. These actions must be reversible to allow cell motion (Huttenlocher, et al., 1995, Ridley, et al., 2003). We hypothesize, that vimentin suppression leads to a diminished phosphorylation of VASP which in turn causes polymerization of actin filaments to form lamellipodia and filopodia. Because VASP is arrested in the unphosphorylated state in cells with decreased vimentin expression, the cyclic depolymerization of actin filaments is inhibited, resulting in decelerated motility of the cell (see

Fig. 6). In conclusion, our study demonstrates for the first time, that an intact vimentin network is essential for the migratory process in endothelial cells and, that it seems to function by regulating the VASP-actin interaction.

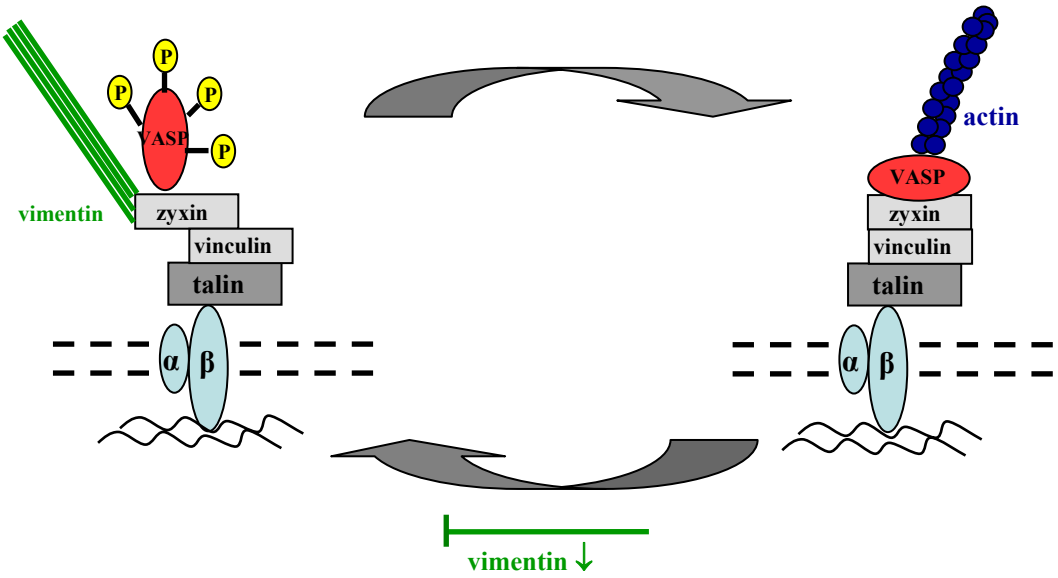


Fig. 6: Schematic presentation of possible role of vimentin in cell migration. Under normal conditions actin polymerizes and depolymerizes alternately to allow cell motion. VASP dephosphorylation is necessary for Actin polymerization. Our results suggest that Vimentin could hold VASP in a conformation in which it can easily be phosphorylated and Actin depolymerizes. Under conditions of suppressed Vimentin expression, the phosphorylation of VASP is inhibited, Actin dynamics are stopped at a polymerized level and the cell is solidified.

The cell adhesion molecule CEACAM-1 plays an important role in vascular remodeling and collateral growth

Cytoskeletal components like Vimentin are directly or (in the case of Vimentin) indirectly connected to the cytoskeleton. According to the Tensegrity model of Donald Ingber the whole cytoskeletal composition consisting of microtubules and intermediate filaments connected to focal adhesions containing various active proteins, cell adhesion molecules and integrins allows the transduction of mechanical forces into biochemical signals and the integration of biochemical signals to elicit mechanical activities of the cell, like migration and cytokinesis (Ingber, 2003). One of the cell adhesion molecules the role of which had not been studied in collateral growth which however appeared to be closely related to endothelial cell migration and proliferation and co-localizes with $\alpha_V\beta_3$ integrin at the invasive front of the extravillous trophoblast was CEACAM1 (Bamberger, et al., 2000, Brummer, et al., 2001, Ergun, et al., 2000). In order to evaluate the *in vivo* impact of CEACAM1 on vascular growth and in particular on arteriogenesis, we used two different murine models: We generated CEACAM1^{endo+} mice on an FVB/N background with additional CEACAM1-L expression under the endothelial cell-specific promoter control of the Tie2 receptor tyrosine kinase (Horst, et al., 2006). To observe the functional consequences of endothelial CEACAM1 deficiency, we also used *Ceacam1*$^{-/-}$ mice with systemic deletion of the *Ceacam1*-gen. For the CEACAM1^{endo+} transgenic line, we modified a construct by T.N. Sato. Transgenic founder lines were identified by Southern blotting, and two transgenic lines were used in the experiments described here. We also

used two independent lines of the *Ceacam1*$^{-/-}$ mice in our experiments. Transgenic and knockout mice were genotyped by PCR (data not shown). To verify CEACAM1 over-expression in the endothelia of CEACAM1^{endo+} transgenic animals, we double-labelled primary endothelial cells from lungs with anti-PECAM1- and anti-CEACAM1-antibodies in flow cytometry. We also confirmed CEACAM1 over-expression of endothelial cells in adult animals by Western blotting and RT-PCR. Macroscopically, no overt vascular damage or alterations were observed under physiological conditions in *Ceacam1*$^{-/-}$ or CEACAM1^{endo+} transgenic mice.

To validate a functional role for CEACAM1 in vascular remodeling in vivo, we investigated vascular growth after induction of ischemia via unilateral femoral artery occlusion in CEACAM1^{endo+} and *Ceacam1*$^{-/-}$ mice and their respective WT littermates. These experiments were based upon the observation that CEACAM1-expression is up- regulated in synergy with other angiogenic factors in ischemic cardiac muscle (Muller, et al., 2005).

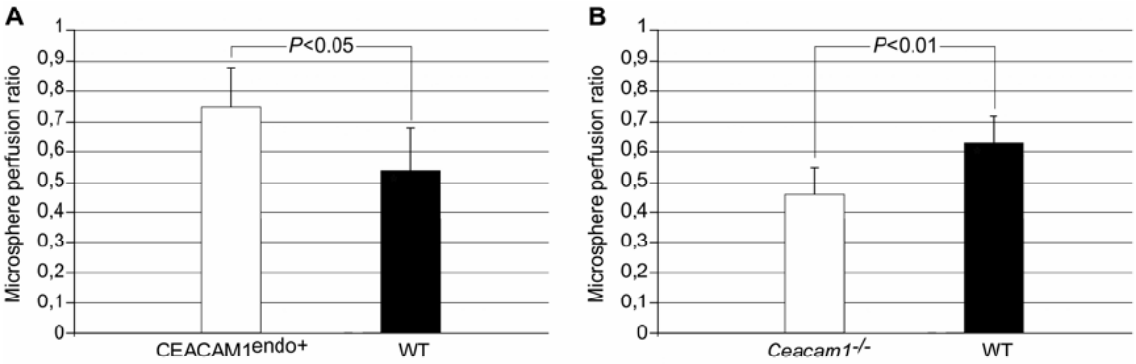


Fig. 7: Collateral blood flow is enhanced in mice overexpressing CEACAM1 under the Tie2 promotor and reduced in mice deficient in CEACAM. Panel A: Enhanced left to right microsphere ratios in calf muscels of mice overexpression CEACAM1 under the Tie2 promotor (CEACAM1endo+) as compared to the respective wild type animals (WT; n=5 in each case). Panel B: reduced left to right microsphere ratios in calf muscles of mice deficient in CEACAM1 (CEACAM 1-/-) as compared to respective wild type animals (WT; n=6 in each case).

As functional parameter we determined collateral blood flows one week after femoral artery occlusion via fluorescent microspheres employing established methods of blood flow determination in mice (Jacobi, et al., 2004). In addition we determined max systolic blood flows via a transonic flow probe placed at the collateral stem region as indirect parameter of collateral conductance. Finally we determined vascular growth via capillary and arteriolar counts in the different calf muscels. The ischemic hindlimb model had the advantage that vascular growth was investigated in a physiologically relevant setting without confounding pathologies and implantation of xenotypic cells or biochemicals as in most mouse tumor models. Perfusion recovery in the ischemic leg was expressed in % of perfusion of the non-ligated leg as described previously (Jacobi, et al., 2004). These data are summarized in Figure 7. Equal perfusion of both kidneys served as internal control of adequate microsphere mixing and injection. Experiments and data analysis were performed by completely blinded investigators. In agreement with the results obtained in neo-vascularization of Matrigel plugs, the relative perfusion of ischemic versus non-ischemic limbs 7 days after surgery was significantly higher in CEACAM1^{endo+} mice compared to their WT litter mates (Fig. 7: CEACAM1^{endo+}: 75 % ± 5%; WT: 54 % ± 6%, $P < 0.05$). Perfusion in ischemic hindlimbs in *Ceacam1*$^{-/-}$ mice was significantly lower opposed to their genetically unaltered siblings (*Ceacam1*$^{-/-}$: 45 % ± 3; WT: 62 % ± 3; $P < 0.01$). Maximal blood flow amplitudes as determined by transonic flow probes also showed a clear tendency towards higher blood flows in CEACAM1^{endo+} mice in the ischemic hindlimb as compared to *Ceacam1*$^{-/-}$ animals. These differences, however, did not reach statistical significances due to large

standard deviations. The alteration in blood flow recovery between the different wild type groups is in accordance with previous studies showing a marked variation in the arteriogenic response among different mouse strains (Scholz, et al., 2002). The lower perfusion rates in *Ceacam1*$^{-/-}$ mice correlated to different vascular densities in ischemic calf muscles after femoral artery ligation, as demonstrated in Figure 7: Immunofluorescent labelling of PECAM1 shows here that vascular densities were markedly increased in CEACAM1^{endo+} compared to *Ceacam1*$^{-/-}$ mice.

A short description of “dead end roads” within our SPP 1069 project

In our initial proposals we planned to develop a method to specifically transfect collateral vessels using a collateral targeting antibody developed in our laboratory. According to the original plan a collateral targeting single chain antibody (ScFv ct) created from hybridoma expressing the original monoclonal collateral targeting antibody (CTA 157-2) would be fused to a single chain antibody binding to the fiber protein of adenoviral vectors (S11 adenobody system). Complexing of adenoviral vectors with this bispecific antibody would render a vector that allowed the targeted transfection of collateral vessels. We succeeded in generating a phage library expressing single chain antibodies based upon the original monoclonal antibody on the phage surface. We also were able to use these libraries to select phages expressing a ScFv ct binding specifically to collateral vessels in vivo. However when trying to generate a recombinant ScFv ct we failed (Mazur, et al., 2003). The reason for this failure became apparent when we modeled the molecular structure of the ScFv ct based upon the peptide sequence. In contrast to other single chain antibodies the heavy and light chain were folded apart at almost 180° rendering the structure very fragile in the linker region. Consequently only parts of the ScFv ct were produced. We planned to continue our attempts in using a "scFv" recognising VEGFR 2 in collaboration Herbert Weich from the GBF in Braunschweig. As desribed previously we were able to produce significant amounts of this scFv and succeeded in selecting VEGFR2 targeting scFv binding to collateral tissue from mice and rats. The further development of the project were halted by the observation that not enough copies of the S11 adenobody could be generated for in vivo experiments. This observation was in accordance with observations of Andrew Baker, Glasgow, who originally had described the S11 adenobody system and who himself had turned to other solutions for the generation of targeting viral vectors. Finally, the reason for developing this instrument became partly obsolete because the questions, we wanted to answer using this system, had largely been answered using other instruments. The differential impact of VEGF A and PlGF on collateral growth was described by Peter Carmeliet, Wolfgang Schaper and Johannes Waltenberger (Autiero, et al., 2003). Furthermore our own results obtained within the SPP 1069 had shifted our attention from the interface of the collateral vessels and circulating cells towards proliferation and differentiation of local cells. Transgenic and knock out models appeared to be much more suitable in answering these questions.

DISCUSSION

The growth of collateral vessels is a complex process involving local as well as remote processes, a number of different cell types as well as altering hemodynamic and biochemical signals, proceeding in different phases (Carmeliet, 2000 Apr, Carmeliet, 2005, Helisch and Schaper, 2003). During the past years several groups including ours have tried to define the different processes, phases, cell types and hemodynamic and biochemical signals. We have concentrated our research on hind-limb models because the anatomical structure of the hindlimb allows to study angiogenesis and the remodeling of preexisting arteriolar shunts into collateral arteries separately. As we started with a fairly simplified hypothesis mentioned in the introduction we had to learn that the process and the dynamics of the process are much more complex. Hemodynamic forces appear to alter in time and

along the growing collateral vessel. At the same time there seems to be a profound influence of the vascularity of the ischemic down-stream region on the hemodynamic forces acting on the growing collateral vessel. This brings angiogenesis as a major albeit indirect contributor to the development of collateral vessels into focus again (Carmeliet, 2005). I have tried to summarize the different processes contributing to collateral vessel development and their interaction as we propose it now in a small cartoon that does not claim to be complete (Fig.8). For the sake of simplicity the arterial circulation is drawn consisting of conductance and resistance vessels as well as the capillary bed. The whole process begins when the main blood supplying vessel is occluded or at least significantly stenosed (Top row second panel). Reduced blood flow in the periphery leads to increased resistance which is flow velocity dependent in blood and to a procoagulant state favoring blood clot formation. Thus, peripheral resistances rise dramatically upon occlusion of the main arterial supply resulting in decreased flow velocities in preexisting arteriolar shunts despite a rise in pressure gradients. Reduced flow velocities allow adhesion and migration of circulating cells (switch 1) into collateral vessels but also (not shown) into peripheral vessels and the capillary bed. Within a short time distal ischemia leads to peripheral vasodilatation (switch 3) increasing flow velocities and shear forces in preexisting arteriolar shunts thereby inhibiting further recruitment of circulating cells (switch 2). Instead, resident precursor cells, part of which originate from or are constantly replenished from the bone marrow, start to proliferate and differentiate into vascular cells and macrophages (switch 2, switch 4).This leads to primary outward remodeling of collateral vessels. At the same time angiogenesis is observed in the ischemic periphery (switch 5). After arterialization of this de-novo formed capillary bed (arteriogenesis in its narrow sence) new resistance vessels are formed resulting in a further decrease of peripheral resistances, which supposedly has a profound influence on the blood supplying collateral vessels (switch 7). At this stage of collateral development, however, hemodynamics appear to promote not only outward- but primarily inward remodeling processes, proliferation slows down considerably and the resulting cork-screw like distortions of collateral vessels impair full restoration of blood flow (switch 6).

As we still struggle to understand the basic physiology and cellular mechanisms of collateral vessel growth our understanding of the molecular mechanisms remains rudimentary (Carmeliet, 2005, Helisch and Schaper, 2003, Tomanek, 2005). Most models are still not differentiated enough to draw a conclusion at which level certain factors influence the growth of collateral vessels. For example, when looking at different studies there is no doubt that VEGF A has an impact on collateral growth but despite many investigations it remains an enigma, how this is achieved (Autiero, et al., 2003, Jacobi, et al., 2004, Lazarous, et al., 1999, Pipp, et al., 2003) There is considerable reason to doubt that it is upregulated in the vicinity of growing collateral vessels (Deindl, et al., 2001). The “arteriogenic” effect of VEGF A is NO dependent (Jacobi, et al., 2004, Murohara, et al., 1998). Thus it is conceivable that the “arteriogenic” effect of this cytokines relies on its effect on peripheral vasodilatation leading to increased flow and shear forces in proliferating collateral vessels and thereby promoting their growth indirectly (switch 2). The same might be achieved by increasing peripheral vascularity via stimulating angiogenesis and later the differentiation of this vascular network into resistance vessels (switch 5 and 7)(Tomanek, 2005). On the other hand on of the VEGF A receptors, FLT 1 has been shown to be present on monocytes and was shown to promote the recruitment of circulating cells (Barleon, et al., 1996, Waltenberger, et al., 2000) and it has been shown that PlGF primarily signaling through FLT1 also promotes collateral growth (Autiero, et al., 2003). Thus the therapeutic effect of VEGF homologues may also be explained by enhanced recruitment of circulating cells (switch 1). Further complexity is added by the fact that not all phases of collateral growth appear to be beneficial. Our investigations on the effect of diabetes mellitus and hypertension on collateral growth demonstrated that diabetes impairs the early proliferative phase of collateral growth (switch 4), whereas arterial hypertension appears to enforce the later remodeling

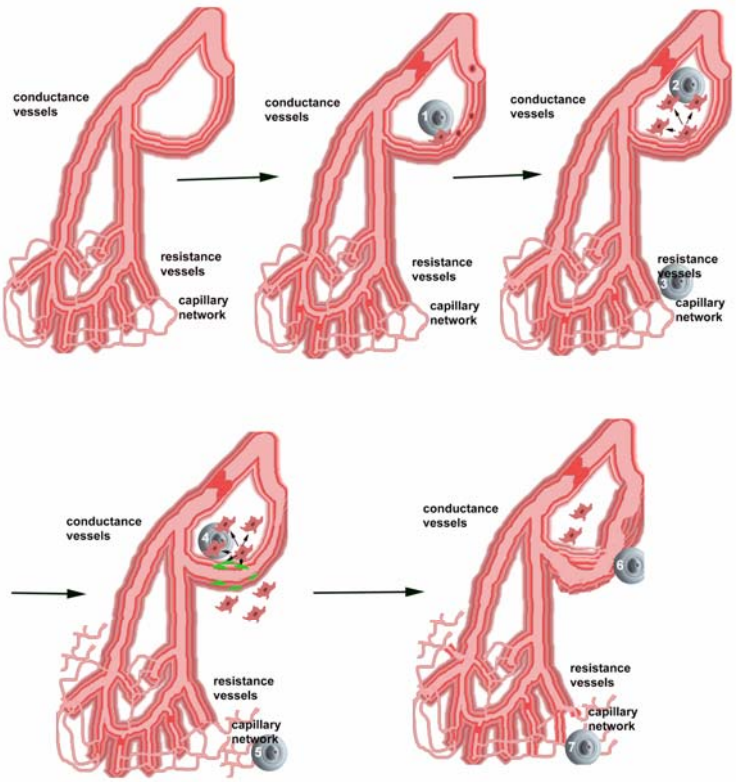


Fig. 8: Schematic presentation of the different key processes involved in collateral development. Key processes are presented as numbered round switches. Each picture represents a typical phase in the time course of collateral development beginning with the normal state in the upper left hand corner and ending in the chronic process of collateral remodeling weeks to month after collateral growth has been induced in the lower right hand corner. A detailed description is found in the text.

phase of collateral growth (switch 6). Only arterial hypertension had a profound negative effect on collateral conductance in the rat hindlimb indicating that perhaps “negative” remodeling constitutes a rate limiting step in collateral vessel formation. The future task will be to define each switch on the molecular level. We have introduced two new candidates to an ever-growing list of possible molecular switches. Yet we cannot definitely say at the moment to which cellular respective physiological switch these molecular candidates belong. The “angiogenesis” switch is the best described at the moment (switch 5)(Carmeliet, 2005, Tomanek, 2005).

I will not go into further details because angiogenesis in its narrow meaning was never the focus of our research and is better described by other members of the SPP 1069 consortium. There are some ideas how the primary network is transformed into an arterialized network and then is remodeled into arteriolar resistance vessels. The former process is called “arteriogenesis” in its narrow meaning whereas the last process remains nameless even for embryologists and is best described with the term “arterial remodeling” (Tomanek, 2005). The same applies to the remodeling of arteriolar or even small arterial shunts into collateral arteries also called “arteriogenesis”, which caused significant confusion (Helisch and Schaper, 2003). The mechanisms of arterial remodeling have extensively been study in the context of diseased vessels with and without interventions like stent implantations (Tomanek, 2005). It remains to be determined how “physiological” remodeling proceeds and what parts of pathological remodeling is “pathologic” and which parts belong the physiological process. Numerous molecular candidates have been claimed to influence physiological remodeling of collateral arteries including NO, GMCSF, FGF family members, VEGF family members, TGF ß, TNF family members, PDGF BB and Angiopoeitins and Angiotensin (Benndorf, et

al., 2003, Buschmann, et al., 2001, Carmeliet, 2003, Helisch and Schaper, 2003, Yang, et al., 1996). They all remain only loosely connected, in theory are capable of perpetuating their influence on collateral growth through several of the above mentioned physiological switches and have not been tested extensively as to the locus of their influence. This certainly also applies to the molecular candidates we have identified. Many would claim that this is an academic discussion of no use in terms of treating patients - good blood flow is good blood flow irrespective where it comes from. The problem is that the dynamics also appear to support reverse remodeling and short-term effects are easily counteracted as the physiological process proceeds. This applies particularly to our patients, who usually suffer from concomitant diseases like diabetes mellitus and arterial hypertension that profoundly influence vascular remodeling. Reverse remodeling of collateral vessels is exactly what appeared to have happened to all clinical trials conducted so far. I want to conclude that we should not mess with the switches unless we at least have a faint idea what each switch is good for. This is the goal of our ongoing and future research.

REFERENCES

Ando J, Tsuboi H, Korenga R, Takada Y, Toyama-Sorimachi N, Miyasaka M, Kamiya A: Shear stress inhibits adhesion of cultured mouse endothelial cells to lymphocytes by downregulating VCAM-1 expression. *Am J Physiol* 36: C679-C687, 1994.

Arras M, Ito WD, Scholz D, Winkler B, Schaper J, Schaper W: Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J Clin Invest* 101: 40-50, 1998.

Autiero M, Waltenberger J, Communi D, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin D, Persico G, Herbert J, Communi D, Shibuya M, Collen D, Conway E, Carmeliet P: Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med* 9: 936-943, 2003.

Bamberger AM, Sudahl S, Loning T, Wagener C, Bamberger CM, Drakakis P, Coutifaris C, Makrigiannakis A: The adhesion molecule CEACAM1 (CD66a, C-CAM, BGP) is specifically expressed by theextravillous intermediate trophoblast. *Am J Pathol* 156: 1165-1170, 2000.

Barleon B, Sozzani S, Zhou D, Weich HA, Mantovani A, Marme D: Migration of human monocytes in response to vascular endothelial growth-factor (vegf) is mediated via the vegf receptor flt-1. *Blood* 87: 3336-3343, 1996.

Bear JE, Loureiro JJ, Libova I, Fassler R, Wehland J and Gertler FB: Negative regulation of fibroblast motility by Ena/VASP proteins. *Cell* 101: 717-728, 2000.

Benndorf R, Boger R, Ergun S, Steenpass A, Wieland T: Angiotensin II type 2 receptor inhibits vascular endothelial growth factor-induced migration and in vitro tube formation of human endothelial cell. *Circ Res* 93: 438-447, 2003.

Brummer J, Ebrahimnejad A, Flayeh R, Schumacher U, Loning T, Bamberger AM, Wagener C: cis Interaction of the cell adhesion molecule CEACAM1 with integrin beta. *Am J Pathol* 159: 537-546, 2001.

Buschmann I, Hoefer I, van Royen N, Katzer E, Braun-Dulleaus R, Heil M, Kostin S, Bode C, Schaper W: GM-CSF: a strong arteriogenic factor acting by amplification of monocyte function. *Atherosclerosis* 159: 343-356, 2001.

Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6: 389-395, 2000.

Carmeliet P: Angiogenesis in health and disease. *Nat Med* 9: 653-660, 2003.

Carmeliet P: Angiogenesis in life, disease and medicine. *Nature* 438: 932-936, 2005.

Deindl E, Buschmann I, Hoefer I, Podzuweit T, Boengler K, Vogel S, van Royen N, Fernandez B, Schaper W: Role of ischemia and of hypoxia-inducible genes in arteriogenesis after femoral artery occlusion in the rabbit. *Circ Res* 89: 779-786, 2001.

Ergun S, Kilic N, Ziegeler G, Hansen A, Nollau P, Gotze J, Wurmbach JH, Horst A, Weil J, Fernando M, Wagener C: CEA-related cell adhesion molecule 1: a potent angiogenic factor and a major effector of vascular endothelial growth factor. *Mol Cell* 5: 311-320, 2000.

Fulton WFM: The Coronary Arteries. Springfield, Illinois: Charles C Thomas; 1965.

Galler AB, Garcia Arguinzonis MI, Baumgartner W, Kuhn M, Smolenski A, Simm A, Reinhard M: VASP-dependent regulation of actin cytoskeleton rigidity, cell adhesion, and detachment. *Histochem Cell Biol* 22: 1-18, 2005.

Harbeck B, Huttelmaier S, Schluter K, Jockusch BM, Illenberger S: Phosphorylation of the vasodilator-stimulated phosphoprotein regulates its interaction with actin. *J Biol Chem* 275: 30817-30825, 2000.

Heil M, Ziegelhoeffer T, Pipp F, Kostin S, Martin S, Clauss M, Schaper W: Blood monocyte concentration is critical for enhancement of collateral artery growth. *Am J Physiol Heart Circ Physiol* 283: H2411-9, 2002.

Helisch A, Schaper W: Arteriogenesis The Development and Growth of Collateral Arteries. *Microcirculation* 10: 83–97, 2003.

Helisch A, Schaper W: Arteriogenesis - The development and growth of collateral arteries. *Microcirculation* 10: 83-97, 2003 Jan.

Herzog S, Sager H, Khmelevski E, Deylig A, Ito W: Collateral arteries grow from preexisting anastomoses in the rat hindlimb. *Am J Physiol Heart Circ Physiol* 283: H2012-H2020, 2002.

Horst A, Ito W, Dabelstein J, Schumacher U, Sander H, Turbide C, Brümmer J, Meinertz T, Beauchemin N, Wagener C: CEACAM1 modulates vascular remodelling in vitro and in vivo. *J Clin Invest*, in press, 2006.

Huttenlocher A, Sandborg RR, Horwitz AF: Adhesion in cell migration. Curr Opin Cell Biol. *7*: 697-706, 1995.

Ingber D. Tensegrity II. How structural networks influence cellular information processing networks. *J Cell Science* 116: 1397-1408, 2003.

Ito W, Weidt C, Köster R, Guckenbiehl M, Schlagner K, Dittmer T, Fiedler W, Gehling U: CCR2 mediates chemoattraction of CD133-positive progenitor cells: a possible mechanism of endothelial progenitor cell homing. Submitted for publication, 2006a.

Ito W, Wenzel U, Becker W, Waltenberger J: Impairment of early collateral proliferation via diabetes mellitus is related to reduced macrophage recruitment. In preparation, 2006b.

Ito WD, Arras M, Scholz D, Winkler B, Htun P, Schaper W: Angiogenesis but not collateral growth is associated with ischemia after femoral artery occlusion. *Am J Physiol* 273: H1255-H1265, 1997a.

Ito WD, Arras M, Winkler B, Scholz D, Schaper J, Schaper W: Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion. *Circ Res* 80: 829-837, 1997b.

Jacobi J, Tam B, Wu G, Hoffman J, Cooke J, Kuo K: Circulation. Adenoviral Gene Transfer With Soluble Vascular Endothelial Growth Factor Receptors Impairs Angiogenesis and Perfusion in a Murine Model of Hindlimb Ischemia. *110*: 2424-2429, 2004.

Khmelewski E, Becker A, Meinertz T, Ito W: Tissue resident cells play a dominant role in arteriogenesis and concomitant macrophage accumulation. *Circ Res* 95: e56-e64, 2004.

Kobler J: Reluctant Surgeon: A biography of John Hunter. New York: Doubleday % Co., Inc.; 1960.

Lazarous D, Shou M, Stiber J, Hodge E, Thirumurti V, Goncalves L, Unger E: Adenoviral-mediated gene transfer induces sustained pericardial VEGF expression in dogs: effect on myocardial angiogenesis. *Cardiovasc Res* 44: 294-302, 1999.

Lloyd P, Hsiao T, Terjung R: Arteriogenesis and angiogenesis in rat ischemic hindlimb: role of nitric oxide. *Am J Physiol Hear Circ Physiol* 281: H2528-H2538, 2001.

Luscinskas FW, Ding H, Tan P, Dumming D, Tedder TF, Gerritsen ME: L- and P-Selectins, but not CD49d (VLA-4) Integrins, mediate monocyte initial attachment to TNF-alpha-activated vascular endothelium under flow in vitro. *J Immunol* 156: 326-335, 1996.

Mazur A, Aurrand-Lions M, Deylig A, Liebau M, Imhof BA, Schaper W, Meinertz T, Ito WD: Collateral targeting with single chain antibodies. *Endothelium* 10: 277-284, 2003.

Muller MM, Singer BB, Klaile E, Obrink B, Lucka L: Transmembrane CEACAM1 affects integrin-dependent signaling and regulates extracellular matrix protein-specific morphology and migration of endothelial cells. *Blood* 105: 3925-3934, 2005.

Murohara T, Asahara T, Silver M, Bauters C, Masuda H, Kalka C, Kearney M, Chen D, Symes J, Fishman M, Huang P, Isner J: Nitric oxide synthase modulates angiogenesis in response to tissue ischemia. *J Clin Invest* 101: 2567-2578, 1998.

Obermeyer N, Janson N, Bergmann J, Buck F, Ito WD: Proteom Analysis of migrating versus non-migrating Rat Heart Endothelial cells reveals distinct expression patterns. *Endothelium* 10: 167-178, 2003.

Obermeyer N, Tiede P, Morbidelli L, Ziche M, Ito W: Vimentin regulates endothelial cell migration, proliferation and VASP phosphorylation. Submitted for publication, 2006.

Patrick CW, McIntire LV: Shear stress and cyclic strain modulation of gene expression in vascular endothelial cells. *Blood Purif* 13: 112-124, 1995.

Pipp F, Heil M, Issbrucker K, Ziegelhoeffer T, Martin S, van den Heuvel J, Weich H, Fernandez B, Golomb G, Carmeliet P, Schaper W, Clauss M: VEGFR-1-selective VEGF homologue PlGF is arteriogenic - Evidence for a monocyte-mediated mechanism. *Circ Res* 92: 378-385, 2003.

Polverini PJ, Cotran RS, Gimbrone MA, Unanue ER: Activated macrophages induce vascular proliferation. *Nature* 269: 804-806, 1977.

Rentrop KP, Feit F, Sherman W, Thornton JC: Serial angiographic assessment of coronary-artery obstruction and collateral flow in acute myocardial-infarction - report from the 2nd mount- sinai-new-york-university reperfusion trial. *Circulation* 80: 1166-1175, 1989.

Resnick N, Gimbrone MAJ: Hemodynamic forces are complex regulators of endothelial gene expression. *FASEB J* 9: 874-882, 1995.

Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, Borisy G, Parsons JT, Horwitz AR: Cell migration: integrating signals from front to back. *Science* 302: 1704-1709, 2003.

Sager H, Rauche K, Middendorf R, Weil J, Ito W: Phase dependent reciprocal regulation of NO synthase expression and macrophage recruitment resolves NO/macrophage paradox during collateral growth. In preparation, 2006.

Sampath R, Kukielka GL, Smith CW, Eskin SG, McIntire LV: Shear stress-mediated changes in the expression of leukocyte adhesion receptors on human umbilical vein endothelial cells in vitro. *Ann Biomed Eng* 23: 247-256, 1995.

Schaper J, Koenig R, Franz D, Schaper W: The endothelial surface of growing coronary collateral arteries. Intimal margination and diapedesis of monocytes A combined SEM and TEM study. *Virchows Arch A Pathol Anat Histol* 370: 193-205, 1976.

Schaper W, Ito WD: Molecular mechanisms of coronary collateral vessel growth. *Circ Res* 79: 911-919, 1996.

Scholz D, Ito W, Fleming I, Deindl E, Sauer A, Wiesnet M, Busse R, Schaper J, Schaper W: Ultrastructure and molecular histology of rabbit hind-limb collateral artery growth (arteriogenesis). *Virchows Arch* 436: 257-270, 2000.

Scholz D, Ziegelhoeffer T, Helisch A, Wagner S, Friedrich C, Podzuweit T, Schaper W: Contributioin of arteriogenesis and angiogenesis to postocclusive hindlimb perfusion in mice. *J Mol Cell Cardiol* 34: 775-787, 2002.

Springer TA: Traffic Signals for Lymphocyte Recirculation and Leukocyte Emigration: The Multistep Paradigm. *Cell* 76: 301-314, 1994.

Tomanek R: Formation of the coronary vasculature during development. *Angiogenesis* 8: 273-284, 2005.

Tsao P, Lewis N, Alpert S, Cooke J: EXPOSURE TO SHEAR STRESS ALTERS ENDOTHELIAL ADHESIVENESS - ROLE OF NITRIC OXIDE. *Circulation* 92: 3513-3519, 1995.

Tsao PS, Buitrago R, Chan JR, Cooke JP: Fluid flow inhibits endothlial adhesiveness. *Circulation* 94: 1682-1689, 1996.

Tsuboi H, Ando J, Korenaga R, Takada Y, Kamiya A: Flow stimulates ICAM-1 expression time and shear stress dependently in cultured human endothelial cells. *Biochem Biophys Res Commun* 206: 988-996, 1995.

Walpola PL, Gotlieb AI, Langille BL: Monocyte adhesion and changes in endothelial cell number, morphology, and F-actin distribution elicited by low shear stress in vivo. *Am J Pathol* 142: 1392-1400, 1993.

Waltenberger J, Lange J, Kranz A: Vascular endothelial growth factor-A-induced chemotaxis of monocytes is attenuated in patients with diabetes mellitus - A potential predictor for the individual capacity to develop collaterals. *Circulation* 102: 185-90, 2000.

Yang HT, Deschenes MR, Ogilvie RW, Terjung RL: Basic Fibroblast Growth Factor Increases Collateral Blood Flow in Rats With Femoral Arterial Ligation. *Circ Res* 79: 62-69, 1996.

Zengin E, Chalajour F, Gehling U, Ito W, Treede H, Lauke H, Weil J, Kilic N, Reichenspurner H, Ergün S: Vascular wall resident progenitor cells: a source for postnatal vasculogenesis. *Development*, in press, 2006.

District-specific Influence of Vascular Endothelial Growth Factor 165 ($VEGF_{165}$) on Arteriogenesis and Angiogenesis of Coronary and Peripheral Arteries as Representative Arteries of Distinct Developmental Origin

Prof. Dr. Sigrid Nikol
Dept. of Molecular Cardiology
Medical Clinic and Policlinics C
University of Münster
Albert-Schweitzer-Str. 33, 48149 Münster, Germany

formerly:
Medical Clinic I, University Hospital Großhadern, LMU Munich

Member of the SPP from 1999 until 2001

SUMMARY

The application of *vascular endothelial growth factor* 165 ($VEGF_{165}$) following arterial occlusion can lead to different responses, arteriogenesis and angiogenesis, particularly if arteries have distinct developmental origins. Pilot studies in pigs with chronically occluded coronary and peripheral arteries and subsequent adventitial delivery of VEGF-DNA demonstrated differential responses in different arterial sites resulting in angiogenesis and arteriogenesis. Smaller coronary arteries tended to demonstrate enlargement of preexisting vessels and formation of new collaterals, whereas in the larger peripheral vasculature exclusively sprouting of larger branch vessels was observed. However, transfer efficiency using liposomes and common optimized transfer conditions was very low (< 1%). Therefore, extensive optimization of liposomal transfection concerning the type of liposomes, DNA/liposome-ratio, cell type, proliferation status and toxicity was performed. The reporter gene for ß-galactosidase is frequently used to determine the efficiency of gene transfer. However, blood is often present in arterial explants and may compromise the results by the presence of hemoglobin. Therefore, the most appropriate method for quantification of ß-galactosidase activity in the presence of blood was investigated. Non-dividing cells represent more realistically *in vivo* conditions for gene therapy. Growth to confluence is sufficient for endothelial cells (Ecs) to reach quiescence in contrast to smooth muscle cells (SMCs). Therefore, alternative techniques were investigated to achieve quiescence also for SMCs. Using these new transfer conditions, markedly increased transfer efficiency was achieved even *in vivo* in the porcine model with chronically occluded coronary and peripheral arteries. In addition, commonly used surgical methods for permanent occlusion are of limited value due to wound healing and may cause angiogenesis by itself. Therefore, different models to achieve long-term occlusions of porcine arteries were evaluated. Among a number of methods investigated, only use of blinded stent grafts resulted in 100% long-term occlusions even after a follow-up period of 6 months. Finally, using this interventional occlusion model and optimized gene transfer conditions in porcine occluded arteries a 2-3fold enhancement of capillary density in myocardium was achieved following VEGF application compared to a 1.5-fold increase of capillary density using the original transfer conditions and compared to a non-treated occlusion control. Nevertheless, no increased collateralization was observed in peripheral occluded arteries compared to non-occluded arteries. Thus, the new optimized transfer and occlusion conditions with augmented effect of VEGF on new vessel formation in peripheral and coronary arteries confirmed our previous

results from the pilot study. Tissues from these animals were also analyzed for VEGF receptor status and using proteomics.
Further investigation of VEGF receptors (VEGFR-1, -2, NP-1, -2) and their signaling pathways in arteries of distinct origin may reveal insights into the role of VEGF in these arteries. However, analysis of genes and related transcription products is often not sufficient to characterize the entire system of interactions within cells or complex organisms. The transcription level of genes does often not correlate with the yield of the final proteins. Posttranslational modifications and protein interaction also determine the function of the protein and can not be captured by DNA and RNA detection efforts. Therefore, proteomics and molecular/functional analysis on the basis of proteomics of peripheral and coronary arteries may help to further understand the role of VEGF or VEGF receptors in angiogenesis/arteriogenesis. In addition, new proteins or factors may be revealed to effect angiogenesis or arteriogenesis per se.

The Role of VEGF in Arteriogenesis

Prof. Dr. Johannes Waltenberger

Dept. of Cardiology
University Hospital Maastricht and
Cardiovascular Research Institute Maastricht (CARIM)
P. Debyelaan 25, P.O.Box 5800, 6202 AZ Maastricht
The Netherlands

Member of the SPP 1069 from 1999 until 2005

SUMMARY

An important compensatory mechanism frequently observed in atherosclerotic disease is the growth of collateral arteries (arteriogenesis). We have established a mouse model of hindlimb ischemia for the functional characterization of the process of arteriogenesis including its consequences on limb perfusion. Using this model we could show that the simultaneous and coordinated activation of VEGFR-1 and VEGFR-2 represents a potent arteriogenic stimulus. These data are consistent with the hypothesis that VEGF-A-induced activation of both monocytes and endothelial cells are necessary to obtain a potent activation of the arteriogenic process. Moreover, we have contributed to the understanding of the molecular and cellular consequences of VEGFR-1 activation in endothelial cells and monocytes. Most importantly, VEGFR-1 is actively involved in VEGF-signalling in endothelial cells by activating VEGFR-2 (molecular transistor) and by forming heterodimers of VEGFR-1 and VEGFR-2. A novel function of VEGFR-2 in tumor angiogenesis could be postulated with the recent finding of an autocrine VEGF-VEGFR-2 loop in primary breast cancer cells and breast cancer cell lines. More recently, we have been able to identify specific signal transduction pathways in human monocytes that are of relevance for monocyte migration and of potential relevance for the process of arteriogenesis. With regard to cardiovascular pathology, we could identify novel signal transduction defects in monocytes from diabetic and from hypercholesterolemic patients, and we could demonstrate a diabetes-related impairment of collateral development in the hindlimb model. Moreover, cholesterol lowering using HMG-CoA reductase inhibitors (statins) appears to be able to revert this functional defect. These data help to better understand the mechanism of action of arteriogenesis and could contribute to the identification of novel therapeutic strategies for the treatment of cardiovascular diseases.

INTRODUCTION

Regional myocardial and peripheral ischemia secondary to an atherosclerotic lesion (arterial stenosis) or occlusion represents one of the most common causes of morbidity and mortality in Western societies. Limited regional blood flow in both situations can be compensated by the growth of collateral arteries, a process called arteriogenesis (Ito *et al.* 1997a, Waltenberger 2001). Angiogenesis and arteriogenesis are the two main mechanisms occurring either in or adjacent to perfusion-compromised tissues. Although angiogenesis might increase the distribution of oxygenated blood in those tissues, arteriogenesis is supposed to be the process that is responsible for enhancing the blood supply (inflow) into ischemic areas.

There are only a few approaches to quantitatively study the development of collateral vessels in humans. In order to systematically study the process of arteriogenesis, there are several animal

models of hindlimb ischemia including a mouse model (Couffinhal *et al.* 1998). The mouse hindlimb model allows both the monitoring of collateral artery growth as well as assessment of hindlimb perfusion. The blood flow distal to the vascular defect can be assessed by different methods including laser Doppler tests, Tc-99m-MIBI scintigraphy for perfusion measurement or by the use of microspheres. Since most of the genetic models are carried out in mice it is very attractive to study the basic mechanisms of arteriogenesis in a mouse model.

The process of arteriogenesis differs significantly from angiogenesis and involves the enlargement of pre-existing collateral arterioles, which includes the proliferation of various cell types such as endothelial cells and smooth muscle cells. This process is triggered by changes in blood flow that lead to increased shear stress in the preexisting arterioles secondary to flow reduction in the stenosed or occluded artery. The exact functional basis, however, underlying the activation of arteriogensis is not fully understood yet (Carmeliet 2000, Schaper *et al.* 2003). Based on the published data at the time of the start of this project (1999), monocytes – adhering to activated endothelial cells out of the bloodstream and evading into the vessel wall - were likely to play a functional role in the process of arteriogenesis. This, however, had not fully been proven. The pieces of evidence that speak in favour of a role of monocytes were *i)* the demonstration of monocyte recruitment at the site of collateral growth (Arras *et al.* 1998), and *ii.)* the positive effect of MCP-1 to stimulate arteriogenesis (Ito *et al.* 1997b). On the other hand, monocyte infiltration could simply reflect an inflammatory environment that might be a prerequisite for certain forms of angiogenesis (Goede *et al.* 1999) or arteriogenesis.

An attractive candidate to stimulate arteriogenesis is vascular endothelial growth factor VEGF as it can activate two of the most important cellular components of this process, namely endothelial cells (Waltenberger *et al.* 1994) and circulating monocytes (Clauss *et al.* 1996). VEGF-A (Ferrara 2002) is an important activator of endothelial cells and can stimulate a variety of endothelial activities including endothelial proliferation, endothelial migration, NO synthase upregulation (Kroll *et al.* 1998) and NO-release in endothelial cells (Kroll *et al.* 1999), vascular permeability, prevention of endothelial apoptosis (Kasahara *et al.* 2000) and angiogenesis. Moreover, vasculogenesis and angiogenesis during embryological development are completely dependent on the action of VEGF (Carmeliet *et al.* 1996, Fong *et al.* 1995, Shalaby *et al.* 1995). The role of VEGF in activating monocytes had not been fully understood, although there were good reasons to believe that monocyte activation/migration during wound repair and arteriogenesis is important (Waltenberger 2001). Moreover, the roles of both VEGFR-1 and VEGFR-2 in endothelial activation were partially unclear at the beginning of this project: VEGFR-2 had been proven to mediate most of the actions of VEGF-A in endothelial cells (Waltenberger *et al.* 1994), while VEGFR-1 was regarded as acting as a scavenger without direct positive action on endothelial cells (Hiratsuka *et al.* 1998).

The final proof for VEGF to be involved in arteriogenesis remains to be provided, however, the following evidence exists: VEGF-A protein can enhance regional perfusion in the rabbit (Takeshita *et al.* 1994) and in the dog (Banai *et al.* 1994). It was unknown, whether PlGF, a member of the VEGF family that activates VEGFR-1, is capable to stimulate arteriogenesis in the mouse model and to enhance regional perfusion. In the human situation, there was no clear evidence that VEGF therapy leads to a significant induction of arteriogenesis at the time of initiation of our project. In brief, there was good evidence for VEGF-A to stimulate angiogenesis and tissue perfusion *in vivo*.

The project was set up to answer the following questions:

1. Can VEGF reliably and quantitatively stimulate arteriogenesis in the hindlimb model?
2. Are monocytes involved in VEGF-stimulated arteriogenesis? Is VEGFR-2 stimulation (using VEGF-E) sufficient to induce arteriogenesis in the absence of a stimulus on monocytes?

3. What is the role of VEGFR-1 in endothelial cell activation? Is there a relation with the activation of VEGFR-2?
4. Is VEGF responsiveness of monocytes affected by the metabolic conditions of the individual? Is it influenced by cardiovascular risk factors such as diabetes mellitus or hypercholesterolaemia? Are these conditions affecting arteriogenesis *in vivo*?
5. If so, what are the underlying molecular defects? Can this information be used for diagnostic purposes? Or can this be used for predicting the function of therapeutic arteriogenesis?
6. What can we learn from our studies on VEGFR activation in the vascular system to be applied to cancer biology, especially with regard to breast cancer, where our group was the first to describe the expression of VEGFR-2 in mammary-derived cells (Kranz *et al.* 1999)?

METHODS

A broad spectrum of methods had been applied for conducting our SPP1069 project:

1) Set up and validation of a mouse hindlimb model for objective and quantitative assessment of regional perfusion using nuclear techniques (99Tc-MIBI-scan, Beta-Camera). *In vivo* studies on arteriogenesis using a mouse hindlimb model.

2) A broad spectrum of molecular cell biology techniques to study the effects of VEGF and related ligands on endothelial cell and monocyte function and signalling.

RESULTS

1. The simultaneous and coordinated activation of VEGFR-1 and VEGFR-2 represents a potent arteriogenic stimulus.

We have established a mouse hindlimb model to induce peripheral ischemia and collateral artery growth (arteriogenesis). In contrast to the majority of research groups, we were initially not convinced that Laser-Doppler flow measurement is the best method to quantitatively assess limb perfusion. In fact, Laser Doppler flow measurement only detects skin blood flow. We therefore pioneered the establishment of the method of Tc-99 MIBI scanning in the mouse, which we detected with a conventional Gamma Camera that was located at the department of Nuclear Medicine at the University of Ulm. A series of validation experiments was performed and the method was validated.
Using this setting, we have asked the question, whether the repetitive application (every 12 hours) of VEGF protein over a period of 7 days results in an improvement of perfusion of the ischemic hindlimb (Babiak *et al.* 2004). In specific, we have used PlGF-1, a ligand for VEGFR-1; VEGF-E, a ligand for VEGFR-2; and VEGF-A, a ligand for both VEGFR-1 and VEGFR-2. Using this setting, we found that only VEGF-A is able to significantly stimulate limb perfusion and arteriogenesis in BalbC mice. This means that both VEGFR-1 and VEGFR-2 need to be activated in order to result in a sufficient stimulus for arteriogenesis. Moreover, the need for VEGFR-1 activation supports our hypothesis that monocyte recruitment is an important component of arteriogenesis. The maximal effect was seen in the presence of VEGF-A stimulation, i.e, when both VEGFR-1 and VEGFR-2 as well as monocytes and endothelial cells were activated.

We furthermore have asked the question, whether VEGF does play a role in endogenous arteriogenesis, i.e. during collateral growth in the absence of a therapeutic stimulus. When applying a receptor-tyrosine-kinase-inhibitor of both VEGFR-1 and VEGFR-2 (such as ZK202650), the process of arteriogenesis was significantly inhibited in a concentrations dependent manner proving a functional role of VEGF in collateral growth.

Additional and thus far unpublished work investigated the question, whether the tyrosine kinase domain of VEGFR-1 is necessary for the induction of arteriogenesis. Using a genetic mouse model with a deficient VEGFR-1 tyrosine kinase (kindly provided by Masabumi Shibuya, Tokyo), we were not able to detect a significant difference to wild type mice (Theses from Patrick Müller and from Thomas Niedhammer, see below). However, these mice are in a Black6 background that has a significantly stronger spontaneous arteriogenesis than other mice strains (own, unpublished data).

Using the same mouse model, we have investigated whether diabetes mellitus and hyperglycemia are effecting the perfusion of an ischemic limb, and whether they can affect arteriogenesis (Thesis Jan Endregat). Preliminary data indicate that diabetes mellitus results in reduced arteriogenesis (Ito *et al.* 2006). These findings are in line with our own hypothesis that diabetes mellitus negatively affects VEGF-dependent monocyte function and VEGF signalling (Waltenberger *et al.* 2000). This seems to be associated with an impaired recruitment of monocytic cells to growing collateral vessels. Moreover, these data are in line with recent clinical findings that the maturation of collateral arteries is significantly impaired and prolonged in diabetic individuals (Werner *et al.* 2003).

With regard to the development of therapeutic anteriogenesis, we had a fruitful collaboration with the group of Peter Boekstegers from the University of Munich. In a common paper, we could demonstrate that the therapeutic application of a peptide growth factor is more efficient, when applied via retroinfusion through the coronary sinus (von Degenfeld *et al.* 2003). This approach was about 10 times more efficient than the antegrade injection into the coronary artery. For this study, we had been labelling fibroblast growth factor-2 protein with iodine (using the chloramine-T method) in our lab in Ulm, which was then transferred to Munich and injected into pigs there. The SPP 1069 had made this collaboration possible.

2. Unravelling the positive signalling of VEGFR-1 in endothelial cells. Identification of a molecular transistor and identification of VEGFR-1/VEGFR-2 heterodimer formation in endothelial cells.

Starting out from the finding that VEGFR-1 activation using PlGF can induce arteriogenesis in PlGF-knockout mice (Carmeliet *et al.* 2001) has triggered an intensive search in our laboratory to find a molecular explanation for this finding. This work had involved virtually our whole lab for a period of more than 2 years. The work was carried out in a fruitful collaboration with the group of Peter Carmeliet in Leuven and resulted in a highly respected joint publication (Autiero*, Waltenberger*, Communi* *et al.* 2003).

Taken together, VEGFR-1 is actively involved in VEGF-signalling in endothelial cells by activating VEGFR-2, which results in a) higher baseline activity of VEGFR-2, and b) in a higher maximal activity of VEGFR-2 following specific stimulation with VEGF-E. We have called this novel signalling mechanism a “molecular transistor” as the activation of a “weak” molecule (VEGFR-1 has a rather poor tyrosine kinase activity) is regulating the activity of a “strong” tyrosine kinase such as VEGFR-2. This was the first clear demonstration of a positive VEGFR-1 signalling in endothelial cells. Moreover, we were able to demonstrate heterodimer formation of VEGFR-1 and VEGFR-2 resulting in a positive signal as well. In this paper, we were also able to provide an explanation why this molecular pathway had remained undetected before: Endothelial cells do produce abundant amounts of PlGF, which is usually stimulating VEGFR-1 in an autocrine fashion. The use of endothelial cells from PlGF knockout mice made it possible to have cells lacking the baseline activation of VEGFR-1, which can easily be overcome by the exogenous addition of PlGF or other VEGFR-1 ligands. In fact, we were able to mimic PlGF knockout cells by challenging human umbilical endothelial cells with neutralizing PlGF antisera. The molecular VEGFR-1/VEGFR-2 transistor could be demonstrated in these cells as well.

Another relevant contribution with regard to endothelial signalling was to provide clear evidence that the anti-apoptotic effect of VEGF-A is mediated via VEGFR-2 (Yilmaz *et al.* 2003). Using endothelial cells *in vitro*, the serum-starvation induced apoptosis could clearly be prevented by stimulation of VEGFR-2 using VEGF-A or VEGF-E, but not by stimulation with the VEGFR-1-specific ligand PlGF-1.

3. VEGFR-1 signal transduction in monocytes. Negative consequences of diabetes mellitus and hypercholesterolaemia on monocyte function.

Given the functional relevance of monocytes and monocyte-derived cells in arteriogenesis (Babiak *et al.* 2004, Ito *et al.* 2006), we have focused on the identification of VEGF-dependent signal transduction pathways in primary human monocytes. These experiments were also triggered by our own previous finding that metabolic diseases and cardiovascular risk factors such as diabetes mellitus is associated with a signal transduction defect (Waltenberger 2005) in the VEGF-triggered and VEGFR-1-mediated signal transduction cascade (Waltenberger *et al.* 2000).

We are currently investigating whether there are abnormalities in the signal transduction system of monocytes that can explain the signal transduction defects that we had postulated early on (Waltenberger *et al.* 2000).

4. VEGF plays a dual role in promoting breast cancer: VEGF-induced autocrine activation of breast cancer cells in addition to stimulating tumor angiogenesis.

We could previously demonstrate the expression pattern of VEGF and its receptors in breast carcinoma (Kranz *et al.* 1999). That paper was the first description of the expression of VEGFR-2 in ductal tissue of the breast. We had been following up on this finding and took advantage of the methodology set up in our lab. We could recently demonstrate a novel autocrine pathway that is active in several mammary carcinoma cell lines as well as in a primary tumor cell line established for this project (Weigand *et al.* 2005). This is a nice example of the interdisciplinary nature of angiogenesis research and that tumor biology can benefit from cardiovascular biology and medicine.

5. The successful identification and characterization of novel receptor tyrosine kinase inhibitors potentially useful to target and inhibit lymphangiogenesis in tumors.

In another successful collaboration within the SPP1069, we were involved in the characterization and functional testing of several novel indolinones (Kirkin *et al.* 2001). The specificity of some of these molecules for VEGFR-2 and VEGFR-3 made them excellent candidates for further preclinical and clinical development. In fact, the substance MAZ51 made it rather far in that respect. In a common publication, we could demonstrate that MAZ51 inhibits endothelial cell and tumor cell growth *in vitro*. It furthermore suppresses tumor growth *in vivo* (Kirkin *et al.* 2004).

DISCUSSION

Our activities of the past 6 years within the SPP1069 have helped us to advance our understanding of a number of issues related to VEGF physiology and pathology. We were able to significantly contribute to the following findings:

1. The simultaneous and coordinated activation of VEGFR-1 and VEGFR-2 represents a potent arteriogenic stimulus. VEGF-A-induces activation of both monocytes and endothelial cells to initiate a potent activation of the arteriogenic process.
2. We have been able to contribute to the understanding of the molecular and cellular consequences of VEGFR-1 activation in endothelial cells. Most importantly, VEGFR-1 is actively involved in VEGF-signalling in endothelial cells by activating VEGFR-2 (molecular transistor) and by forming heterodimers with VEGFR-2.
3. We have been able to characterize several signal transduction pathways in human monocytes that are of relevance for monocyte migration and of potential relevance for the process of arteriogenesis.
4. We could identify novel signal transduction defects in monocytes from diabetic and from hypercholesterolemic patients. Likewise, we could demonstrate a diabetes-related impairment of collateral development in the hindlimb model.
5. Cholesterol lowering using HMG-CoA reductase inhibitors (statins) is capable of reverting the hypercholesterolaemia-related signal transduction defect resulting in a restoration of monocyte responsiveness to VEGF stimulation.
6. We have been able to identify a novel function of VEGFR-2 in tumor angiogenesisby identifying an autocrine VEGF-VEGFR-2 loop in primary breast cancer cells and breast cancer cell lines *in vitro*.
7. We could characterize MAZ51 as a promising agent for the inhibition of tumor angiogenesis and lymphangiogenesis by selectively inhibiting VEGFR-2 and VEGFR-3 tyrosine kinase activity.

These findings help to better understand the mechanism of action of arteriogenesis and could contribute to future developments in cardiovascular medicine, both with regard to novel diagnostic as well as novel therapeutic strategies. The initial hype related to angiogenesis and gene therapy in the mid and late 1990s had been replaced by solid data and a much better basis for future clinical developments.

The involvement in an active national network such as SPP1069 – with its numerous international connections – was of great advantage for carrying out the own research projects. In specific, the network provided an excellent basis for formal and informal cooperations and exchange, which was of advantage for the projects. Moreover, the interactions were beneficial for all scientists, PhD and MD students involved. Speaking for my own group, some of the students felt at home in the network and had some “graduate school substitute”.

My own move to Maastricht, The Netherlands, for taking a Chair in Cardiology and Invasive Cardiology, had major consequences for my research group and for my own projects. My involvement in the SPP1069 represented continuity and was of great benefit for the group at the new location. Moreover, participating in the SPP from abroad was without any obstacle, but with benefits for both sides. In conclusion, the SPP1069 has contributed its part for integration of science in Europe. I wish to encourage the DFG and its reviewers to support this process of European integration, as I am sure that we can learn a great deal from each other; in the end, all parties including German scientist can benefit from the structures and collaboration opportunities in Europe.

REFERENCES

Arras M, Ito WD, Scholz D, Winkler B, Schaper J, Schaper W: Monocyte activation in angiogenesis and collateral growth in the rabbit hindlimb. *J Clin Invest* 101: 40-50, 1998.

Autiero M*, Waltenberger J*, Communi D*, Kranz A, Moons L, Lambrechts D, Kroll J, Plaisance S, De Mol M, Bono F, Kliche S, Fellbrich G, Ballmer-Hofer K, Maglione D, Mayr-Beyrle U, Dewerchin M, Dombrowski

S, Stanimirovic D, Van Hummelen P, Dehio C, Hicklin DJ, Persico G, Herbert JM, Shibuya M, Collen D, Conway EM, Carmeliet P: Role of PlGF in the intra- and intermolecular cross talk between the VEGF receptors Flt1 and Flk1. *Nat Med* 9: 936-943, 2003.

Babiak A, Schumm AM, Wangler C, Loukas M, Wu J, Dombrowski S, Mattuschek C, Kotzerke J, Dehio C, Waltenberger J: Coordinated activation of VEGFR-1 and VEGFR-2 is a potent arteriogenic stimulus leading to enhancement of regional perfusion. *Cardiovasc Res* 61: 789-795, 2004.

Banai S, Jaklitsch MT, Shou M, Lazarous DF, Scheinowitz M, Biro S, Epstein SE, Unger EF: Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs. *Circulation* 89: 2183-2189, 1994.

Böldicke T, Tesar M, Griesel C, Rohde M, Gröne H-J, Waltenberger J, Kollet O, Lapidot T, Yayon A, and Weich H: Single-chain antibodies recognizing the human vascular endothelial growth factor receptor-2 (VEGFR-2/flk-1) on the surface of primary endothelial cells and preselected CD34$^+$ cells from cord blood. *Stem Cells* 19:24-36, 2001.

Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6: 389-395, 2000.

Carmeliet P, Mackman N, Moons L, Luther T, Gressens P, van Vlaenderen I, Demunck H, Kasper M, Breier G, Evrard P, Müller M, Risau W, Edgington T, Collen D: Role of tissue factor in embryonic blood vessel development. *Nature* 383: 73-75, 1996.

Carmeliet P, Moons L, Luttun A, Vincenti V, Compernolle V, De Mol M, Wu Y, Bono F, Devy L, Beck H, Scholz D, Acker T, DiPalma T, Dewerchin M, Noel A, Stalmans I, Barra A, Blacher S, Vandendriessche T, Ponten A, Eriksson U, Plate KH, Foidart JM, Schaper W, Charnock-Jones DS, Hicklin DJ, Herbert JM, Collen D, Persico MG: Synergism between vascular endothelial growth factor and placental growth factor contributes to angiogenesis and plasma extravasation in pathological conditions. *Nat Med* 7: 575-583, 2001.

Clauss M, Weich H, Breier G, Knies U, Rockl W, Waltenberger J, Risau W: The vascular endothelial growth factor receptor Flt-1 mediates biological activities. Implications for a functional role of placenta growth factor in monocyte activation and chemotaxis. *J Biol Chem* 271: 17629-17634, 1996.

Couffinhal T, Silver M, Zheng LP, Kearney M, Witzenbichler B, Isner JM: Mouse model of angiogenesis. *Am J Pathol* 152: 1667-1679, 1998.

Ferrara N: Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. *Semin Oncol* 29: 10-14, 2002.

Fong G-H, Rossant J, Gertsenstein M, Breitman ML: Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. *Nature* 376: 66-70, 1995.

Goede V, Brogelli L, Ziche M, Augustin HG: Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1. *Int J Cancer* 82: 765-770, 1999.

Hiratsuka S, Minowa O, Kuno J, Noda T, Shibuya M: Flt-1 lacking the tyrosine kinase domain is sufficient for normal development and angiogenesis in mice. *Proc Natl Acad Sci USA* 95: 9349-9354, 1998.

Ito WD, Arras M, Scholz D, Winkler B, Htun P, Schaper W: Angiogenesis but not collateral growth is associated with ischemia after femoral artery occlusion. *Am J Physiol* 273: H1255-1265, 1997a.

Ito WD, Arras M, Winkler B, Scholz D, Schaper J, Schaper W: Monocyte chemotactic protein-1 increases collateral and peripheral conductance after femoral artery occlusion. *Circ Res* 80: 829-837, 1997b.

Ito WD, Wenzel U, Endregat J, Niedhammer T, Becker W, Waltenberger J: Impairment of early collateral proliferation via diabetes mellitus is related to reduced macrophage recruitment. In preparation, 2006.

Kasahara Y, Tuder RM, Taraseviciene-Stewart L, Le Cras TD, Abman S, Hirth PK, Waltenberger J, Voelkel NF: Inhibition of VEGF receptors causes lung cell apoptosis and emphysema. *J Clin Invest* 106: 1311-1319, 2000.

Kirkin V, Mazitschek R, Krishnan J, Steffen A, Waltenberger J, Pepper MS, Giannis A, Sleeman JP: Characterization of indolinones which preferentially inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2. *Eur J Biochem* 268: 5530-5540, 2001.

Kirkin V, Thiele W, Baumann P, Mazitschek R, Rohde K, Fellbrich G, Weich H, Waltenberger J, Giannis A, Sleeman JP: MAZ51, an indolinone that inhibits endothelial cell and tumor cell growth in vitro, suppresses tumor growth in vivo. *Int J Cancer* 112: 986-993, 2004.

Kranz A, Mattfeldt T, Waltenberger J: Molecular mediators of tumor angiogenesis: enhanced expression and activation of vascular endothelial growth factor receptor KDR in primary breast cancer. *Int J Cancer* 84: 293-298, 1999.

Kroll J, Waltenberger J: VEGF-A induces expression of eNOS and iNOS in endothelial cells via VEGF receptor-2 (KDR). *Biochem Biophys Res Commun* 252: 743-746, 1998.

Kroll J, Waltenberger J: A novel function of VEGF receptor-2 (KDR): rapid release of nitric oxide in response to VEGF-A stimulation in endothelial cells. *Biochem Biophys Res Commun* 265: 636-639, 1999.

Schaper W, Scholz D: Factors Regulating Arteriogenesis. *Arterioscler Thromb Vasc Biol* 3: 3, 2003.

Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu X-F, Breitman ML, Schuh AC: Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. *Nature* 376: 62-66, 1995.

Takeshita S, Zheng LP, Brogi E, Kearney M, Pu L-Q, Bunting S, Ferrara N, Symes JF, Isner JM: Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model. *J Clin Invest* 93: 662-670, 1994.

von Degenfeld G, Raake P, Kupatt C, Lebherz C, Hinkel R, Gildehaus FJ, Munzing W, Kranz A, Waltenberger J, Simoes M, Schwaiger M, Thein E, Boekstegers P: Selective pressure-regulated retroinfusion of fibroblast growth factor-2 into the coronary vein enhances regional myocardial blood flow and function in pigs with chronic myocardial ischemia. *J Am Coll Cardiol* 42: 1120-1128, 2003.

Waltenberger J: Impaired collateral vessel development in diabetes: potential cellular mechanisms and therapeutic implications. *Cardiovasc Res* 49: 554-560, 2001.

Waltenberger J: Growth factor signal transduction defects in the cardiovascular system. *Cardiovasc Res* 65: 574-580, 2005.

Waltenberger J, Claesson-Welsh L, Siegbahn A, Shibuya M, Heldin CH: Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor. *J Biol Chem* 269: 26988-26995, 1994.

Waltenberger J, Lange J, Kranz A: Vascular endothelial growth factor-A-induced chemotaxis of monocytes is attenuated in patients with diabetes mellitus: A potential predictor for the individual capacity to develop collaterals. *Circulation* 102: 185-190, 2000.

Weigand M, Hantel P, Kreienberg R, Waltenberger J: Autocrine vascular endothelial growth factor signalling in breast cancer. Evidence from cell lines and primary breast cancer cultures in vitro. *Angiogenesis* 8: 197-204, 2005.

Werner GS, Richartz BM, Heinke S, Ferrari M, Figulla HR: Impaired acute collateral recruitment as a possible mechanism for increased cardiac adverse events in patients with diabetes mellitus. *Eur Heart J* 24: 1134-1142, 2003.

Yilmaz A, Kliche S, Mayr-Beyrle U, Fellbrich G, Waltenberger J: The antiapoptotic role of VEGFR-2 is mediated via inhibition of p38 MAPK. *Biochem Biophys Res Commun* 306: 730-736, 2003.

Tumor angiogenesis

Identification of molecular mechanisms of oncogen-induced, Sp1-mediated activation of the VEGF promoter

Dr. Günter Finkenzeller
Dept. of Surgery
University of Freiburg
Hugstetter Str. 55, 79106 Freiburg, Germany

Member of the SPP 1069 from 1999 until 2001

This project has been transferred in the course of its funding period. See project of Dr. Ulrike Fiedler and Prof. Dr. Dieter Marmé (page 172).

Role of Matrix Metalloproteinases in the Regulation of Tumor Angiogenesis and Invasion

Prof. Dr. Norbert E. Fusenig
Dr. Margareta M. Müller

Deutsches Krebsforschungszentrum (DKFZ)
Abt. Differenzierung und Carcinogenese
Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Member of the SPP 1069 from 1999 until 2003

SUMMARY

Angiogenesis inhibitory studies have documented a causal relationship between tumor vascularization and invasion suggesting a crucial role of stromal proteases. Angiogenesis inhibition caused remodelling of the tumor stroma both at the cellular and extracellular matrix (ECM) level leading to the reformation of a basement membrane. These effects presumably resulted from reduced degradation of ECM components due to decreased protease expression and activation. Of the different matrix metalloproteinases (MMPs) MMP-9 and MMP-13 were identified to play a pivotal role in an in vivo tumor invasion model. Thus, studies were focussed on stromal MMP-9 and MMP-13 by further analyzing their expression and cellular localization during activated and blocked angiogenesis, respectively. Special emphasis had been put on the evaluation of distinct MMP activity in situ, which were measured both directly (zymograms) and by identifying ECM breakdown products by immunostaining with specific antibodies. A functional proof of the role of distinct MMPs has to be accomplished by use of novel more specific protease inhibitors and interference with their expression utilizing MMP-9 and/or MMP-13 knockout animals as well as by using antisense oligonucleotide and siRNA strategies, both in situ and in 3D coculture assays. Finally, efforts for the understanding of the molecular mechanisms underlying the stromal alterations following angiogenesis inhibition were initiated by analyzing differential expression of resting and activated endothelial cells to identify and functionally characterize signalling factors inducing or repressing stromal cell MMP expression.

INTRODUCTION

Evidences of the decisive role of tumor-stroma interactions for tumor development and progression have further accumulated as highlighted in a special issue of Differentiation in 2002 (Cunha et al, 2002). Alterations in the stromal microenvironment, including enhanced vascularization (angiogenesis), modified extracellular matrix (ECM) composition, influx and action of inflammatory cells and unbalanced protease activity are subtantiated as essential regulatory factors of tumor growth, invasion and metastasis (Cunha et al, 2003; Mueller et al, 2002; Bissell et al, 2002; Lynch et al, 2002; van Kempen et al, 2002; Fidler 2002). Matrix metalloproteinases (MMPs) – due to their multipotential activity in the degradation of extracellular matrix as well as in the release of matrix-bound growth factors – play a significant role in angiogenesis and tumor progression (van Kempen et al, 2002; Bergers et al, 2000). Strict regulation of MMP expression and activity is critical in order to maintain proper ECM homeostasis, but in diseased states such as cancer there is often a high level of MMP activity at the tumor-stroma interface. In addition to their role in ECM turnover, MMPs are implicated in a wide variety of roles that can assist tumor initiation, growth, migration, angiogenesis, invasion, and metastasis. Thus, MMPs are no longer considered only as ECM degrading enzymes

but as a part of an elegant communication system by which the tumor interacts with the stroma (Fidler 2002; Bissell et al, 2002; Lynch et al, 2002; van Kempen et al, 2002). One member of the MMP family predominantly linked to the invasive processes of endothelial and tumor cells is MMP-9/gelatinase B. Recent studies on transgenic mouse models suggest that MMP-9 produced by infiltrating inflammatory cells functionally contributes to cancer development by ECM degradation but, more importantly, by release and activation of matrix-sequestered growth factors thus enabling their function as regulators of epithelial, endothelial and stromal cell growth (van Kempen et al, 2002; Bergers et al, 2000). In addition, MMP-9 acts as regulator and effector in leukocyte biology and both MMP-9 as well as MMP-2 and MT1-MMP can be released by shedding of cell membrane vesicles (Opdenakker et al, 2001; Taraboletti et al, 2002). Furthermore, one of the physiologic MMP inhibitors (TIMP-3) inhibits VEGF-meditated angiogenesis by blocking its binding to the VEGF receptor 2 (Qi et al, 2003). The importance of MMPs in ECM remodelling was further emphasized by recent data on the binding of pro MMP-9 to collagen type IV components as well as to cell membranes via CD44. MMPs are sequestered as inactive zymogenes in the ECM as a reservoir of latent enzymes which can be rapidly and focally released (Zucker et al, 2000).

To study the cellular and molecular mechanisms of tumor stroma interactions, we have established and refined in vivo (matrix-inserted surface transplantation) and in vitro (organotypic coculture) models as well as developed and characterized keratinocyte cell lines representing different stages of skin epithelial carcinogenesis from immortal (HaCaT) to highly malignant and metastatic cells (for review see (Fusenig et al, 1998; Fusenig et al, 2002; Mueller et al, 2004; Mueller et al, 2002). In the surface transplantation assay, a model well suited to study early tumor-stroma interactions, distinct dynamics of angiogenesis and stromal activation could be identified (see Fig .1). Whereas both phenomena were transient in premalignant and benign tumor transplants, they persisted in malignant tumors and were enhanced in metastatic transplants (Mueller et al, 2001; Skobe et al, 1997).

Although such detailed kinetics of angiogenesis and stromal induction cannot be identified in established human tumors, we have documented a clear correlation between different tumor stages and the intensity of vascularization (mean vessel density, MVD) in different stages of epithelial human skin tumors (SCC) (Strieth et al, 2000). In this study, computer-assisted morphometric methods were established for quantitation of MVD in tumor sections and a close correlation between depth of penetration of tumors and level of MVD has been substantiated. The functional basis for this correlation seems to be that the depth of infiltration of tumor cells may be indicative for the intensity of tumor-stroma interactions and thus of enhanced angiogenesis. Similar correlations were observed in a variant of human skin keratoacanthomas, generally considered as benign tumors, which exhibit signs of malignant progression and also a high MVD comparable to that of late stage SCCs (Strieth et al, 2002).

Further evidence of the significance of tumor-stroma interactions for the tumor phenotype was obtained in earlier tumor transplantation studies. Whereas benign tumor transplants, exhibiting transient stromal activation and angiogenesis, formed a well structured basement membrane, this structure was lost in malignant transplants as soon as the activated stroma came in close vicinity to the tumor cells (Tomakidi et al, 1999). Comparably, the persistently activated stroma in malignant transplants which interfered with the assembly of a structured basement membrane had also significant influence on the differentiation program of the tumor cells resulting in enhanced dedifferentiation and expression of atypical differentiation markers in malignant epithelia. On the other hand, atypical differentiation in benign transplants normalized to a rather regular epidermal phenotype coincident with regression of stromal activation (Tomakidi et al, 2003).

More importantly, a quasi-benign phenotype of malignant transplants was induced by modulating the tumor stroma and inhibition of tumor vascularization, respectively. This could be achieved by two different approaches:

(i) Blockade of VEGF-R2 signalling, one of the major regulators of ongoing angiogenesis in malignant transplants, by the neutralizing antibody DC101 resulted in abrogation of tumor vascularization and restriction of tumor cell invasion (Skobe et al, 1997; Vosseler et al, 2005; Vosseler 2001).

(ii) By disturbing the proteolytic balance of a serine protease system in the tumor stroma using plasminogen activator inhibitor (PAI-1) knockout mice, both tumor vascularization and tumor-cell invasion were efficiently inhibited (Bajou et al, 1998). Restoration of PAI-1 activity in the deficient animals via adenoviral vectors using constructs with different site-specific mutations, clearly demonstrated that the lack of protease inhibitory efficacy of PAI-1 and not its interaction with the ECM component vitronectin was responsible for the blockade of tumor vascularization and invasion (Bajou et al, 2001). In both assays, the quasi-benign (non-invasive) and differentiated phenotype of the tumor epithelium in transplants with inhibited tumor vascularization was associated with the continuous immunostaining of major basement membrane components (laminin and collagen type IV) suggesting a normalization of the tumor-stroma border zone (Bajou et al, 1998; Bajou et al, 2001; Skobe et al, 1997).

These observations evidenced a crucial and causal role of stromal modifications in tumor invasion. Furthermore, they pointed to an essential contribution of the activation of matrix proteases during stimulated vascularization with enhanced ECM turnover, whereas angiogenesis inhibition and interference with protease activity resulted in normalized ECM and epithelial structures.

Comparable to the different dynamics of angiogenesis induction by benign and malignant tumor cells, respectively, a drastic difference in the induction of stromal proteases by these different tumor cells were observed. Whereas malignant tumor cells induced quite rapidly the expression of stromal MMPs after transplantation, only a weak and transient induction was observed in transplants of benign tumor cells (Airola et al, 2001; Fusenig et al, 2002) and Fig. 1). This observation was in contrast to the MMP expression data obtained in monolayer cultures, where no significant difference was noticed between benign and malignant HaCaT cells as analyzed in different cooperative studies (Ala-aho et al, 2000; Bachmeier et al, 2000; Johansson et al, 2000; Meade-Tollin et al, 1998). On the other hand, whereas both early- (II-4) and late-stage (A-5RT3) malignant HaCaT cells showed a mutual induction of MMPs in coculture with fibroblasts, this was not observed in cocultures of benign tumor cells with fibroblasts. This indicated that only malignant tumor cells were able to interact with stromal cells resulting in the induction of MMPs (Airola et al, 2001; Borchers et al, 1997). More importantly, in the stroma of malignant transplants, distinct dynamics of expression of different MMPs were noticed starting with MMP-9 (gelatinase B) within the first week after transplantation coinciding with beginning of angiogenesis and stromal infiltration in the collagen matrix. This was followed by the expression of MMP-13 (the interstitial collagenase of the mouse), after two weeks, and MMP-3 (stromelysin-1) as latest MMP being induced when invasive tumors had established (Fusenig et al, 2002; Mueller et al, 2002) and Vosseler, S, Airola, K. and Fusenig, N.E., in preparation). These data clearly indicated that benign and malignant epithelia elicit qualitatively different stromal responses evident by the distinct dynamics of induction of angiogenesis and MMP expression.

The kinetics of induction of stromal MMPs seen in the malignant tumor transplants suggests different functions for these proteases in tumor angiogenesis, vascularization and invasion. Initial attempts to identify the stromal cells producing the different MMPs, in particular MMP-9, were only partially successful. In frozen sections of transplants, the localization of MMP-9 expression by radioactive in situ hybridization indicated that the producer cells were perivascular and most probably

not the endothelial cells. Preliminary double immunostaining studies had suggested that a subpopulation of macrophages stained positive for MMP-9, although it was not clear whether these cells were also the producer cells. Further studies revealed a differential accumulation of inflammatory cells in the tumor stroma in benign and malignant transplants indicating that they might play an essential role in tumor stroma interactions, as already suggested from data of experimental carcinogenesis studies (van Kempen et al, 2002; Bergers et al, 2000).

METHODS

The low-grade malignant tumorigenic clone (A-5) was derived from the immortalized human keratinocyte cell line HaCaT (Boukamp et al, 1988) after transfection with the c-Ha-ras oncogene and by recultivation of heterotransplants in nude mice the highly malignant tumorigenic clone (A-5RT3) was established (Fusenig et al, 1998; Mueller et al, 2001; Boukamp et al, 1990). All cells were grown in enriched minimal essential medium (4x MEM) supplemented with 5% FCS and 200µg/ml geneticin.

Cells were transplanted onto the dorsal muscle fascia of 7- to 9-week-old nude mice (Swiss/c nu/nu back crosses) as monolayer cultures growing on collagen type 1 gels using a silicone chamber device (Skobe et al, 1998; Fusenig et al, 1983). Transplants were dissected en bloc, embedded in Tissue-Tek (Miles Laboratories, Elkhart, IN), and frozen in liquid nitrogen vapor for preparation of cryostat sections. For labelling of proliferating cells, mice received tail vein injections of 5-Bromodeoxyuridine (BrdU) and 2-deoxycytidine (65mM each) in 0.9% NaCl (100µl) 1.5h before being killed. Transplants were dissected after different timepoints. The experiments were repeated three times.

$2x10^5$ A-5RT3 cells in 100 µl culture medium were injected s.c. in the back of 7- to 9-week-old nude mice (Swiss/c nu/nu back crosses). Tumor formation was assayed weekly and two diameters determined by calipers over different observation periods and tumor size were calculated. Tumors were removed en bloc and processed like the transplants.

The in vivo anti-angiogenic activity of the VEGFR-2 neutralizing antibody DC101 from ImClone Systems Incorporated (New York, USA) was tested in mice carrying s.c. tumors and transplants of the highly malignant keratinocyte clone HaCaT-ras A-5RT3 starting 2 and 14d after transplantation, respectively. Mice received intraperitoneal injections of the mAb DC101 (500 to 800µg per mouse as indicated in 150µl PBS) or PBS at different time intervals (from twice a week to every 2nd day) for a total of 10 weeks. Transplants were dissected after different timepoints.

Rat mAb against mouse CD31 was obtained from BD PharMingen (Heidelberg, Germany), guinea pig polyclonal anti-serum against cytokeratins (pan) from Progen (Heidelberg, Germany), sheep polyclonal antibody against BrdU from NatuTec (Frankfurt a. M., Germany), rabbit polyclonal antibody against tenascin-C from Telios Pharmaceuticals (San Diego, USA), biotinylated mouse mAb against a-smooth muscle actin from Progen (Heidelberg, Germany), rat monoclonal antibody against mouse neutrophil granulocytes from Serotec (Düsseldorf, Germany), sheep polyclonal antibody against mouse MMP-9 and -13 were a gift from Prof. Gillian Murphy (University of Cambridge, UK), rabbit polyclonal antibody against mouse collagen type IV was from Novotec (Lyon, France). Secondary antibodies were obtained from Dianova (Hamburg, Germany) and Hoechst 33258 bisbenzimide for nuclear staining from Sigma-Aldrich (Taufkirchen, Germany).

For in situ hybridization mouse cDNAs encoding MMP-9 and MMP-13 were used (a gift from Prof. Gillian Murphy, University of Cambridge, UK).

For immunfluorescence staining, frozen sections were fixed for 5min in 80% methanol at 4°C and 2min in acetone at -20°C, and rehydrated in PBS. For BrdU localization in DNA, sections were additionally denatured in 2M HCl for 10min at room temperature and washed (3x10min). Primary antibodies were incubated in 12% BSA/PBS at RT for 2h or 4°C over night. After washing (3x10min) sections were incubated with appropriate secondary antibodies together with 5µg/ml Hoechst bisbenzimide for staining of cell nuclei. Prior to embedding in Permafluor (Immunotech, Marseille, France) sections were washed again (3x10min) in PBS. Stained sections were examined and photographed with a Olympus AX-70 microscope fitted with epifluorescence optics.

In situ hybridization was performed as described. In brief, DIG-labelled RNA probes for mouse MMP-9, -and -13 were prepared using T7, SP6 or T3 RNA-polymerase (for antisense and sense, respectively) according to the manufacturer's instructions (Roche, Mannheim, Germany). Cryostat sections were fixed in 4% paraformaldehyde, pre-treated, hybridized and washed at high stringency as described. DIG was labelled by anti-DIG-AP (Roche, Mannheim, Germany) and alkaline phosphatase reaction was detected by NBT/BCIP (Gibco-Life Technologies/ Invitrogen, Eggenstein-Leopoldshafen, Germany). After DIG in situ hybridization of MMPs counterstaining was performed by indirect immunfluorescence with antisera against pankeratin and collagen type IV. Sections were photographed with different channels being assigned different colors for further analysis using an Olympus AX-70 microscope fitted with epifluorescence optics and Analysis Imaging Software.

RESULTS

Enhanced dynamics of angiogenesis and MMP expression in late-stage malignant cell transplants

Comparable to our earlier observations of distinct dynamics of angiogenesis induction in benign and early malignant keratinocyte transplants (Skobe et al, 1997), we confirmed even more discriminating kinetics between another benign (HaCaT A-5) and a highly malignant and metastasizing HaCaT variant (A-5RT3; (Mueller et al, 2001). For these studies, we continued to use the matrix-inserted transplantation assay that we established and which proved to be a highly sensitive and useful in vivo assay to study tumor-stroma interactions (for review see (Fusenig et al, 2002; Mueller et al, 2001) and Fig. 1). Whereas in transplants of benign cells, a short and transient induction of angiogenesis and stromal activation occurred which declined after 3-4 weeks, this induction was earlier and stronger in late-stage malignant transplants and persisted throughout the whole observation period (10 weeks). The stromal reaction became manifested by the beginning of invasion of vessels and other stromal cells into the collagen matrix and the enhanced proliferative activity of endothelial and other stromal cells resulting in the formation of a reactive granulation tissue. As a characteristic indicator of endothelial cell activation, the upregulation of VEGFR-1 and -2 was persistent in malignant transplants but transient resulting in a downregulation in the benign stroma, prior to the reduction of cell proliferation and vessel density (Mueller et al, 2002; Skobe et al, 1997; Vosseler et al, 2005; Vosseler 2001). Similar dynamics as seen with angiogenesis were distinguished in the expression of selected MMPs in the stroma of benign and malignant transplants by in situ hybridization (see Fig. 1). Focussing on MMP-9 (gelatinase B), we confirmed earlier observations of a very early onset of induction of this protease in a distinct linear zone of stromal cells infiltrating the collagen matrix, just in front of the sprouting vessels both by in situ hybridization and immunostaining. This labelling of perivascular areas in stromal regions close to and within the tumor tissue was further enhanced and maintained in the malignant transplants throughout the observation period of 10 weeks. A comparable expression profile was observed for stromal MMP-13 using a mouse-specific cDNA probe and specific antibodies, but the onset of expression was delayed

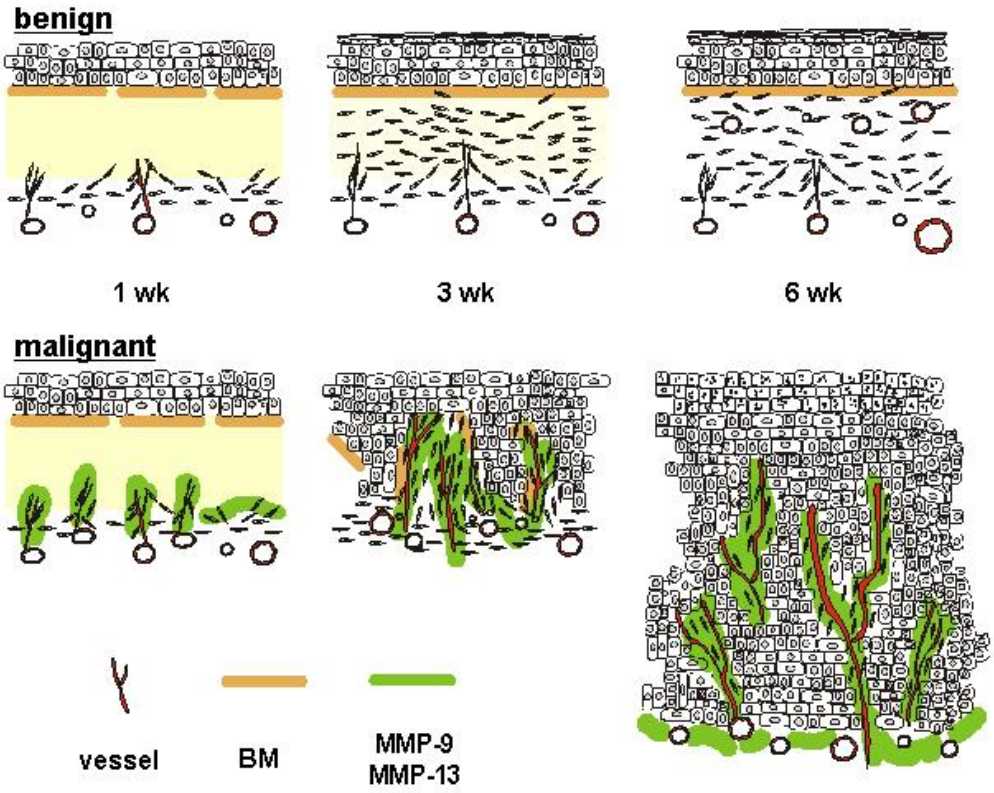


Fig. 1: Protease Expression in Tumor Transplants: Schematic cross-section through surface transplants at different timepoints showing benign tumor transplants with transient angiogenesis, complete basement membrane without invasion and no stromal MMP-9 and -13 expression in the upper panel. The lower panel indicates malignant transplants with persistent angiogenesis, interrupted basement membrane with invasion and high MMP-9 and -13 expression.

by 1-2 weeks and only started when the newly formed granulation tissue got into contact with the tumor epithelium.

Comparable to the kinetics of angiogenesis, the induction of MMP-9 expression was weaker and transient in transplants of benign cells starting at week 2, declining after week 3, and being absent after 4 weeks. Preliminary studies reported in the previous work report describing virtually negative results concerning MMP-9 expression in benign transplants obviously missed these short time periods of transient expression. This transient and weak stimulation of MMP-9 expression in benign transplants was also confirmed by immunofluorescent microscopy using a mouse-specific antibody (obtained from Prof. G. Murphy, Cambridge, UK). On the other hand, a consistent staining by this antibody was observed in stromal areas close to the tumor parenchyma in malignant transplants (Vosseler 2001); S. Vosseler, K. Airola and N.E. Fusenig, in preparation).

Cellular localization of MMP-9 protein and distribution of inflammatory cells

As reported in the last work report, the initial colocalization of MMP-9 antibody reaction with cells labelled with the Mac-1 (CD11b) antibody suggested a subfraction of macrophages as major producers or carriers for this protease. However, unexpected staining reactions on mouse blood smears and comparable reactivity of this antibody with areas in transplants identified as neutrophil granulocytes by a neutrophil specific antibody (Serotec, MCA771G, clone 7/4) which also stained neutrophil granulocytes in blood smears, raised serious doubts on the macrophage specificity of the

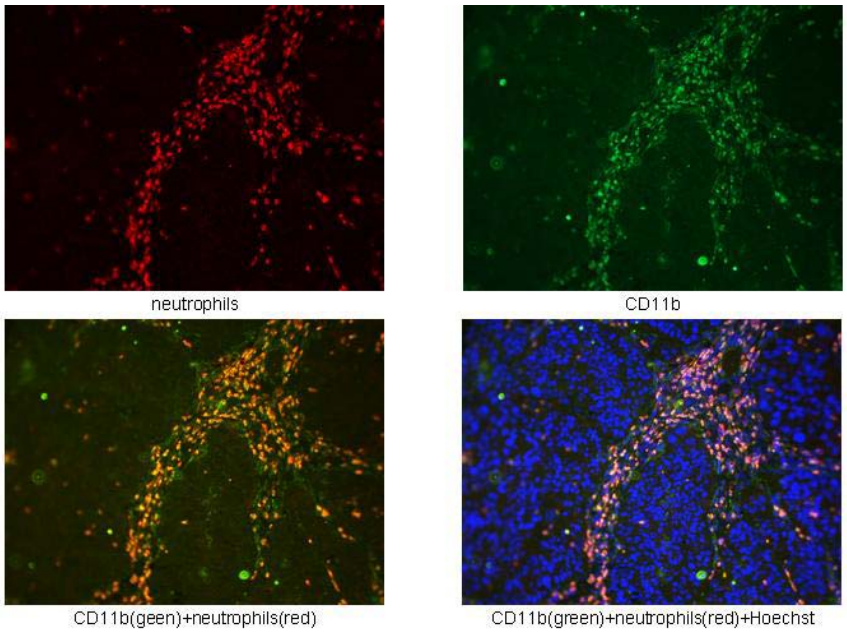


Fig. 2: Differential identification of neutrophil granulocytes (neutrophils) and monocytes/macrophages (CD11b) by double immunostaining in 6wk transplants of A5 RT3 cells (nuclear staining by Hoechst 33258)

Mac-1 antibody, although it is frequently used and cited as such in literature. Double immunostaining with Mac-1 and the neutrophil antibody was not possible with indirect immunofluorescence technique, since both are mouse monoclonal antibodies. However, with directly fluorochrome-coupled antibodies, co-immunolocalization was feasible and surprisingly, both antibodies colocalized on a large cell population considered as neutrophil granulocytes, although some cells were stained only by CD11b (Fig. 2). When double staining was performed with the MMP-9 and the neutrophil antibody, nearly all cells were again double stained, clearly indicating that the MMP-9 positive cells were, at least in their vast majority, neutrophil granulocytes (Fig. 3). In addition, some MMP-9 staining was obviously not direct cell-associated, but diffusely localized in stromal areas within the tumor parenchyma suggesting adsorption of the proenzyme to ECM components, as also demonstrated by others (Opdenakker et al, 2001; Zucker et al, 2000). On the other hand, when using another tissue-macrophage-specific antibody ERMP-23 (targeted to galactose-N-acetyl galactosamin) not recognizing monocytes in blood smears, a strict dissociation between ERMP-23-positive macrophages and MMP-9 staining cells was observed (Fig. 3). This codistribution of neutrophils and MMP-9 correlated well with the accumulation of MMPs and neutrophils in the tissues on one side and macrophages on the other throughout (see Fig. 3). Whereas neutrophils were rare and loosely dispersed in the stroma of benign transplants – comparable to the transient expression and weak staining of MMP-9 – they were numerous in malignant transplants and particularly accumulated in the stromal areas adjacent and infiltrating into the tumor parenchyma. In contrast, ERMP-23-positive macrophages were more frequent in the stroma beneath benign transplants and here closely associated to the epithelium, while in malignant transplants they were less frequent and localized more distantly from the epithelium. Thus, costaining with antibodies to MMP-9 and neutrophils and macrophages, respectively, a distinct discrimination of the two inflammatory cell types and the association of MMP-9 staining to the neutrophil granulocyte fraction was obvious. Therefore, neutrophils had to be considered the major carrier and possibly producer of MMP-9 in the stroma of malignant transplants (S. Vosseler, K. Airola, and N.E. Fusenig, in preparation).

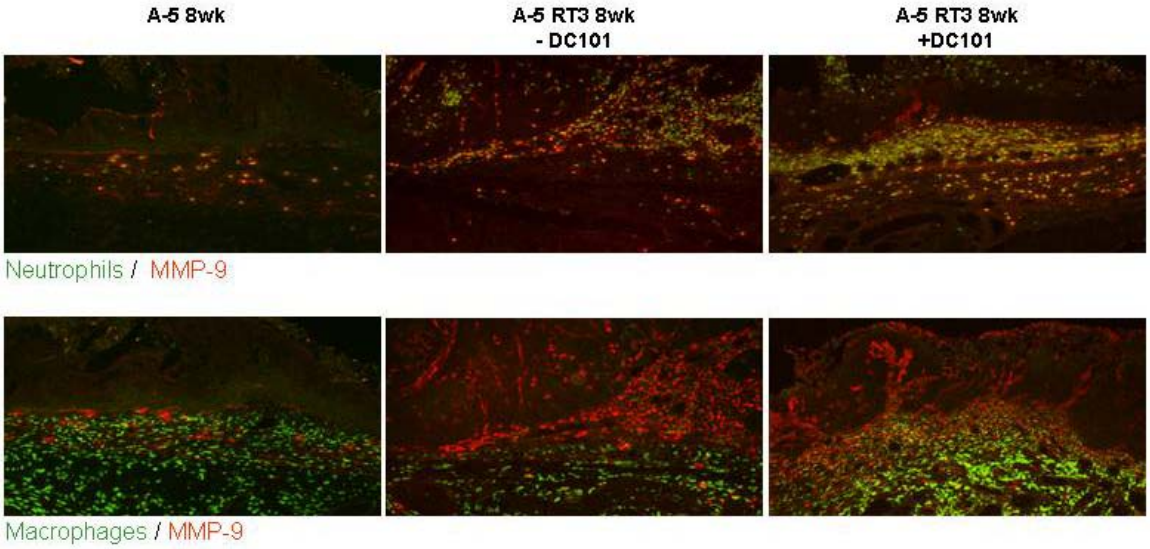


Fig. 3: Colocalization by double immunofluorescence microscopy of neutrophil granulocytes (upper column) and macrophages (lower column), respectively, in transplants of benign (A-5) and malignant (A-5RT3) cells in control (-DC101) and following angiogenesis inhibition by VEGFR-2 blocking antibody (+DC101).

Identification of inducing factors of MMP-9 expression and its producer cells

Although it was not clear whether neutrophils were induced by the malignant cells to express MMP-9 or are just attracted as carriers of this protease, their predominant localization in the tumor-near stroma of malignant transplants prompted us to search for chemotactic factors released by the malignant epithelia and not the benign ones. Neutrophils are well known to express and to transport ample amounts of MMP-9 in their granules and secrete it via degranulation (Opdenakker et al, 2001). In previous studies we had detected differential and constitutive expression of the hematopoietic growth factors granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-colony stimulating factor (GM-CSF) exclusively by late-stage malignant HaCaT cells following tumor progression (Mueller et al, 1999). Since the tumor cells also expressed the corresponding receptors for both factors, an autocrine stimulation of tumor cell growth and migration was postulated and confirmed in *in vitro* assays. Due to the correlation of enhanced malignancy of different HaCaT variants (Mueller et al, 2001) as well as late-stage human tumors (Mueller et al, 1999) with expression of G-CSF and GM-CSF and their receptors, a crucial role of these factors for tumor progression was postulated. On the one hand, both factors are primarily known for their stimulatory effects on granulocytes and monocytes and as functional chemoattractants for these cells. Their expression further corresponds to a stronger angiogenesis and stromal activation with a profound influx of inflammatory cells in the more aggressive tumor cell transplants (Fusenig et al, 2002; Mueller et al, 2002; Mueller et al, 2001). This suggested additional paracrine functions of G-CSF and GM-CSF through attraction of inflammatory cells and by stimulation of angiogenesis, stromal activation and protease expression (Obermueller et al, 2004). To better understand the functional significance of G-CSF and GM-CSF in tumor progression, each factor was stably transfected into benign (non-expressing) HaCaT A-5 cells. Although the transfected cells showed no alteration of proliferation in culture, G-CSF-expressing cells exhibited invasive tumor growth with strong angiogenesis and enhanced recruitment of granulocytes and macrophages in heterotransplants. On the other hand, GM-CSF-expressing cells grew to benign, non-invasive tumors with a typical transient effect on angiogenesis and leukocyte recruitment (Obermueller et al, 2004). Subsequent recultivation of cells from G-CSF-expressing tumor transplants, however, resulted in cells with

enhanced malignancy and, remarkably, additional and constitutive expression of GM-CSF. This suggested a crucial role and a cooperative function of both factors in tumor progression and modulation of the tumor microenvironment. The detailed analysis of the consequences of stable expression of G-CSF and GM-CSF on stromal protease expression, leukocyte infiltration and angiogenesis is still ongoing.

Inhibition of tumor angiogenesis causes vessel regression

As shown earlier, inhibition of angiogenesis by blocking VEGFR-2 signalling resulted in abrogation of tumor vascularization and blockade of invasion in transplants of an early-stage malignant keratinocyte cell line (Skobe et al, 1997). As a consequence of this anti-angiogenic treatment, the tumor epithelium acquired a quasi-benign phenotype with normalized architecture and enhanced epidermal differentiation so that the malignant nature of these cells was questioned. It was further argued that the inhibition of tumor invasion was dependent on the early stage of malignancy of these cells.

Therefore, we used the highly malignant and metastatic HaCaT variant A-5RT3 to demonstrate that in transplants of these aggressive cells repeated application of the VEGFR-2 blocking antibody DC101 drastically inhibits angiogenesis and tumor growth in a dose-dependent manner (Fig. 4). More importantly, DC101 also caused vessel regression in an already vascularized tumor tissue and induces vascular maturation (Vosseler et al, 2005; Vosseler 2001). Vessel regression in stromal strands penetrating the tumor epithelium is evident by the rapid disappearance of vessels within 2-4 days after start of treatment, demonstrated at the immunohistochemical level by the loss of CD31 staining.

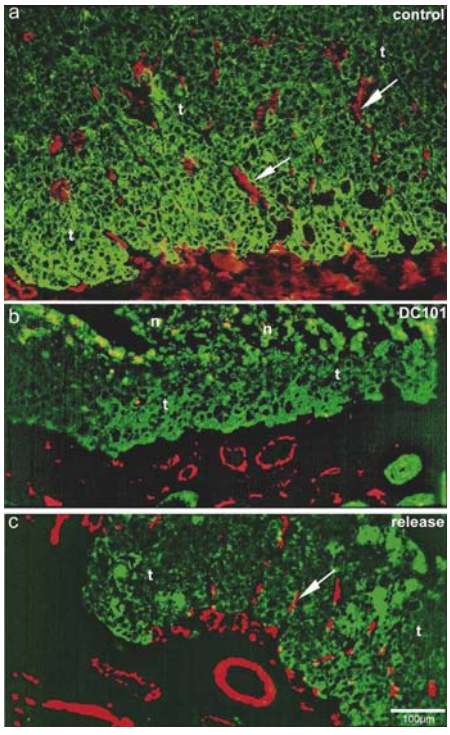


Fig. 4: DC101 abrogates intratumoral vascularization a) Control A-5RT3 subcutaneous tumor (t), heavily vascularized, 31 days after inoculation. b) After six weeks of DC101 treatment (720µg every 3rd day; start 2 days after inoculation), intratumoral (t) vessels had disappeared and necrotic areas (n) increased. c) Vascularization of tumor tissue (t) was restored three weeks after discontinuation of treatment. Double immunfluorescence microscopy of frozen sections stained with a antiserum against cytokeratins (green) to mark epithelial tumor cells (t) and CD31 (red) to label endothelial cells. Vessels in intratumoral stromal strands are marked by arrows (all panels same magnification, bar 100µm).

On the other hand, these stromal areas were clearly stained with an antibody to tenascin-C, a provisional ECM component which is prominent in tumor stroma (Miller et al, 2005).

This vessel regression is also functionally detectable by non-invasive imaging methods which we recently established for small experimental tumors in cooperation with Drs. Fabian Kiessling and Martin Krix, Department of Radiological Diagnostics at the DKFZ, utilizing both dynamic nuclear magnetic resonance tomography (MRT) and contrast-enhanced ultrasound (US) techniques (Kiessling et al, 2002; Kiessling et al, 2003; Krix et al, 2003). Within 2 days after start of DC101 application to nude mice carrying subcutaneous tumors of A-5 RT3 cells, effects on vascularization function, with a drastic decrease in perfusion are clearly obvious, before tumor size reduction is observed (Kiessling et al, 2003; Krix et al, 2003). These non-invasive techniques have turned out to be very valuable methods to examine functional parameters of tumor vascularization and sensitive diagnostic means to monitor anti-angiogenic therapy both in experimental animals and in patients.

VEGFR-2 inhibition affects maturation of vessels and stroma

At the cellular level, blockade of VEGFR-2 signalling caused a rapid downregulation of endothelial proliferative activity, as anticipated, with enhanced apoptosis on one side but also followed by phenomena of vessel maturation on the other. The earliest changes noticed were a rapid and complete downregulation of expression of VEGFR-2 but also of VEGFR-1, both preceding vessel regression and reduction of vessel density. This may be interpreted as indirect evidence that both receptors are regulated by VEGF through the interaction with VEGFR-2. Furthermore, the dilated and leaky vessel walls observed in control transplants had obviously matured to rather normal capillaries with a continuous endothelial lining being encircled by a structured basement membrane and associated with pericytes, as detected at the ultrastructural level (Vosseler et al, 2005). Another consequence of DC101 treatment was the nearly complete retraction of vessels from the small stromal strands penetrating the tumor parenchyma and, as a consequence, a drastic reduction of the vital tumor parts with a corresponding increase of necrotic tumor areas (Fig. 4). Interestingly, tumor cell proliferation in the vital areas was not strongly inhibited (Skobe et al, 1997; Vosseler et al, 2005). The most dramatic effect, however, was a nearly complete prevention and even reversion of invasion of these highly aggressive cancer cells, a phenomenon being comparable to what has been seen in early malignant cell transplants (Skobe et al, 1997; Vosseler et al, 2005). This blockade and reversion of tumor cell invasion is most probably a consequence of maturation processes in the stromal regions immediately adjacent to the tumor cells. At the lightmicroscopical level, a small zone just beneath the tumor parenchyma was nearly completely devoid of blood vessels when stained with CD31.

At the ultrastructural level, myofibroblasts and thick bundles of collagen fibers accumulated in this zone immediately beneath the tumor cells. More impressively, the highly irregular tumor-stroma border zone with multiple large tumor cell membrane protrusions and vesicles shed into this area had disappeared and a rather normal basal cell membrane was visible exhibiting hemidesmosomes which connected the cell membrane with stretches of a reformed lamina densa (Vosseler et al, 2005). This dramatic normalization of the tumor stroma border is supposedly a consequence of a reduced degradation of the respective basement membrane components due to inhibition of protease expression and/or activation. The synthesis and partial deposition of the major components of the basement membrane was still detected by immunohistochemistry, even in highly invasive areas (Tomakidi et al, 1999). There is evidence from other studies that de novo synthesis of basement membrane constituents in tumor and adjacent stromal cells is enhanced (Hagedorn et al, 2001). Thus, we hypothesized that the structural reorganization of the epithelial basement membrane

which probably prevented tumor cell invasion, was due to altered turnover of its constituents caused by reduced protease expression and activity.

We have observed a comparable phenotypic normalization of the tumor-stroma border in another experimental setting when both early (II-4) and late-stage (A-5RT3) malignant HaCaT cells were transplanted onto a modulated stromal bed. This was achieved by preimplantation of a biodegradable hyaluronan-based meshwork which rapidly induced the formation of a granulation tissue which later developed into a foreign body granulation tissue. This fibrotic tissue nearly completely inhibited invasion of II-4 and considerably delayed infiltration of A-5RT3 cells (M. Willhauck, H.-J. Stark, N. Mirancea, S. Vosseler, N.E. Fusenig, submitted). A modulation of the tumor-stroma border was first evident by the accumulation of myofibroblasts and, again, thick collagen filament bundles beneath the tumor cells as well as the reappearance of stretches of a structured basement membrane with hemidesmosome-like condensations in the epithelial ventral plasma membrane. This alteration in tumor stroma was probably causal for the observed inhibition of tumor invasion. Thus, stromal modulation can either positively (as shown with PDGF-transfected HaCaT cells, (Skobe et al, 1998) or negatively influence tumor progression and tumor phenotype in experimental model systems.

The next obvious question to answer was whether inhibition of angiogenesis and the resulting normalization of tumor-associated stroma was due to an altered expression/activation of MMPs, in particular of the gelatinase MMP-9. However, when DC101-treated transplants were stained for stromal MMP-9 with a mouse-specific antibody, no substantial reduction of MMP-9 protein was observed throughout the observation period up to 8 weeks of treatment. Similarly, the number of neutrophil granulocytes was not reduced but rather increased in the stroma of treated malignant transplants which exhibited blockade of invasion. The density of macrophages stained with ERMP-23 was also clearly increased and cells were localized more closely to the tumor parenchyma. Again, MMP-9 immunoreactivity was colocalized within the area populated by neutrophils and costained these cells, but also associated with tenascin-C positive, but CD31-negative stretches of stroma strand penetrating into marginal tumor areas. Interestingly, granuloctyes were no longer positively stained with the MMP-9 antibody when localized in the necrotic tumor areas. This observation strongly indicated that staining was preferentially caused by MMP-9 stored in the neutrophil granules and that this staining was lost after degranulation (see Fig. 3). This release of stored MMP-9 obviously did not occur at the tumor-stroma border but only in the necrotic tumor areas, or, alternatively, immunostaining was masked in this environment.

This discrepancy between a morphologically evident reduction of ECM turnover and the persistent localization of MMP-9 protein prompted us to intensify our attempts to visualize MMP-9 expression by non-radioactive in situ hybridization and identify the synthesizing cell types by immunostaining on the same tissue section. Using serial sections for in situ hybridization and immunolabelling, larger tissue areas may be identified but at the single cell level, in particular in a poorly organized structure, such as tumor stroma, a clear localization at the single cell level is very difficult with this procedure. Double labelling of the same frozen sections, however, with in situ hybridization and immunostaining caused major problems, because most antigens were too sensitive to survive the rather harsh treatment required for in situ hybridization so that only a few antigenic sites could be visualized. So far, we found two which tolerated the procedure and these were cytokeratins to visualize tumor cells and collagen type IV to highlight blood vessels by staining of their basement membrane (Fig. 5). The results obtained in these studies were rather surprising in that mouse-MMP-9 mRNA localization was restricted to small spots at the tumor-stroma margin, mostly in the intratumoral septae but clearly in the stromal compartment, as should be expected when using a mouse-specific cDNA probe (Fig. 5). These spots were in the vicinity of collagen type IV antibody-stained vessel areas but not within

endothelial cells, though all available endothelial cell-specific antibodies did not maintain their reactivity. Clearly, the areas of MMP-9 mRNA expression were more restricted than indicated on sections used for radioactive in situ hybridization and even much more than the immunostained areas. Also, most parts of stromal regions populated with granulocytes did not exhibit MMP-9 expression signals.

Following DC101 treatment, these spots of MMP-9 expression were drastically reduced and only visible in a few small remaining stromal invaginations into the tumor parenchyma. From these still preliminary data we conclude that the immunostaining of MMP-9 mostly labelled stored material in granules of neutrophils and that this was not or only marginally affected by DC101 in contrast to the newly synthesized protease. We further conclude that the local MMP-9 synthesis most probably does not occur in granulocytes but in other vessel-associated cells, possibly myofibroblasts, or other functional states of stromal cells.

Studies on the expression of mouse MMP-13 mRNA by non-radioactive in situ hybridization demonstrated its localization in defined spots at the immediate border of the epithelial tumor tissue, whereas immunolocalization was both cellular, but mostly extracellular in the intratumoral stromal strands. Long-term treatment with DC101 (6 weeks) drastically reduced the expression of MMP-13 mRNA (Fig. 5 e, f) comparable to that seen with MMP-9. On the other hand, it is also reduced significantly the immunolocalization of the MMP-13 protein which was now restricted to individual perivascular cells.

In order to further identify MMP-9 and MMP-13 expressing stromal cells, fibroblasts have been isolated from tumor tissues from nude mouse heterotransplants representing tumor-modulated reactive granulation tissue cells. Furthermore, in order to obtain more reliable tumor-associated

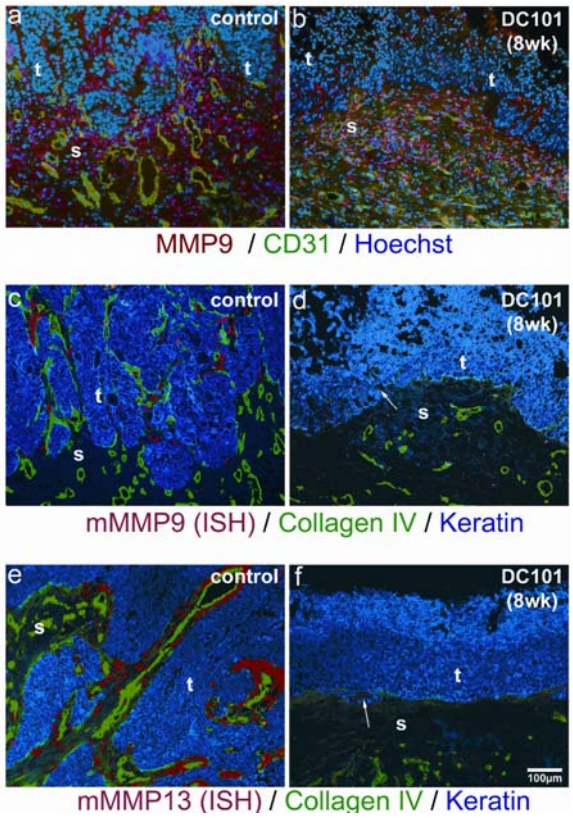


Fig. 5: DC101 abrogates stromal expression of MMP-9 and MMP-13. a) At the protein level, mouse MMP-9 is localized by immunostaining (in red) to the stromal areas beneath and within the tumor (t) epithelium in 10-week control transplants. b) Staining is not significantly reduced following DC101 treatment (800µg/animal, every 2nd day, start 14d after transplantation) for 8 weeks. Double immunofluorescence staining of vessels with CD31 antibody (green) and mouse MMP-9 antibody (red). Nuclei are counterstained with Hoechst DNA stain (blue). c) At the RNA level, murine MMP-9 expression is localized by non radioactive in situ hybridization (ISH, red color) to distinct spots adjacent to vessels (green) and tumor cells (blue), predominantly in intratumoral stromal strands in transplants. d) DC101-treatment (800µg/animal, every 2nd day, start 14d after transplantation) (for 8 weeks) reduces stromal MMP-9 expression at the tumor stroma border to a few spots (arrow). e) Mouse MMP-13 (ISH, red color) is strongly expressed in the intratumoral stromal strands close to vessels (green) and to the tumor border (blue) and (f) dramatically downregulated after 8 week-inhibition of VEGFR-2 signalling. Only a few spots in the adjacent stroma (s) (arrow) are visible. Double immunofluorescence microscopy staining of tumor cells (t) with a cytokeratin antibody (blue) and vessel basement membrane by a collagen type IV antibody (green) as well as non-radioactive in situ hybridization signals (red) in the same frozen sections. All panels same magnification, bar 100µm.

stromal cells, fibroblasts have been isolated from human skin SCCs, purified by magnetobead selection using the human fibroblast-specific antibody AS02 and propagated in vitro. Characterization of these tumor-derived fibroblasts is ongoing concerning their expression of ECM components, growth factors, and cytokines, both in mono- and coculture with tumor cells. Surprisingly, these cells, even after a few passages in vitro, have maintained a property of enhancing tumor growth as demonstrated by the induction of tumor growth in heterotransplants of the non-tumorigenic immortal HaCaT keratinocytes when coinjected subcutaneously (J. Mertens, M. Willhauck, K. Kleinschmidt, H.-J. Stark and N.E. Fusenig, in preparation). These "primed" stromal cells represent appropriate candidates to examine the induction of MMPs by tumor cells in 3D organotypic cultures.

Rapid effects of VEGFR-2 Inhibition

In follow-up studies done in 2004 we observed rapid reduction in vascularization and reversion of the tumor phenotype after short-term treatment with DC101. Beginning 24 hours and progressively continuing through 96 hours of DC101 treatment, vascularization of the tumor tissue was rapidly reversed and tumor invasion was strongly inhibited. In particular, stromal areas localized within the tumor parenchyma showed reduced numbers of CD31 stained vessels. This suggested that VEGFR-2 inhibition not only limited the formation of new vessels but may have also caused rapid regression of pre-existing vessels. This was confirmed by quantifying vessels in tumor-associated stromal strands which were identified by immunostaining against tenascin-c, a major component of the tumour’s stromal ECM. Treatment with DC101 progressively reduces vessels within the strands of the tumor stroma. To better define the effects of VEGFR-2 inhibition on vascular physiology, endothelial proliferation was analyzed. Whereas endothelial proliferation was reduced in those vessels that remained after treatment, no detectable apoptotic endothelial cell could be found. This data suggests that VEGFR-2 inhibition is not only capable of limiting the formation of new vessels but may have also caused rapid regression of preformed vessels leading to reduction in microvessel density as soon as 24 hours after initial DC101 treatment and continuing throughout the duration of theses studies. Ultrastructural analysis of the tumor vasculature revealed that treatment with DC101 results in a rapid regression of immature vessels leading to the predominance of the mature vascular phenotype. Simultaneously, stromal expression of MMP-9 and MMP-13 was drastically reduced 96 hours after VEGFR-2 inhibition as detected by in situ hybridization and in situ zymography indicated decreased gelatinase activity. Moreover, the morphology of the tumor-stroma border changed from a highly invasive carcinoma to a well-demarcated, pre-malignant phenotype. The latter was characterized by the appearance of a regular basement membrane in immunostaining and ultra-structural analysis (Fig. 6). These findings suggest that VEGFR-2 inhibition by DC101 evokes very rapid reduction of preformed vessels and decrease in stromal protease expression and gelatinolytic activity resulting in the modulation of the tumor-stroma border zone and reversion of the tumor phenotype. Thus, short-term inhibition of VEGF signalling results in complex stromal alterations with crucial consequences for the tumor phenotype.

Functional consequences of MMP inhibition on angiogenesis and tumor invasion

The originally planned transplantation experiments using MMP-9 knockout nude mice have not been performed so far for two reasons:

1. MMP-9 knockout mice incrossed into a nude background were not readily available, but attempts have been made to obtain these animals.

2. Studies of another laboratory (Prof. A. Noel, Liège, Belgium, personal communication) with immunocompetent MMP-9 knockout mice and transplanting syngeneic mouse carcinoma cells with our surface transplantation assay did not reveal any substantial alterations of tumor

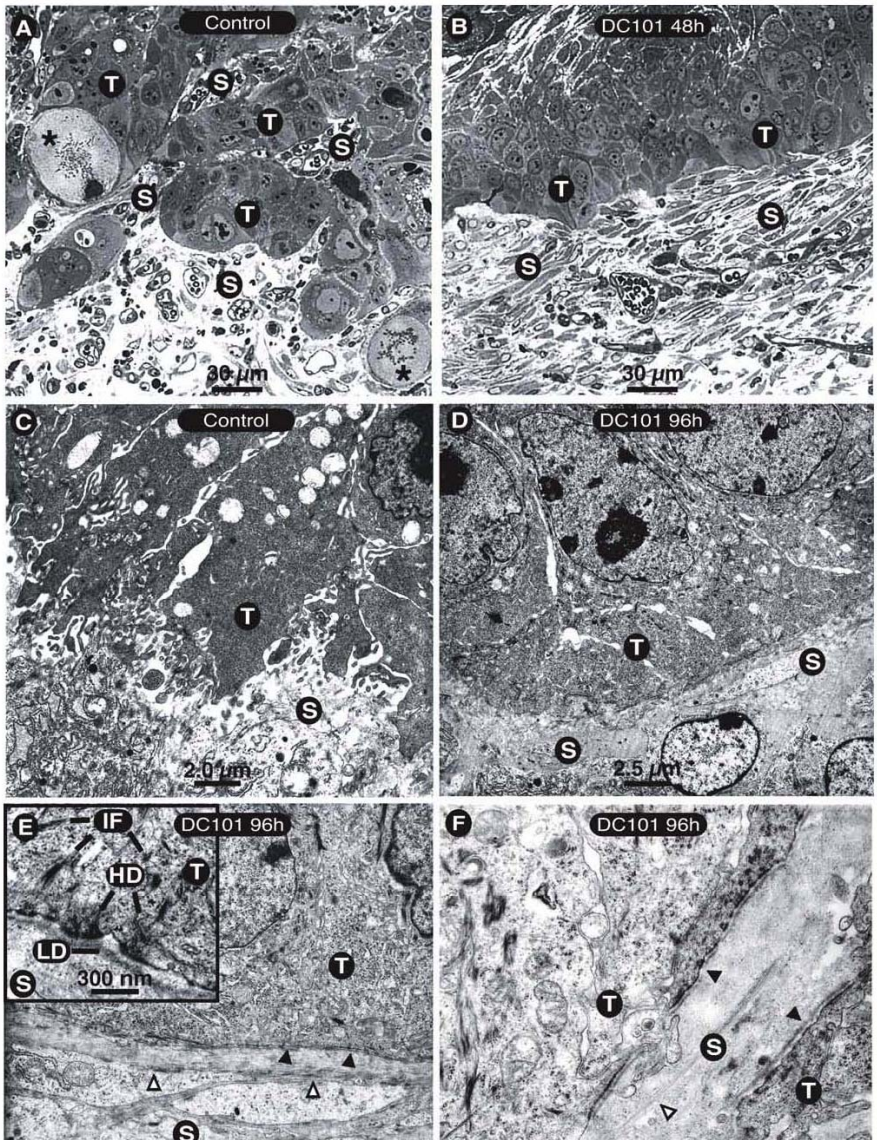


Fig. 6: Ultra-Structure of the Tumor Stroma Border Zone. Semi-thin sections of control (A) and 48 hours DC101 treated (B) surface transplants. A shows a control with irregular tumor (T) stromal (S) borders with mitotic tumor cells (*). 48 hours after DC101 treatment (B) there is a regular tumor stromal border with a reduction of vessels within the underlying stroma as compared to the control (A). These changes were pronounced at the ultra-structural level in controls (C) with numerous cellular extensions invading the surrounding stroma. After 96 hours of DC101 treatment (D-F) there is a regular tumor stromal border with collagen bundles (open arrowheads). The treated sample in E shows a continuous basement membrane (filled arrowheads) along the tumor-stromal border with collagen bundles present in the stroma (open arrowheads). The insert in E shows a higher magnification of the basement membrane after 96h DC101 treatment showing a distinct lamina densa (LD) as well as hemidesmosomes (HD) connected to intermediate filaments (IF). F shows a 96h DC101 treated sample with a fingerlike stromal projection with regular tumor-stromal borders, collagen bundles (open arrowheads), and an adjacent basement membrane (filled arrowheads) between 2 tumor areas.

vascularization or invasion as compared to wildtype animals. On the other hand, there has been a recent report on the reduction of intraperitoneal tumor growth of an ovarian carcinoma associated with decreased macrophage infiltration in MMP-9 knockout mice (Fidler 2002). Thus, it has to be determined whether the genetic background of the respective knockout animals played a major

role in causing these different results and whether MMP-9 alone plays a major role in tumor angiogenesis and invasion. We are in contact with both groups to get the respective MMP-9 knockout nude incrosses and will then perform the transplantation studies.

As additional proof for the functional role of MMP-9 as well as other MMPs in tumor angiogenesis and invasion, we performed several inhibitory studies in vivo with established and newly developed MMP inhibitors, although with rather low success. These studies included:

a) Experiments with the established broad-range inhibitor batimastat (BB94, British Biotec) applied daily to nude mice carrying s.c. transplants of the aggressive A-5RT3 HaCaT cell line. When treatment was started 2 days after tumor cell inoculation, an average of 40% growth inhibition was noticed after 3 and 4 weeks as compared to controls. Nevertheless, treated tumors were still rapidly enlarging so that the experiment was terminated. By immunohistochemistry, no major reduction in the vessel density was noticed in the treated as compared to the control tumors.

b) The second study was performed with a synthetic MMP inhibitor (RO28-2653, Roche Diagnostics, Penzberg) with high specificity for MMP-2, -9 and MT-1MMP using again s.c. heterotransplants of A-5RT3 cells and starting treatment 2 days after cell inoculation. From week 2 to 5 a significant reduction in tumor growth was noticed in the treated group by 50%. Histologically, no major differences between treated and control tumors were observed and the same was true for the immunohistochemical visualization of vascularization. Immunostaining with the mouse-specific MMP-9 antibody showed strong staining of stromal strands, however, no difference between control and treated tumors was seen. The only difference observed was a tendency to a reduced frequency of (ERMP-23-positive) macrophages in the treated tumors.

c) In a third attempt, a novel phosphinic-type inhibitor (RX P03 from Dr. Vicent Dive, Paris, France) has been tested. This inhibitor showed in vitro high affinity to MMP-2, -8, -9 and -11 and had demonstrated in another laboratory moderate inhibitory effect on the growth of heterotransplanted tumors. Using again A-5RT3 heterotransplants in nude mice and systemic treatment with this inhibitor, we only observed minor growth inhibitory effects throughout an observation period of 4 weeks when the experiment had to be terminated due to the size of the tumors.

Thus, due to the actual lack of better and more specific protease inhibitors, this type of studies has been terminated and the interest in the next period will be focussed on the functional role of specific proteases in remodelling of the ECM in the tumor stroma.

DISCUSSION

VEGFR-2 blockade causes normalization of tumor stroma and reverts tumor phenotype

The results of our studies demonstrate that in a mouse model of tumor invasion using the human skin SCC line A-5RT3 inhibition of VEGF signalling by blocking its interaction with VEGFR-2 has wide-ranged consequences for tumor-stroma interactions leading to reversion of the tumor phenotype. Blockade of VEGFR-2 resulted in inhibition of tumor growth and in abrogation of tumor cell invasion. This not only applies to low-grade malignant tumor cells as shown earlier (Skobe et al, 1997) but is even more impressive in transplants of the high-grade and metastatic SCC cell line A-5RT3 used in this study. VEGFR-2 blockade induced a phenotypic shift from a highly malignant to a premalignant, i.e. non-invasive tumor phenotype. This was associated with regression of immature and maturation of preexisting vessels as well as with the remodelling of the activated tumor stroma into a stabilized connective tissue. Most importantly, blockade of VEGFR-2 resulted in normalization of the tumor-stroma border with restoration of mature basement membrane structures including

hemidesmosomes at the basal pole of tumor cells. This maturation of the tumor-stroma border zone with accumulation of collagen bundles and reformation of basement membranes is most likely due to a downregulation of matrix-degrading proteases in the stroma, as shown for mRNA expression of murine MMP-9 and MMP-13. This decrease in essential ECM-degrading enzymes could then result in a reduced turnover of crucial BM-constituents enabling their accumulation and structural organization. As a consequence, structured basement membranes and a stable connective tissue were formed indicated by the accumulation of collagen bundles.

The surprising normalizing effects on tumor cells with disappearance of membrane protrusions and vesicles and reformation of hemidesmosomes was not caused by blockade of a direct VEGF effect on tumor cells. Thus, the normalizing effect on the tumor cell phenotype must be caused indirectly by stromal alterations resulting in the formation of features reminiscent of a fibrotic tissue and highlighting again the essential role of the tumor microenvironment (Olumi et al, 1999; Tlsty 2001; Bissell et al, 2002). Because these stromal alterations were initiated by downregulation of endothelial-cell activity, endothelial cell activation and maturation, respectively, may have essential functional consequences for the pathophysiology of the tumor stroma. While these features of stromal maturation and tumor phenotype reversion are also seen in subcutaneous tumors, they are particularly well demonstrated in surface transplants with their distinct geometry and the well-defined stromal development (Mueller et al, 2002).

Abrogation of VEGF signalling results in vessel regression and maturation

The consequences of blocking VEGF signal transduction observed in this studies are clearly exceeding the classical role of VEGF as mitogenic, motogenic and survival factor for endothelial cells (Folkman et al, 1992; Carmeliet et al, 2000; Ferrara et al, 1999; Carmeliet 2000; Ferrara 2002). For more than a decade, the central role of VEGF in the regulation of angiogenesis, in particular in tumor angiogenesis has been substantiated by many studies (Carmeliet 2000; Ferrara 2002; Dvorak et al, 1995; Klement et al, 2000; Prewett et al, 1999). However, questions concerning its functional significance in the interactions between tumor cells and the surrounding stroma have remained largely untouched.

Due to the elimination of VEGF signalling as a critical survival factor for endothelial cells of immature, pericyte-free small vessels (Benjamin et al, 1999), DC101 treatment causes vessel regression with retraction of preformed blood vessels, particularly visible in the stromal projections within the tumor parenchyma. This vessel regression and the associated blockade of formation of new vessels was enforced by the downregulation of expression of both VEGF receptors (VEGFR-1 and -2) starting one week after DC101-treatment (i.e. in 3-week-old transplants), and resulting in a complete loss of signal between week 2 and 3 of treatment. Since ligand interaction is considered a major regulator of VEGFR-2 expression (Elvert et al, 2003) blocking this interaction may have been causal for VEGFR-2 downregulation as shown recently (Zhang et al, 2002). This would, however, not explain why VEGFR-1 is also downregulated, because DC101 specifically blocks ligand binding to VEGFR-2 (Witte et al, 1998) and VEGF expression in tumor cells is ongoing at a high level (Javaherian et al, 1998). Downregulation of both VEGF receptors coincident with the enhanced association of vessels with α-sm actin-positive perivascular cells, that we considered to represent pericytes, can be understood as hallmarks of vessel maturation (Jain 2003). The interpretation of vessel normalization is further supported by the substantial change of vessel ultrastructure from typical tumor vessels with dilatated, thin, and interrupted endothelial lining to mature pericyte-associated and basement membrane-surrounded capillaries embedded in a collagen fiber-rich ECM. Whether this normalization of vessels was induced by withdrawal of VEGF signalling exclusively or additionally mediated indirectly in the context of stromal maturation, i.e. by downregulated protease activity

awaits further analysis. Further data on normalization of tumor vessels as a result of blockade of VEGF signalling have been reported demonstrating reduced vascular permeability (Tong et al, 2004) and reversion of vascular fenestration (Inai et al, 2004).

Stromal maturation by reduced MMP expression and ECM turnover

The most remarkable consequences of DC101-treatment were the changes in the tumor-stroma border zone. Here a massive accumulation of collagen bundles on the stromal side and a regular structure of the basal pole of tumor cells were prominent features resulting in a smooth and straight tumor surface which strongly contrasted the irregular border zone in control transplants. This was second only to the reappearance of long stretches of a well-structured basement membrane typically anchored to the basal cell pole on one side through hemidesmosomes and by anchoring fibrils to the collagen fibers on the other. This surprising feature of a normalized epithelial stromal border zone formed by a poorly differentiating and metastasizing carcinoma cell line (Mueller et al, 2001) was similarly seen in DC101-treated subcutaneously growing A-5RT3 tumors (not shown here). Whereas immunohistological staining of malignant tumor-stroma borders with antibodies specific to BM components, such as collagen type IV or laminin is observed, though often as interrupted labelling in well-differentiated carcinomas (Tomakidi et al, 1999; Tomakidi et al, 2003; Tomasek et al, 2002) a structured basement membrane with hemidesmosomes has not been reported to date. Formation of a structured BM is a diagnostic feature of premalignant tumor lesions and classically seen in benign tumor stages. In addition, considering the inverse correlation observed between the amount of retained peritumoral basement membrane and the degree of tumor aggressiveness (Barsky et al, 1983), reformation of basement membrane structures is a clear indication for a phenotypic shift of the invasive malignant transplants to a premalignant, well delineated tumor phenotype following VEGFR-2 blockade.

Whether these characteristics of stromal maturation are due to altered expression levels of ECM components or their reduced turnover as consequence of lowered protease activity, requires further detailed studies (Hagedorn et al, 2001). However, our data indicate that altered protease expression may play an important role. One of the matrix-degrading proteases most frequently associated with malignant tumors is MMP-9 (gelatinase B) which is known to cleave components of the ECM, particularly of the BM (Huang et al, 2002; Yu et al, 2000) thereby facilitating endothelial cell migration as well as tumor cell invasion (Nelson et al, 2000; Stetler-Stevenson 1999). Blockade of VEGFR-2 by a tyrosine kinase inhibitor reduced MMP-2 and -3 secretion in endothelial cells and inhibited their migration (Wagner et al, 2003). In MMP-9-deficient mice reduced angiogenesis, tumor progression and metastasis have been reported, highlighting the role of this protease for angiogenesis and cancer (Werb et al, 1999; Coussens et al, 2000; Itoh et al, 1999). However, the molecular mechanisms by which MMPs promote tumor invasion and angiogenesis are still poorly understood (Quaranta 2000). More recent studies suggest that ECM-proteolysis contributes to angiogenesis by exposing cryptic regulatory elements within ECM components (Xu et al, 2001; Kalluri 2003) and by release of ECM-sequestered angiogenesis factors (Coussens et al, 2000).

In our study, localization of MMP-9 protein in the stroma of malignant transplants revealed a rather diffuse perivascular staining in the stromal strands and predominantly localized to neutrophil granulocytes as seen by counterstaining (S.V. unpublished results). Neutrophil granulocytes are known to store considerable amounts of MMP-9 in their granules (Opdenakker et al, 2001) and sequestration of proMMP-9 as well as other MMPs to ECM components has been demonstrated (Olson et al, 1998). Following DC101-treatment there was some decrease in protein staining in the stromal strands in the tumor tissue but staining was not significantly reduced in the underlying stroma where granulocytes were still abundant (not shown here, S.V. in preparation). However, when RNA

expression was analyzed MMP-9 expression was restricted to distinct spots in intratumoral stromal strands, in close association to vessels and tumor cells. Whether the expressing cells were endothelial cells, pericytes (Nielsen et al, 1997) or other stromal cells awaits further investigations. In any case, this expression was drastically reduced in DC101-treated transplants with downregulation of endothelial activation by blocking VEGF signalling (Sweeney et al, 2002). We hypothesize that this reduced expression of MMP-9 was responsible for reduced degradation of BM components thus allowing their accumulation and structural reorganization. In particular, laminin is a critical determinant of morphogenesis and differentiation and directs tissue-specific gene expression in tissue-type in vitro models (Streuli et al, 1995). Its reduced turnover due to MMP-9 downregulation may contribute not only to BM reconstitution but also to normalization of epithelial polarity and thus to the reverted tumor phenotype.

Comparably, the reduced expression of murine MMP-13 in the tumor adjacent stroma indicated decreased turnover of its substrate collagen type I which supposedly resulted in the accumulation of bundles of collagen fibers in the tumor-neighboring stroma. Although the MMP-13 synthesizing cell type has not yet been identified, the localization of RNA signals indicates that perivascular cells, most probably fibroblasts, are promising candidates. Their localization close to endothelial cells and tumor cells suggests that MMP-13 expression is induced by paracrine signals from both neighboring cells, a regulatory mechanism that may well extend to MMP-9. Blockade of endothelial cell activation obviously resulted in downregulation of both MMPs suggesting a major paracrine role of endothelial cell-derived factors to control these stromal MMPs.

This rather complex process of stromal maturation into a dense fibrotic tissue together with the reformation of a structural BM obviously resulted in phenotypic reversion of the invasive malignant tumor into a premalignant, non-invasive tissue. This is in line with a rapidly increasing body of evidence from several groups demonstrating that the microenvironment has crucial regulatory functions on the tumor phenotype (Bissell et al, 2002; Kenny et al, 2003; Tomasek et al, 2002; Javaherian et al, 1998). We and others have thus demonstrated with different tumor cell types using in vitro and in vivo model systems that signals from the microenvironment can enhance or revert the malignant phenotype without affecting the abnormal genotype of tumor cells. These results further document the regulatory potential of the stroma on the tumor phenotype and suggest promising new tools for tumor therapy.

Follow-up studies done in 2004 showed the rapid effects of VEGFR-2 inhibition upon vascular regression and reversion of the tumor-phenotype in an experimental model of angiogenesis and tumor invasion using surface heterotransplants of the highly malignant human SCC cell line A-5RT3. Here, the initial phases of a phenotypic shift from a malignant to a pre-malignant phenotype after VEGFR-2 inhibition were associated with both vascular regression and quiescence and remodeling of the tumor-stromal border. Normalization of the tumor-stromal border was detected after DC101 treatment on an ultra-structural level with reduction of tumor cell protrusions from the surrounding stroma and formation of a typical basement membrane at the tumor surface. Additionally, as a consequence of immature vessel regression DC101 treatment resulted in a normalization of the microvasculature with capillaries exhibiting complete endothelial and pericytic basement membranes and interendothelial junctions as well as close association with surrounding pericytes.

On a histological level, normalization of the tumor-stromal border was characterized by the disappearance of stromal projections from the tumor and reversion of the invasive phenotype. These findings were especially striking at the ultra-structural level, with accumulation of collagen bundles in tumor adjacent stroma and the formation of a well structured basement membrane attached to the basal tumor cell pole by hemidesmosomes. This was striking considering the aggressive growth characteristics of the SCC cell line A-5RT3 (Mueller et al, 2001). Formation of a structured basement

membrane is a diagnostic feature of benign or pre-malignant tumors. Additionally, there has been an inverse correlation reported between the amount of tumor-associated basement membrane and tumor aggressiveness (Barsky et al, 1983). Therefore, the normalization of the basement membrane shown here is a clear indication of a phenotypic shift in the invasive malignant transplants to a pre-malignant tumor phenotype after VEGFR-2 inhibition.

The extent to which basement membrane and stromal maturation are due to altered expression of extracellular matrix components and/or their reduced turnover through lowered protease activity remains to be studied (Hagedorn et al, 2001). However, our data showing reduced expression of both MMP-9 (gelatinase B) and MMP-13 (collagenase), with reduced levels of gelatinase activity as seen by in situ zymography indicates that altered protease expression may play an important role in the maturation process. MMP-9 is one of the matrix-degrading proteases most frequently associated with malignant tumors and is known to cleave components of the extracellular matrix, particularly those of the basement membrane (Huang et al, 2002; Yu et al, 2000). It has been shown to facilitate endothelial cell migration as well as tumor cell invasion (Nelson et al, 2000; Stetler-Stevenson 1999). Furthermore, MMP-9 deficient mice display inhibited angiogenesis, tumor progression, and metastasis (Werb et al, 1999; Coussens et al, 2000; Itoh et al, 1999). Our studies not only showed a reduction in the expression of MMP-9 after VEGFR-2 inhibition but also a reduction in general gelatinase activity, which suggested their inhibition plays a crucial role in the stromal maturation process after DC101 treatment. Comparably, reduced MMP-13 expression in tumor adjacent stroma most likely contributed to decreased turnover of collagenase type I. Additionally, blockade of VEGFR-2 by a tyrosine kinase inhibitor also reduces the secretion of other matrix metalloproteases such as MMP-2 and MMP-3 in endothelial cells, inhibiting their migration (Wagner et al, 2003). However, the molecular mechanisms by which MMPs promote tumor invasion and angiogenesis are still poorly understood (Quaranta 2000). We hypothesize that reduced expression of MMP-9 and MMP-13, with subsequently reduced gelatinase activity was responsible for reduced degradation of BM components, therefore allowing their accumulation and structural reorganization. In particular, laminin is a critical determinant of morphogenesis and differentiation and directs tissue-specific gene expression in tissue-type in vitro models (Streuli et al, 1995). Its reduced turnover due to MMP-9 downregulation may contribute not only to BM reconstitution but also to normalization of epithelial polarity and, thus, to the reverted tumor phenotype. Significantly, these normalization effects were not caused by the direct blockade of VEGF on tumor cells. The human A-5RT3 tumor cells express VEGFR-2 in vitro at the RNA level (data not shown), however, the mouse-specific DC101-antibody does not cross-react with the human VEGFR-2 (Witte et al, 1998). Thus, the normalizing effect on the tumor cell phenotype must be caused indirectly by stromal alterations (Olumi et al, 1999; Bissell et al, 2002; Tlsty 2001). Although the MMP-9 and MMP-13 synthesizing cell types have not yet been identified, the localization of RNA signals indicated that perivascular cells, most probably fibroblasts, were the most likely candidates. Their localization close to endothelial cells and tumor cells suggests that their expression was induced by paracrine signals from both neighboring cells. Blockade of endothelial cell activation resulted in downregulation of both MMPs, suggesting a major paracrine role of endothelial cell–derived factors to control these stromal MMPs.

Because these stromal alterations were seen after alteration of VEGF signaling by DC101 it can be assumed that endothelial cell activity may have essential functional consequences for the pathophysiology of the tumor stroma. While features of stromal maturation and tumor phenotype reversion are also seen in subcutaneous tumors, they are particularly well demonstrated in the surface transplants used here with their distinct geometry and the well-defined stromal development (Mueller et al, 2002). In this in vivo model, early changes in the tumor-stroma border are manifest and available for detailed analysis. As with other in vivo models, quantification of results is still difficult and remains restricted to morphometric analysis of vascularization by determining mean

vessel density (Klement et al, 2000; Carmeliet et al, 2000; Kim et al, 2002) or by counting nuclei with incorporated BrdU as mean of cell proliferation.

This work demonstrates the rapid effects of VEGFR-2 inhibition upon reduction in vascular density, protease expression and modulation of tumor stromal morphology. The decreased expression of a major gelatinase represented by MMP-9 and an interstitial collagenase (MMP-13) as well as the regression of preformed vessels after treatment with DC101 was thought to aid in the reversion of the tumor phenotype from a malignant to a pre-malignant one beginning as soon as 24 hours after treatment. Further studies into the detailed mechanisms of VEGF inhibition upon MMP-9 and MMP-13 expression and its consequences on the tumor-stroma phenotype are currently underway.

Abbreviations used

DC101	blocking antibody to mouse VEGFR-2
ECM	extracellular matrix
ERMP-23	antibody targeted to galactose-N-acetyl galactosamin, specific for tissue macrophages
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte/macrophage colony-stimulating factor
HaCaT A-5	benign keratinocyte cell line
HaCaT II-4	low grade malignant keratinocyte cell line
HaCaT A-5 RT3	highly malignant and metastatic keratinocyte cell line
MMP	matrix metalloproteinase
MMP-9	gelatinase B
MMP-13	mouse interstitial collagenase
PAI	plasminogen activator inhibitor
PDGF	platelet-derived growth factor
PECAM	platelet-endothelial cell adhesion molecule / CD31
TIMP	tissue inhibitor of matrix metalloproteinases
VEGFR-2	VEGF receptor 2 (flk1/mouse-KDR/human)
VEGF	vascular endothelial growth factor

REFERENCES

Airola K, Fusenig NE: Differential stromal regulation of MMP-1 expression in benign and malignant keratinocytes. *J Invest Dermatol* 116: 85-92, 2001.

Ala-aho R, Johansson N, Grenman R, Fusenig NE, Lopez-Otin C, Kahari VM: Inhibition of collagenase-3 (MMP-13) expression in transformed human keratinocytes by interferon-gamma is associated with activation of extracellular signal-regulated kinase-1,2 and STAT1. *Oncogene* 19: 248-257, 2000.

Andriani F, Garfield J, Fusenig NE, Garlick JA: Basement membrane proteins promote progression of intraepithelial neoplasia in 3-dimensional models of human stratified epithelium. *Int J Cancer* 108: 348-357, 2004.

Bachmeier BE, Boukamp P, Lichtinghagen R, Fusenig NE, Fink E: Matrix metalloproteinases-2,-3,-7,-9 and-10, but not MMP-11, are differentially expressed in normal, benign tumorigenic and malignant human keratinocyte cell lines. *Biol Chem* 381: 497-507, 2000.

Bajou K, Noel A, Gerard RD, Masson V, Brunner N, Holst-Hansen C, Skobe M, Fusenig NE, Carmeliet P, Collen D, Foidart JM: Absence of host plasminogen activator inhibitor 1 prevents cancer invasion and vascularization. *Nat Med* 4: 923-928, 1998.

Bajou K, Masson V, Gerard RD, Schmitt PM, Albert V, Praus M, Lund LR, Frandsen TL, Brunner N, Dano K, Fusenig NE, Weidle U, Carmeliet G, Loskutoff D, Collen D, Carmeliet P, Foidart JM, Noel A: The plasminogen activator inhibitor PAI-1 controls in vivo tumor vascularization by interaction with proteases, not vitronectin. Implications for antiangiogenic strategies. *J Cell Biol* 152: 777-784, 2001.

Barsky SH, Siegal GP, Jannotta F, Liotta LA: Loss of basement membrane components by invasive tumors but not by their benign counterparts. *Lab Invest* 49: 140-147, 1983.

Benjamin LE, Golijanin D, Itin A, Pode D, Keshet E: Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal. *J Clin Invest* 103: 159-165, 1999.

Bergers G, Brekken R, McMahon G, Vu TH, Itoh T, Tamaki K, Tanzawa K, Thorpe P, Itohara S, Werb Z, Hanahan D: Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis. *Nat Cell Biol* 2: 737-744, 2000.

Bissell MJ, Radisky DC, Rizki A, Weaver VM, Petersen OW: The organizing principle: microenvironmental influences in the normal and malignant breast. *Differentiation* 70: 537-546, 2002.

Bleuel K, Popp S, Fusenig NE, Stanbridge EJ, Boukamp P: Tumor suppression in human skin carcinoma cells by chromosome 15 transfer or thrombospondin-1 overexpression through halted tumor vascularization. *Proc Natl Acad Sci USA* 96: 2065-2070, 1999.

Boukamp P, Petrussevska RT, Breitkreutz D, Hornung J, Markham A, Fusenig NE: Normal keratinization in a spontaneously immortalized aneuploid human keratinocyte cell line. *J Cell Biol* 106: 761-771, 1988.

Boukamp P, Stanbridge EJ, Foo DY, Cerutti PA, Fusenig NE: c-Ha-ras oncogene expression in immortalized human keratinocytes (HaCaT) alters growth potential in vivo but lacks correlation with malignancy. *Cancer Res* 50: 2840-2847, 1990.

Breitkreutz D, Schoop VM, Mirancea N, Baur M, Stark HJ, Fusenig NE: Epidermal differentiation and basement membrane formation by HaCaT cells in surface transplants. *Eur J Cell Biol.* 75: 273-286, 1998.

Breitkreutz D, Mirancea N, Schmidt C, Beck R, Werner U, Stark HJ, Gerl M, Fusenig NE: Inhibition of basement membrane formation by a nidogen-binding laminin gamma1-chain fragment in human skin-organotypic cocultures. *J Cell Sci* 117: 2611-2622, 2004.

Carmeliet P, Jain RK: Angiogenesis in cancer and other diseases. *Nature* 407: 249-257, 2000.

Carmeliet P: Mechanisms of angiogenesis and arteriogenesis. *Nat Med* 6: 389-395, 2000.

Chiquet-Ehrismann R: Tenascin and other adhesion-modulating proteins in cancer. *Semin Cancer Biol* 4: 301-310, 1993.

Coussens LM, Tinkle CL, Hanahan D, Werb Z: MMP-9 supplied by bone marrow-derived cells contributes to skin carcinogenesis. *Cell* 103: 481-490, 2000.

Cunha GR, Matrisian LM: It's not my fault, blame it on my microenvironment. *Differentiation* 70: 469-472, 2002.

Cunha GR, Hayward SW, Wang YZ, Ricke WA: Role of the stromal microenvironment in carcinogenesis of the prostate. *Int J Cancer* 107: 1-10, 2003.

Dvorak HF, Brown LF, Detmar M, Dvorak AM: Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis. *Am J Pathol* 146: 1029-1039, 1995.

Elvert G, Kappel A, Heidenreich R, Englmeier U, Lanz S, Acker T, Rauter M, Plate K, Sieweke M, Breier G, Flamme I: Cooperative interaction of hypoxia-inducible factor-2alpha (HIF-2alpha) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *J Biol Chem* 278: 7520-7530, 2003.

Ferrara N, Alitalo K: Clinical applications of angiogenic growth factors and their inhibitors. *Nat Med* 5: 1359-1364, 1999.

Ferrara N: VEGF and the quest for tumour angiogenesis factors. *Nat Rev Cancer* 2: 795-803, 2002.

Fidler IJ: The organ microenvironment and cancer metastasis. *Differentiation* 70: 498-505, 2002.

Folkman J, Shing Y: Angiogenesis. *J Biol Chem* 267: 10931-10934, 1992.

Fusenig NE, Boukamp P: Multiple stages and genetic alterations in immortalization, malignant transformation, and tumor progression of human skin keratinocytes. *Mol Carcinog* 23: 144-158, 1998.

Fusenig NE: Significance of Angiogenesis and Vascularization for Tumor Invasion and Expansion. In: *Molecular Basis for Invasion and Metastasis*, Fidler IJ, Niitsu Y, Seiki M, Yokota J (Eds.), Tokyo, pp 111-117, 1999.

Fusenig NE, Skobe M, Vosseler S, Hansen M, Lederle W, Airola K, Tomakidi P, Stark HJ, Steinbauer H, Mirancea N, Boukamp P, Breitkreutz D: Tissue models to study tumor-stroma interactions. In: *Proteases and their Inhibitors in Cancer Metastasis*, Muschel RJ, Foidart JM, Dordrecht, The Netherlands, Kluwer Academic Publishers, pp 205-223, 2002.

Hagedorn HG, Bachmeier BE, Nerlich AG: Synthesis and degradation of basement membranes and extracellular matrix and their regulation by TGF-beta in invasive carcinomas (Review). *Int J Oncol* 18: 669-681, 2001.

Huang S, Van Arsdall M, Tedjarati S, McCarty M, Wu W, Langley R, Fidler IJ: Contributions of stromal metalloproteinase-9 to angiogenesis and growth of human ovarian carcinoma in mice. *J Natl Cancer Inst* 94: 1134-1142, 2002.

Inai T, Mancuso M, Hashizume H, Baffert F, Haskell A, Baluk P, Hu-Lowe DD, Shalinsky DR, Thurston G, Yancopoulos GD, McDonald DM: Inhibition of vascular endothelial growth factor (VEGF) signaling in cancer causes loss of endothelial fenestrations, regression of tumor vessels, and appearance of basement membrane ghosts. *Am J Pathol* 165: 35-52, 2004.

Itoh T, Tanioka M, Matsuda H, Nishimoto H, Yoshioka T, Suzuki R, Uehira M: Experimental metastasis is suppressed in MMP-9-deficient mice. *Clin Exp Metastasis* 17: 177-181, 1999.

Jain RK: Molecular regulation of vessel maturation. *Nat Med* 9: 685-693, 2003.

Javaherian A, Vaccariello M, Fusenig NE, Garlick JA: Normal keratinocytes suppress early stages of neoplastic progression in stratified epithelium. *Cancer Res* 58: 2200-2208, 1998.

Johansson N, Ala-aho R, Uitto V, Grenman R, Fusenig NE, Lopez-Otin C, Kahari VM: Expression of collagenase-3 (MMP-13) and collagenase-1 (MMP-1) by transformed keratinocytes is dependent on the activity of p38 mitogen-activated protein kinase. *J Cell Sci* 113 Pt 2: 227-235, 2000.

Jones FS, Jones PL: The tenascin family of ECM glycoproteins: structure, function, and regulation during embryonic development and tissue remodeling. *Dev Dyn* 218: 235-259, 2000.

Kalluri R: Basement membranes: structure, assembly and role in tumour angiogenesis. *Nat Rev Cancer* 3: 422-433, 2003.

Karen J, Wang Y, Javaherian A, Vaccariello M, Fusenig NE, Garlick JA: 12-O-tetradecanoylphorbol-13-acetate induces clonal expansion of potentially malignant keratinocytes in a tissue model of early neoplastic progression. *Cancer Res* 59: 474-481, 1999.

Kiessling F, Fink C, Hansen M, Bock M, Sinn H, Schrenk HH, Krix M, Egelhof T, Fusenig NE, Delorme S: Magnetic resonance imaging of nude mice with heterotransplanted high-grade squamous cell carcinomas: use of a low-loaded, covalently bound Gd-Hsa conjugate as contrast agent with high tumor affinity. *Invest Radiol* 37: 193-198, 2002.

Kiessling F, Farhan N, Lichy M, Vosseler S, Heilmann M, Krix M, Bohlen P, Miller D, Mueller MM, Kauczor HU, Fusenig NE, Delorme S: Dynamic contrast enhanced magnetic resonance imaging rapidly indicates vessel regression in human squamous carcinomas grown in nude mice caused by VEGF-receptor 2 blockade with DC101. *Neoplasia* 6: 213-23, 2004.

Kiessling F, Heilmann M, Vosseler S, Lichy M, Krix M, Fink C, Kiessling I, Steinbauer H, Schad L, Fusenig NE, Delorme S: Dynamic T1-weighted monitoring of vascularization in human carcinoma heterotransplants by magnetic resonance imaging. *Int J Cancer* 104: 113-120, 2003.

Kim ES, Serur A, Huang J, Manley CA, McCrudden KW, Frischer JS, Soffer SZ, Ring L, New T, Zabski S, Rudge JS, Holash J, Yancopoulos GD, Kandel JJ, Yamashiro DJ: Potent VEGF blockade causes regression of coopted vessels in a model of neuroblastoma. *Proc Natl Acad Sci USA* 99: 11399-11404, 2002.

Klement G, Baruchel S, Rak J, Man S, Clark K, Hicklin DJ, Bohlen P, Kerbel RS: Continuous low-dose therapy with vinblastine and VEGF receptor-2 antibody induces sustained tumor regression without overt toxicity. *J Clin Invest* 105: R15-R242000.

Krix M, Kiessling F, Vosseler S, Kiessling I, Le Huu M, Fusenig NE, Delorme S: Comparison of intermittent-bolus contrast imaging with conventional power Doppler sonography: quantification of tumour perfusion in small animals. *Ultrasound Med Biol* 29: 1093-1103, 2003.

Krix M, Kiessling F, Vosseler S, Farhan N, Mueller MM, Bohlen P, Fusenig NE, Delorme S: Sensitive noninvasive monitoring of tumor perfusion during antiangiogenic therapy by intermittent bolus-contrast power Doppler sonography. *Cancer Res* 63: 8264-8270, 2003.

Lynch CC, Matrisian LM: Matrix metalloproteinases in tumor-host cell communication. *Differentiation* 70: 561-573, 2002.

Maas-Szabowski N, Szabowski A, Stark HJ, Andrecht S, Kolbus A, Schorpp-Kistner M, Angel P, Fusenig NE: Organotypic cocultures with genetically modified mouse fibroblasts as a tool to dissect molecular mechanisms regulating keratinocyte growth and differentiation. *J Invest Dermatol* 116: 816-820, 2001.

Maas-Szabowski N, Starker A, Fusenig NE: Epidermal tissue regeneration and stromal interaction in HaCaT cells is initiated by TGF-alpha. *J Cell Sci* 116:2937-2948, 2003.

Miller DW, Vosseler S, Mirancea N, Hicklin DJ, Bohlen P, Volcker HE, Holz FG, Fusenig NE: Rapid vessel regression, protease inhibition, and stromal normalization upon short-term vascular endothelial growth factor receptor 2 inhibition in skin carcinoma heterotransplants. *Am J Pathol* 167: 1389-1403, 2005.

Mudgil AV, Segal N, Andriani F, Wang Y, Fusenig NE, Garlick JA: Ultraviolet B irradiation induces expansion of intraepithelial tumor cells in a tissue model of early cancer progression. *J Invest Dermatol* 121: 191-197, 2003.

Mueller MM, Herold-Mende CC, Riede D, Lange M, Steiner HH, Fusenig NE: Autocrine growth regulation by granulocyte colony-stimulating factor and granulocyte macrophage colony-stimulating factor in human gliomas with tumor progression. *Am J Pathol* 155: 1557-1567, 1999.

Mueller MM, Fusenig NE: Constitutive expression of G-CSF and GM-CSF in human skin carcinoma cells with functional consequence for tumor progression. *Int J Cancer* 83: 780-789, 1999.

Mueller MM, Peter W, Mappes M, Huelsen A, Steinbauer H, Boukamp P, Vaccariello M, Garlick J, Fusenig NE: Tumor progression of skin carcinoma cells in vivo promoted by clonal selection, mutagenesis, and autocrine growth regulation by granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. *Am J Pathol* 159: 1567-1579, 2001.

Mueller MM, Fusenig NE: Tumor-stroma interactions directing phenotype and progression of epithelial skin tumor cells. *Differentiation* 70: 486-497, 2002.

Mueller MM, Fusenig NE: Friends or foes - bipolar effects of the tumour stroma in cancer. *Nat Rev Cancer* 4: 839-849, 2004.

Nelson AR, Fingleton B, Rothenberg ML, Matrisian LM: Matrix metalloproteinases: biologic activity and clinical implications. *J Clin Oncol* 18: 1135-1149, 2000.

Nielsen BS, Sehested M, Kjeldsen L, Borregaard N, Rygaard J, Dano K: Expression of matrix metalloprotease-9 in vascular pericytes in human breast cancer. *Lab Invest* 77: 345-355, 1997.

Obermueller E, Vosseler S, Fusenig NE, Mueller MM: Cooperative autocrine and paracrine functions of granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor in the progression of skin carcinoma cells. *Cancer Res* 64: 7801-7812, 2004.

Olson MW, Toth M, Gervasi DC, Sado Y, Ninomiya Y, Fridman R: High affinity binding of latent matrix metalloproteinase-9 to the alpha2(IV) chain of collagen IV. *J Biol Chem* 273: 10672-10681, 1998.

Olumi AF, Grossfeld GD, Hayward SW, Carroll PR, Tlsty TD, Cunha GR: Carcinoma-associated fibroblasts direct tumor progression of initiated human prostatic epithelium. *Cancer Res* 59: 5002-5011, 1999.

Opdenakker G, Van den Steen PE, Dubois B, Nelissen I, Van Coillie E, Masure S, Proost P, Van Damme J: Gelatinase B functions as regulator and effector in leukocyte biology. *J Leukoc Biol* 69: 851-859, 2001.

Prewett M, Huber J, Li Y, Santiago A, O'Connor W, King K, Overholser J, Hooper A, Pytowski B, Witte L, Bohlen P, Hicklin DJ: Antivascular endothelial growth factor receptor (fetal liver kinase 1) monoclonal antibody inhibits tumor angiogenesis and growth of several mouse and human tumors. *Cancer Res* 59: 5209-5218, 1999.

Qi JH, Ebrahem Q, Moore N, Murphy G, Claesson-Welsh L, Bond M, Baker A, Anand-Apte B: A novel function for tissue inhibitor of metalloproteinases-3 (TIMP3): inhibition of angiogenesis by blockage of VEGF binding to VEGF receptor-2. *Nat Med* 9: 407-415, 2003.

Quaranta V: Cell migration through extracellular matrix: membrane-type metalloproteinases make the way. *J Cell Biol* 149: 1167-1170, 2000.

Schoop VM, Mirancea N, Fusenig NE: Epidermal organization and differentiation of HaCaT keratinocytes in organotypic coculture with human dermal fibroblasts. *J Invest Dermatol* 112:343-353, 1999.

Skobe M, Rockwell P, Goldstein N, Vosseler S, Fusenig NE: Halting angiogenesis suppresses carcinoma cell invasion. *Nat Med* 3: 1222-1227, 1997.

Skobe M, Fusenig NE: Tumorigenic conversion of immortal human keratinocytes through stromal cell activation. *Proc Natl Acad Sci USA* 95: 1050-1055, 1998.

Smola H, Stark HJ, Thiekotter G, Mirancea N, Krieg T, Fusenig NE: Dynamics of basement membrane formation by keratinocyte-fibroblast interactions in organotypic skin culture. *Exp Cell Res* 239: 399-410, 1998.

Strieth S, Hartschuh W, Pilz L, Fusenig NE: Angiogenic switch occurs late in squamous cell carcinomas of human skin. *Br J Cancer* 82: 591-600, 2000.

Strieth S, Hartschuh W, Pilz L, Fusenig NE: Carcinoma-like vascular density in atypic keratoacanthoma suggests malignant progression. *Br J Cancer* 87: 1301-1307, 2002.

Stetler-Stevenson WG: Matrix metalloproteinases in angiogenesis: a moving target for therapeutic intervention. *J Clin Invest* 103: 1237-1241, 1999.

Streuli CH, Schmidhauser C, Bailey N, Yurchenco P, Skubitz AP, Roskelley C, Bissell MJ: Laminin mediates tissue-specific gene expression in mammary epithelia. *J Cell Biol* 129: 591-603, 1995.

Szabowski A, Maas-Szabowski N, Andrecht S, Kolbus A, Schorpp-Kistner M, Fusenig NE, Angel P: c-Jun and JunB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin. *Cell* 103: 745-755, 2000.

Sweeney P, Karashima T, Kim SJ, Kedar D, Mian B, Huang S, Baker C, Fan Z, Hicklin DJ, Pettaway CA, Dinney CPN: Anti-vascular endothelial growth factor receptor 2 antibody reduces tumorigenicity and metastasis in

orthotopic prostate cancer xenografts via induction of endothelial cell apoptosis and reduction of endothelial cell matrix metalloproteinase type 9 production. *Clin Cancer Res* 8: 2714-2724, 2002.

Taraboletti G, D'Ascenzo S, Borsotti P, Giavazzi R, Pavan A, Dolo V: Shedding of the matrix metalloproteinases MMP-2, MMP-9, and MT1-MMP as membrane vesicle-associated components by endothelial cells. *Am J Pathol* 160: 673-680, 2002.

Tlsty TD: Stromal cells can contribute oncogenic signals. *Semin Cancer Biol* 11:97-104, 2001.

Tomakidi P, Mirancea N, Fusenig NE, Herold-Mende C, Bosch FX, Breitkreutz D: Defects of basement membrane and hemidesmosome structure correlate with malignant phenotype and stromal interactions in HaCaT-Ras xenografts. *Differentiation* 64: 263-275, 1999.

Tomakidi P, Stark HJ, Herold-Mende C, Bosch FX, Steinbauer H, Fusenig NE, Breitkreutz D: Discriminating expression of differentiation markers evolves in transplants of benign and malignant human skin keratinocytes through stromal interactions. *J Pathol* 200: 298-307, 2003.

Tomasek JJ, Gabbiani G, Hinz B, Chaponnier C, Brown RA: Myofibroblasts and mechano-regulation of connective tissue remodelling. *Nat Rev Mol Cell Biol* 3: 349-363, 2002.

Tong RT, Boucher Y, Kozin SV, Winkler F, Hicklin DJ, Jain RK: Vascular normalization by vascular endothelial growth factor receptor 2 blockade induces a pressure gradient across the vasculature and improves drug penetration in tumors. *Cancer Res* 64: 3731-3736, 2004.

Vaccariello M, Javaherian A, Wang Y, Fusenig NE, Garlick JA: Cell interactions control the fate of malignant keratinocytes in an organotypic model of early neoplasia. *J Invest Dermatol* 113: 384-391, 1999.

van Kempen LC, Rhee JS, Dehne K, Lee J, Edwards DR, Coussens LM: Epithelial carcinogenesis: dynamic interplay between neoplastic cells and their microenvironment. *Differentiation* 70: 610-623, 2002.

Vosseler S, Mirancea N, Bohlen P, Mueller MM, Fusenig NE: Angiogenesis inhibition by vascular endothelial growth factor receptor-2 blockade reduces stromal matrix metalloproteinase expression, normalizes stromal tissue, and reverts epithelial tumor phenotype in surface heterotransplants. *Cancer Res* 65: 1294-1305, 2005.

Wagner S, Fueller T, Hummel V, Rieckmann P, Tonn JC: Influence of VEGF-R2 inhibition on MMP secretion and motility of microvascular human cerebral endothelial cells (HCEC). *J Neurooncol* 62: 221-231, 2003.

Werb Z, Vu TH, Rinkenberger JL, Coussens LM: Matrix-degrading proteases and angiogenesis during development and tumor formation. *APMIS* 107: 11-18, 1999.

Witte L, Hicklin DJ, Zhu Z, Pytowski B, Kotanides H, Rockwell P, Bohlen P: Monoclonal antibodies targeting the VEGF receptor-2 (Flk1/KDR) as an anti-angiogenic therapeutic strategy. *Cancer Metastasis Rev* 17: 155-161, 1998.

Xu J, Rodriguez D, Petitclerc E, Kim JJ, Hangai M, Moon YS, Davis GE, Brooks PC, Yuen SM: Proteolytic exposure of a cryptic site within collagen type IV is required for angiogenesis and tumor growth in vivo. *J Cell Biol* 154: 1069-1079, 2001.

Yu Q, Stamenkovic I: Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 14: 163-176, 2000.

Zhang W, Ran S, Sambade M, Huang X, Thorpe PE: A monoclonal antibody that blocks VEGF binding to VEGFR2 (KDR/Flk-1) inhibits vascular expression of Flk-1 and tumor growth in an orthotopic human breast cancer model. *Angiogenesis.* 5: 35-44, 2002.

Zucker S, Cao J, Chen WT: Critical appraisal of the use of matrix metalloproteinase inhibitors in cancer treatment. *Oncogene* 19: 6642-6650, 2000.

Importance of Angiopoietin-1 and Angiopoietin-2 during vascularisation of malignant tumors

Dr. Georg Martiny-Baron
Novartis Inc.
CH-4002 Basel, Switzerland

Member of the SPP 1069 from 1999 until 2001

This project has been transferred in the course of its funding period. See project of Dr. Ulrike Fiedler and Prof. Dr. Dieter Marmé (page 172).

Impact of the Tumor Suppressor Gene DPC4/Smad4 on Regulators of Angiogenesis

PD Dr. Irmgard Schwarte-Waldhoff
Prof. Dr. Wolff Schmiegel

Medical University Clinic
Knappschaftskrankenhaus
Ruhr-Universität Bochum
In der Schornau 23-25, 44892 Bochum, Germany

Member of the SPP 1069 from 1999 until 2001

SUMMARY

Smad4/*DPC4* (deleted in pancreatic carcinoma, locus 4) is a tumor suppressor gene lost at high frequency in cancers of the pancreas and other gastrointestinal organs. *Smad4* encodes a key intracellular messenger in the transforming growth factor β (TGF-β) signaling cascade. TGF-β is a potent inhibitor of the growth of epithelial cells; thus, it has been assumed that loss of Smad4 during tumor progression relieves this inhibition. Herein, we show that restoration of Smad4 to human pancreatic carcinoma cells suppressed tumor formation *in vivo*, yet it did not restore sensitivity to TGF-β. Rather, Smad4 restoration influenced angiogenesis, decreasing expression of vascular endothelial growth factor and increasing expression of thrombospondin-1. In contrast to the parental cell line and to control transfectants that produced rapidly growing tumors *in vivo*, Smad4 revertants induced small nonprogressive tumors with reduced vascular density. These data define the control of an angiogenic switch as an alternative, novel mechanism of tumor suppression for Smad4 and identify the angiogenic mediators vascular endothelial growth factor and thrombospondin-1 as key target genes.

INTRODUCTION

Angiogenesis in tumor biology

Neovascularization through the induction of angiogenesis is a prerequisite for growth of solid tumors beyond a microscopic size (Hanahan and Folkman, 1996) and, in addition, is a key event for metastasis (Woodhouse et al., 1997). Angiogenesis is an invasive multistep process comprised of proteolytic degradation of the extracellular matrix, migration and proliferation of endothelial cells and differentiation and maturation of the newly formed vessels (Risau, 1997). A wide variety of molecules are involved in these processes, including extracellular matrix molecules, proteases and their inhibitors as well as growth factors. In normal adult tissues the physiological control of angiogenesis is regulated by a balance of inducers and inhibitors. Accordingly, two types of events may disturbe this delicate balance in the process of tumorigenesis and cause an angiogenic phenotype: induction of angiogenesis-activating factors and loss or down-regulation of angiogenesis inhibitors (Hanahan and Folkman, 1996; Bouck et al., 1996).

Angiogenic growth factors such as VEGF, aFGF, bFGF, TGFα and TGFß are upregulated in many tumor cells. Angiogenic growth factors are supposed to act predominantly via paracrine mechanisms. This has been unequivocally shown for VEGF, which specifically stimulates chemotaxis and mitogenesis in vascular endothelial cells, but has no effect on tumor cell growth in vitro.

Thrombospondin is one of the most studied natural inhibitors of angiogenesis, whose antiangiogenic activity has been confirmed in a number of in vitro and in vivo approaches. Thrombospondin is a large secreted glycoprotein, which interacts with cell surface receptors, growth factors and with other matrix components and is able to influence different cell types. Thrombospondin induces apoptosis in activated endothelial cells.

Angiogenesis requires interactions among tumor cells, endothelial cells, stromal cells and the extracellular matrix. These interactions are governed by extracellular matrix molecules such as fibronectin, by proteases such as the matrix metalloproteinases MMP2 and MMP9 and the serine protease urokinase and by their inhibitors such as the TIMP's and PAI-1. Thus, all of these molecules are implicated in the angiogenic process through their role in tissue remodelling, release and activation of growth factors through proteolytic processing and production of angiogenesis inhibitors such as angiostatin (proteolytic fragment of plasminogen) and endostatin (proteolytic fragment of collagen 18).

Angiogenesis is known to be regulated by environmental influences as well as by genetic mechanisms. The nature of the molecular changes that induce the angiogenic switch in tumors remains poorly defined. Tumorigenesis at the molecular level is known to be due to the accumulation of genetic alterations which comprise the activation of oncogenes and inactivation or loss of tumor suppressor genes. Consequently, it has been suggested, that these same genetic alterations may contribute to the acquisition of an angiogenic phenotype by induction of proangiogenic factors and down-regulation of angiogenesis inhibitors. Precedents for this concept are upregulation of TGF-α, TGFß and VEGF by mutant ras oncogenes (Rak et al., 1995; Okada et al., 1998) and by mutationally inactivated p53 (Kieser et al., 1994), regulation of thrombospondin by p53 (Volpert et al., 1997; Grossfeld et al., 1997) and control of VEGF by the von Hippel-Lindau gene (Schoenfeld et al., 1998). The complexity of the angiogenic process implies, that multiple regulators and control mechanisms remain to be identified.

Tumor suppressor DPC4

The DPC4 gene has been isolated as a candiadate tumor suppressor gene located on chromosome 18q21.1 (Hahn et al., 1996). DPC4 has been shown to be functionally inactivated in one half of pancreatic adenocarcinomas, in pancreatic endocrine tumors, in a subset of colon and biliary tract carcinomas and in rare cases of other tumor types. Germline mutations of DPC4 have been reported in juvenile polyposis.

The product of DPC4 belongs to the evolutionary conserved family of SMAD proteins which have been shown to transfer signals of the TGF-ß superfamily of cytokines (Massaguè et al., 1997; Heldin et al., 1997; Derynck and Feng, 1997). The TGF-ß superfamily includes the TGF-ß's, the activins/inhibins and the bone morphogenetic proteins, all of them regulators of a broad range of developmental and cellular processes (Roberts and Sporn, 1990; Kingsley, 1994). TGF-ß signals through the sequential activation of two homodimeric cell-surface receptors - type I and type II receptors - both of which are serine-threonine kinases. Activated type I receptors phorphorylate class 1 pathway-restricted Smad proteins, which then associate with the shared partner DPC4. Heteromeric SMAD complexes translocate to the nucleus, where they function as transcriptional regulators. DPC4 target genes relevant for its tumor suppressive function have not yet been identified. Binding of Smad3 and DPC4/Smad4 proteins to specific DNA sequences has been shown in different experimental settings (Yingling et al., 1997; Dennler et al., 1998; Vindevoghel et al., 1998; Zawel et al., 1998). However, the reports disagree on the consensus binding sequences. It seems likely, that other transcription factors, some possibly expressed only in certain kinds of cells, complex with Smads and through their binding sequences further specify the transcriptional response.

Cooperation of Smad proteins with the ubiquitous transcription factors AP1, Sp1, TFE3 and with the transcriptional coactivators p300/CBP and MSG1 have been reported. The finding, that Smad proteins also transduce signals from receptor tyrosine kinases further adds to the complexity of Smad dependent transcriptional regulation. Consequently, the identification of Smad target genes may not be feasible through biochemical approaches. Analysis of expression profiles in tumor-suppressed cell clones stably reexpressing DPC4, in contrast, provides the opportunity to identify target genes, which are affected by DPC4 in a quasi-physiological situation.

Roles of DPC4 and TGFß in tumorigenesis and angiogenesis

TGFß is well known as a prominent negative growth factor for normal epithelial cells. Numerous human tumor cell lines, however, have lost sensitivity towards TGFß mediated growth inhibition. Molecular mechanisms for this resistance such as mutation of the TGFß type II receptor or downregulation of TGFß receptor levels have been unravelled in certain tumors. When DPC4's function in signal transduction of TGFß was identified, functional inactivation of DPC4 was assumed to contribute to tumorigenesis through abrogation of the growth-inhibitory TGFß signal pathway. The role of TGFß in tumorigenesis, however, is complex. Besides its growth inhibitory effect on normal epithelial cells, TGFß exerts various functions which could result in promotion of tumor growth: TGFß induces matrix deposition, supports the growth of certain mesenchymal cell types, is a strong inhibitor of the immune system and a potent inducer of angiogenesis (Roberts and Sporn, 1990; Massagué, 1990).

The dual functions of TGFß are most clearly reflected in a model of heterozygous TGFß1 knock-out mice. TGFß's tumor suppressive function has been confirmed by the finding, that these mice exhibit increased sensitivity towards chemically induced carcinogenesis. Further analysis showed, that in tumors the second wild-type TGFß1 allele was always retained and its expression upregulated, indicating a tumor-promoting TGFß function. These data correspond to findings in many advanced human tumors, where high expression levels of TGFß have been reported and furthermore have been correlated with decreased survival.

Based on our findings in the tumor suppressed DPC4 reconstituted cells we hypothesize that DPC4 may be involved in the negative regulation of "oncogenic" TGFß effects. Inactivation of DPC4 in this model would release TGFß signals from negative control and would result in unrestricted or unbalanced angiogenic activity. Though we have not yet formally proven TGFß involvement in the DPC4 effects discussed here, this model is supported by the fact that several of the genes constitutively showing DPC4 dependent down-regulation in our cell culture system are known to be induced by TGFß.

Functional analysis of DPC4 through DPC4 reexpression in DPC4-deficient human carcinoma cell lines

In an approach to prove DPC4's function as a tumor suppressor gene we have performed stable transfections with a DPC4 expression construct in pancreas and colon carcinoma cell lines exhibiting loss of DPC4. We could establish a number of cell clones, each, derived from the SW480 colon carcinoma cell line and derived from the Hs766T pancreas carcinoma cell line, which stably reexpress DPC4 RNA and protein. In vitro analysis did not provide evidence for a significant DPC4 effect on cell growth in these lines. Analysis of tumor formation in nude mice, however, provided ultimate proof of DPC4's tumor suppressor activity: tumor formation from DPC4 reconstituted SW480 cell clones was completely suppressed, and tumors derived from DPC4 reconstituted Hs766T cell clones stopped to grow when they had reached a size of few millimeters while tumor take rate was not affected.

These findings prompted us to analyse the expression of genes associated with tumorigenesis and led to the identification of target genes for DPC4 mediated suppression of tumorigenicity:
Comparison of steady-state RNA levels in DPC4 reconstituted Hs766T cell clones with DPC4 negative controls showed DPC4 dependent down-regulation of VEGF, MMP2, uPAR and fibronectin and reciprocal up-regulation of thrombospondin, TIMP2, TIMP3 and PAI-1.

In conclusion, reexpression of DPC4 in a pancreas carcinoma cell line at the same time increased the expression levels of angiogenesis inhibitors and decreased the expression of angiogenesis inducers or angiogenesis promoting factors. Thus DPC4 exerts a double effect to switch the balance of angiogenesis inducers and inhibitors to the “off“ position. These findings are consistent with a prominent role for DPC4 in the regulation of angiogenesis in the tumorigenic process.

METHODS

The focus of the laboratory during the funding period (1999-2001) was on functional analysis of the tumor suppressor Smad4. Functional loss of Smad4 is a late step, genetically, in the carcinogenic process, suggesting that the tumor promoting effects through loss of Smad4 depend on a prior accumulation of (other) early genetic alterations. This genetic context can hardly be established in mouse models. Thus, we concentrated on Smad4-deficient human cancer cell lines, in which Smad4 was restored through stable transfection. Sets of Smad4 +/- cell clones were functionally and molecularly characterized. These analyses included expression profiling on the mRNA and later on the protein level. Smad4 target genes are assessed in primary human colorectal and pancreatic tumors.

The establishment of Smad4 +/- cell systems was extended to restoration of Smad4 through retroviral transduction in further cell lines. Moreover, in vitro transformation systems are established through knockdown of Smad4 i.e. in adenoma cells, harbouring APC and Ki-ras mutations.

Based on the emerging concept, that Smad4 alters interactions of tumor cells with their environment, we later in cooperation with the Medical Proteome-Center Bochum developed a specialized proteomic approach, tentatively termed (differential) secretomics. As cell cultures provide direct access to proteins released by tumor cells, this approach to our opinion bears great potential not only to decipher further Smad4 targets but also to detect putative tumor biomarkers.

RESULTS

The finding that restoration of the tumor suppressor DPC4 in Hs766T human pancreatic adenocarcinoma cells mediated tumor suppression in nude mice and affected expression levels of prominent angiogenesis regulators provided the basis for this project. The main objectives were to

- analyse the angiogenic activity of DPC4 reconstituted cells by *in vitro* and *in vivo* assays
- analyse the functional activity and the contribution of the diverse mediators and effectors to this activity
- dissect components of the signal pathways involved
- investigate DPC4’s presumed role as a negative regulator of tumor promoting TGFß responses
- decipher mechanisms through which DPC4 mediated transcription control works at the promoters of its target genes

Results are published in Schwarte-Waldhoff et al., 2000.

Analysis of the angiogenic activity of DPC4 reconstituted and control cell clones in nude mice

The initial finding that Hs766T cells reexpressing low levels of DPC4 exhibit reduced tumor growth have been reproduced several times. Whereas tumor take rates were unaffected, tumor growth was retarded as compared to tumors derived from DPC4 negative control clones and came to an arrest after reaching few millimeters in diameter. The mean tumor mass of eight tumors each from three independent DPC4 positive clones was around 40 to 100 mg at 17 days after subcutaneous injection. At this time point mice with tumors derived from DPC4 negative clones had to be killed as the largest tumors in this group grew bigger than 10 mm in one dimension. The mean tumor mass derived from DPC4 negative cells was roughly 350 and 400 mg then. An independent experiment in Scid mice confirmed DPC4 mediated suppression of tumor growth to a similar degree.

DPC4 expression in nude mouse tumors was confirmed at the RNA level. Comparison of RNA levels in vitro with a number of DPC4 positive cell lines and tissues as well as with other cells reexpressing DPC4 after transfer of constitutive or inducible vector constructs revealed, that DPC4 expression levels in reconstituted Hs766T cells are low and correspond to roughly one fifth to one third of normal endogenous levels. Protein levels in all samples examined, whether DPC4 positive tissues and cell lines or lines with stable or inducible reexpression of DPC4 in general directly corresponded to RNA levels. So, as tumor material is very limited we did not yet confirm DPC4 reexpression in Hs766T derived nude mouse tumors at the protein level by Western blotting. Rather, we established staining of DPC4 first in human tissue sections by immunohistochemistry using a commercially available anti-DPC4 antibody. Immuno-histochemical staining will then be established for the nude mouse tumors.

Examination of nude mouse tumors by standard histology did not reveal DPC4 induced changes of differentiation, both DPC4 negative and positive tumors were poorly differentiated. The rate of apoptosis did not differ significantly in both tumor entities, as indicated by TUNEL staining. In contrast, investigation of the proliferating cell compartment by Ki67 staining indicated strongly different rates: roughly 30% of the tumor cells stained positive in the DPC4 negative tumors as compared to 5-10% in DPC4 positive tumors. This result corresponds to the differences in tumor size and growth behavior.

Determination of microvessel density in nude mouse tumors was performed by CD31 staining. Whereas we could not detect significant differences in densities of cords and capillaries throughout the tumors, medium-sized and large vessels at the tumor periphery of DPC4 reconstituted cells were reduced to 68% and 50% of the controls, respectively. This pattern in nude mouse tumors presumably reflects a DPC4 mediated shift in angiogenic activity, as demonstrated by angiogenesis assays.

Functional analysis of presumptive DPC4 targets

Expression analysis of candidate target genes in DPC4 reconstituted Hs766T cells showed DPC4 dependent down-regulation of VEGF, MMP2 and uPAR and reciprocal up-regulation of thrombospondin, TIMP2, TIMP3 and PAI-1. These differences, though moderate in their extent (two- to threefold), were reproduced in independent RNA preparations. Corresponding differences were detected at the protein level, as shown by ELISA for uPAR, PAI-1, VEGF and MMP2, by zymography for MMP2 and by Western blotting of concentrated conditioned media for TIMP2, VEGF and thrombospondin. Whereas the proteases and their inhibitors are implicated in diverse functions during the tumorigenic process like angiogenesis, invasion and metastasis, effects of changes in expression levels of VEGF and TSP-1 presumably are confined to angiogenesis. Thus, we have performed angiogenesis assays using conditioned media from DPC4 reconstituted Hs766T cells and

negative controls, respectively. Activation of endothelial cell migration in a modified Boyden chamber assay by conditioned media derived from DPC4 reconstituted cells did not exceed background levels. In contrast, the chemotactic reponse of endothelial cells to conditioned media from DPC4 negative control clones was as high as their response to saturating levels of recombinant VEGF. This response could be suppressed by addition of a neutralising anti-VEGF antibody, indicating, that VEGF levels in the conditioned media are necessary and sufficient to induce a full angiogenic response in this assay. The lack of response to conditioned media from DPC4 reexpressing cells was not only due to limiting VEGF levels: addition of recombinant VEGF to conditioned media was not sufficient to exert a significant effect. Neutralization of TSP-1 from conditioned media by an anti-TSP antibody enabled a moderate response of endothelial cells, suggesting that TSP-1 produced by DPC4 reconstituted cells has inhibiting activities in endothelial cell migration. When this inhibitory activity was depleted and VEGF was added, endothelial cells again showed strong chemotactic responses. These results suggested, that VEGF and TSP-1 are the main players to determine angiogenic activities of Hs766T cells and that DPC4 induced an angiogenic shift by changing the balance of inducing and inhibiting activities through down-regulation of VEGF and up-regulation of TSP-1. Angiogenesis assays reflect different aspects of the angiogenic process. Though endothelial cell migration assays are believed to closely reflect angiogenic activities we wished to confirm the DPC4 induced angiogenic shift in an independent in vivo assay and chose to examine vessel ingrowth in the rat cornea. Again, the response to conditioned media from DPC4 negative cells was as strong as to recombinant VEGF or bFGF, whereas there was no vessel ingrowth in response to supernatants from DPC4 reexpressing cells. As with endothelial cell migration assays, VEGF and TSP-1 could be identified as the factors responsible for this difference in behavior through depletion by neutralizing antibodies and addition of recombinant proteins. In summary, two basically different angiogenesis assays revealed clear-cut suppression of angiogenic activities of DPC4 reexpressing Hs766T cells.

Dissection of the signal transduction pathway(s) mediating DPC4 dependent shifts in expression levels of target genes

DPC4 mediated changes in expression levels of target genes are controlled in a cell-autonomous way: expression was analysed in standard growth media as well as in serum free media. Consequently, constitutive expression levels of target genes are either independent from extracellular cytokines or are subject to control through the endogenously produced cytokines and reflect the net outcome of signaling cascades after signal integration. DPC4 is potentially involved in signal transmission for all TGF-ß superfamily cytokines and limiting levels of receptors, Smads and further interacting transcription factors may determine the cellular response to DPC4 reexpression. Consequently, to dissect pathways implicated in DPC4 mediated effects in reconstituted Hs766T cells, we startet to analyse expression of the main players in TGF-ß signaling cascades, comprising TGF-ß cytokines, receptors, Smads and further transcription factors at the RNA level. TGF-ß1, TGF-ß2 and TGF-ß3 as well as TßRI (Alk5) and TßRII were examined by Northern blotting and by RNAse protection assays using riboquant kits. Hs766T cells express abundant levels of TGF-ß1, but rarely TGF-ß2 or TGF-ß3. Whereas expression of TßRI is barely detectable, TßRII levels are abundant, consistent with published data. Expression of R-Smads (receptor-Smads) was shown in RPA analyses indicating that the levels of Smads 2 and 3, implicated in transduction of the TGF-ß/activin/inhibin subclass exceed the levels of Smads 1, 5 and 8 implicated in transmission of BMP signals. Smads6 and 7, reported to be induced by TGF-ß cytokines, are constitutively expressed and TGF-ß induction of Smad7 was confirmed. In contrast to DPC4 reconstituted SW480 cells where DPC4 reexpression affected constitutive expression levels of several of these players we did not detect significant changes in DPC4 reexpressing Hs766T cells as compared to the negative controls.

However, preliminary results from expression profiling through hybridization of microarrays indicate, that several cytokines, growth factors, receptors, signal transduction components and transcription factors are affected by reexpression of DPC4. In line with this, experiments in DPC4 reconstituted SW480 suggested, that the DPC4 status may more strongly affect responses to cytokines or growth factors unrelated to the TGF-ß superfamily than to TGF-ß cytokines themselves. This finding also implies, that expression profiles in vitro may strongly differ from those in vivo, where the cells are subject to very different microenvironmental influences. Preliminary analyses of expression profiles from nude mouse tumors, though comprised of human and mouse RNA's derived from infiltrating mouse cells, apparently confirm this expectation. In conclusion, these analyses on the RNA level only already suggest, that future examination of DPC4 effects will require the analysis of signaling networks rather than single linear pathways. Nevertheless, as this is a central question, we started the analysis of DPC4 target genes in response to TGF-ß addition or TGF-ß depletion. A number of DPC4 target genes, like VEGF and MMP2 had been reported previously to be TGF-ß inducible. Preliminary results suggest, that VEGF and MMP2 in Hs766T cells may be slightly induced by TGF-ß. This finding, however, has to be confirmed and DPC4 dependent as well as putative DPC4 independent mechanisms have to be separated.

Analyses approaching the elucidation of mechanisms for DPC4 dependent regulation of target genes

DPC4 has been characterized as a transcription factor or transcriptional co-modulator. Smad binding sequence elements (SBE) have been identified to which Smad3 or Smad4/DPC4 can specifically bind. Other sequence elements like the CAGA box have been defined in specific promoters, to which Smads also have been shown to bind. These sequence elements, however, are short and Smad binding is not strong enough on its own to confer specificity on target gene regulation. Rather, Smads have to cooperate with further transcription factors which may bind to their respective recognition sites neighbouring SBE's. In addition, mechanisms of Smad dependent target gene regulation may exist, where Smads do not directly bind to DNA in the promoter region but are components of transcription factor complexes present at the promoter. Moreover, as most target genes are subject to complex control through interaction of factors regulated by a variety of signaling cascades, Smads may also impinge on target gene expression levels through a variety of indirect mechanisms. These may comprise transcriptional induction or repression of factors directly involved in target gene regulation or competition for transcriptional coregulators at the protein level.

As a first approach to investigate whether Smad4 has a direct effect on VEGF and TSP-1 promoters, we have performed transient transfection assays with reporter constructs, where the luciferase gene is under control of the VEGF or TSP-1 promoter, respectively. VEGF promoter constructs were kindly provided by Prof. Marmé (Freiburg), TSP-1 promoter constructs by Prof. Bouck (Chicago). The full length VEGF promoter showed relatively low baseline activity, the TSP-1 promoter exhibited much higher activity in transiently transfected HeLa cells. Cotransfection of a DPC4 expression construct had no significant effect on luciferase activity. In order to increase transcriptional responses to transiently transfected DPC4, we produced a chimeric construct comprised of the Smad4 DNA binding domain and the VP16 transactivation domain. Again, cotransfection of this construct with the VEGF or TSP-1 promoter reporter constructs did not affect luciferase activity. In contrast, luciferase expression driven by a series of six SBE sites was induced by a factor of 10 upon cotransfection with the DPC4-VP16 chimeric construct. In conclusion, these preliminary experiments did not provide evidence for direct Smad4 binding to the VEGF or TSP-1 promoter, respectively. These preliminary reporter transfections have been performed in HeLa cells, a well established cell model for transient transfection analyses. However, it has become increasingly clear that DPC4 is a transcriptional co-modulator, cooperating with and fine-tuning the activity of

further transcription factors. So, we consider it imperative to perform corresponding analyses in Hs766T cells, in order to provide the appropriate molecular context.

DISCUSSION

Functional inactivation of the tumor suppressor gene Smad4 plays a prominent role in pancreatic carcinogenesis. Defining the mechanisms whereby Smad4 functions as a tumor suppressor is critical to address its potential as a therapeutic or diagnostic target. Smad4 transmits signals of the TGF-ß superfamily of cytokines and functions as a transcriptional co-modulator. The prevailing view suggests that Smad4‘s tumor suppressor function primarily resides in its capability to mediate TGF-ß growth inhibitory responses. Our results, however, show that Smad4-mediated tumor suppression is independent from TGF-ß induced growth inhibition. Rather, they show that Smad4 affects interactions of the tumor cells with the microenvironment. Smad4-mediated suppression of angiogenesis was one of the first examples illustrating this principle. In this project, we could demonstrate that Smad4 in human pancreatic cancer cells works as a transcriptional modulator of VEGF and TSP-1 expression, leading to downregulation of VEGF and induction of TSP-1. These alterations moderated reversion of the angiogenic phenotype, ultimatey resulting in the suppression of tumor growth.

Due to the fact that this project was funded for one period, only, the underlying molecular mechanisms and pathways could not be further deciphered and the scope of our findings could not be evaluated. However, some effort was put since then into the further elucidation of the emering concept, that Smad4 affects interactions of the tumor cells with their environment: We have developed a novel proteomic approach, termed differential secretomics, in which we directly analyse proteins released by tumor cells in vitro. This approach received funding by the DFG, has provided interesting results (Volmer et al., 2004; Volmer et al., 2005; Diehl et al., submitted) and will hopefully be continued with some success.

REFERENCES

Bouck N, Stellmach V, Hsu S: How tumors become angiogenic. *Adv Cancer Res* 69: 135-174, 1996.

Dennler S, Itoh S, Vivien D, ten Dijke P, Huet S, Gauthier J: Direct binding of Smad3 and Smad4 to critical TGF-ß-inducible elements in the promoter of human plasminogen activator inhibitor-type 1 gene. *EMBO J* 17: 3091-3100, 1998.

Derynck R, Feng X-H: TGF-ß receptor signaling. *Biochim Biophys Acta* 1333: F105-F150, 1997.

Diehl H, Stühler K, Klein-Scory S, Volmer M, Schöneck A, Schmiegel W, Meyer HE, Schwarte-Waldhoff I: Mining the colorectal cancer “secretome” for biomarker discovery, submitted.

Grossfeld GD, Ginsberg DA, Stein JP: Thrombospondin-1 expression in bladder cancer: association with p53 alterations, tumor angiogenesis, and tumor progression. *J Natl Cancer Inst* 89: 219-227, 1997.

Hahn SA, Schutte M, Hoque ATM, Moskaluk CA, da Costa LT, Rozenblum E, Weinstein CL, Fischer A, Yeo CJ, Hruban RH, Kern SE: *DPC4*, a candidate tumor suppressor gene at human chromosome 18q21.1. *Science* 271: 350-353, 1996.

Hanahan D, Folkman J: Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 86: 353-364, 1996.

Heldin C-H, Miyazono K, ten Dijke P: TGF-b signalling from cell membrane to nucleus through SMAD proteins. *Nature* 390: 465-471, 1997.

Kieser A, Weich HA, Brandner G, Marme D, Kolch W: Mutant p53 potentiates protein kinase C induction of vascular endothelial growth factor expression. *Oncogene* 9: 963-969, 1994.

Kingsley DM: The TGF-ß superfamily: new members, new receptors, and new genetic tests of function in different organisms. *Genes Dev* 8: 133-146, 1994.

Massague J: The transforming growth factor-ß family. *Ann Rev Cell Biol* 6: 597-641, 1990.

Massague J, Hata A, Liu F: TGF-ß signalling through the Smad pathway. *Trends Cell Biol* 7: 187-192, 1997.

Okada F, Rak JW, St. Croix B, Lieubeau B, Kaya M, Roncari L, Shirasawa S, Sasazuki T, Kerbel RS: Impact of oncogenes in tumor angiogenesis: mutatn k-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells. *Proc Nat Acad Sci USA* 95: 3609-3614, 1998.

Rak J, Mitsuhashi Y, Bayko L, Filmus J, Shirasawa S, Sasazuki T, Kerbel RS: Mutant ras oncogenes upregulate VEGF/VPF expression: implications for induction and inhibition of tumor angiogenesis. *Cancer Res* 55: 4575-4580, 1995.

Risau W: Mechanisms of angiogenesis. *Nature* 386: 671-674, 1997.

Roberts AB, Sporn MB: The transforming growth factor-betas. Peptide growth factors and their receptors. Sporn MB, Roberts AB (Eds.). Heidelberg, Springer-Verlag, pp 419-472, 1990.

Schoenfeld A, Davidowitz EJ, Burk RD: A second major native von Hippel-Lindau gene product, initiated from an internal translation start site, functions as a tumor suppressor. *Proc Natl Acad Sci USA* 95: 8817-8822, 1998.

Schwarte-Waldhoff I, Volpert O, Bouck N, Sipos B, Hahn S, Klein-Scory S, Luttges J, Kloppel G, Graeven U, Eilert-Micus C, Hintelmann A, Schmiegel W: Smad4/DPC4-mediated tumor suppression through suppression of angiogenesis. *Proc Natl Acad Sci USA* 97: 9624-9629, 2000.

Schwarte-Waldhoff I, Schmiegel W: Smad4 transcriptional pathways and angiogenesis. *Int J Gastrointest Cancer* 31:47-59, 2002.

Vindevoghel L, Kon A, Lechleider R, Uitto J, Roberts A, Mauviel A: Smad-dependent transcriptional activation of human type VII collagen gene (COLL7A1) promoter by transforming growth factor-ß. *J Biol Chem* 273: 13053-13057, 1998.

Volmer MW, Radacz Y, Hahn SA, Klein-Scory S, Stuhler K, Zapatka M, Schmiegel W, Meyer HE, Schwarte-Waldhoff I: Tumor suppressor Smad4 mediates downregulation of the anti-adhesive invasion-promoting matricellular protein SPARC: Landscaping activity of Smad4 as revealed by a "secretome" analysis. *Proteomics* 4: 1324-1334, 2004.

Volmer MW, Stuhler K, Zapatka M, Schoneck A, Klein-Scory S, Schmiegel W, Meyer HE, Schwarte-Waldhoff I: Differential proteome analysis of conditioned media to detect Smad4 regulated secreted biomarkers in colon cancer. *Proteomics* 5: 2587-2601, 2005.

Volpert OV, Dameron KM, Bouck N: Sequential development of an angiogenic phenotype by human fibroblasts progressing to tumorigenicity. *Oncogene* 14: 1495-1502, 1997.

Woodhouse EC, Chuaqui RF, Liotta LA: General mechanisms of metastasis. *Cancer* 80: 1529-1537, 1997.

Yingling JM, Datto MB, Wong C, Frederick JP, Liberati NT, Wang X-F: Tumor Suppressor Smad4 is a transforming growth factorß-inducible DNA binding protein. *Mol Cell Biol* 17: 7019-7028, 1997.

Zawel L, Dai J, Buckhaults S, Zhou S, Kinzler K, Vogelstein B, Kern S: Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol Cell* 1: 611-617, 1998.

Lymphatic angiogenesis

Functional Analysis of the Lymphatic System in Normal and Diseased Tissue

PD Dr. Thomas Hawighorst

Dept. of Gynecology and Obstetrics
University of Göttingen
Robert-Koch-Str. 40, 37075 Göttingen, Germany

Prof. Dr. Manuel Koch

Center for Biochemistry
University of Cologne
Joseph-Stelzmann-Str. 52, 50931 Cologne, Germany

Members of the SPP 1069 from 2004 until 2006

SUMMARY

LVYE-1 is widely used as a molecular marker to distinguish blood from lymphatic vessels. LYVE-1 has been shown to function as a specific high-affinity receptor for hyaluronan (HA) in lymphatic vessel endothelium by mediating the endocytosis of HA. Hyaluronan has been shown to be a key mediator of tumor cell migration and increased amounts of HA in the tumor interstitium have been reported to predict poor survival of patients. However, the overall biological function of LYVE-1 in lymphatic vessels, in particular with regard to tumorigenesis, has remained unknown. To analyze the role of LYVE-1 in tumor progression and metastasis we performed mouse xenotransplantation studies using MDA-MB-435 breast cancer cells which overexpress the soluble ectodomain of LYVE-1. We provide evidence for an inhibitory role of soluble LYVE-1 on breast carcinoma growth and progression. Both overexpression of sLYVE-1 in transfected breast cancer cells as well as systemic application of recombinant soluble LYVE-1 protein resulted in a significant inhibition of tumor growth in vivo. Based on these data we have very recently generated a transgenic mouse line for the targeted overexpression of sLYVE-1 in the basal epidermal layer of the skin using a keratin K14 expression cassette. This transgenic mouse line represents an exciting tool which will allow us to further study the role of soluble LYVE-1 for tumorigenesis by performing either two step chemically-induced carcinogenesis or by crossing these transgenic mice into a HPV-8 expressing mouse line yielding a great number of tumors within a couple of weeks.

Extracellular matrix proteins are important components of all multicellular organisms. To study the importance of this group of molecules with regard to the lymphatic system, different approaches were chosen. First, expression analysis of mouse lymph endothelial cells sorted by magnetic beads was performed. The isolation of this cell type has been established and the expression analysis is ongoing. Secondly, the description of secreted proteins by a proteonomic approach is ongoing. A further major achievement is the generation of a lymphatic specific Cre recombinase expressing transgenic mouse line. To test this Cre-lox system, the integrin $\beta 1$ chain will be tissue specifically removed. Since integrins are the classic extracellular matrix receptors, removal of this particular receptor from lymphatic endothelial cells will correspond to the loss of several different ligands. These tools will without any doubt lead to exciting and relevant research in the field of lymphatic vasculature biology.

1. INTRODUCTION

The lymphatic vasculature forms a vessel network that drains interstitial fluid from tissue and returns it to the blood. Lymphatic vessels play also an important role in the mediation of the immune response. They have an important role in physiological processes such as regeneration and wound healing and in the pathogenesis of several diseases, such as cancer, lymphedema and various inflammatory conditions (for review see Wilting et al, 2005 and Alitalo et al, 2005). Defining the functional role of lymphatic vessels in normal and diseased tissue is a long-term goal of our project.

Understanding the mechanisms of lymphatic metastasis, including the identification of stromal and tumor determinants that are important for tumor progression, represents a major challenge in which we have placed great emphasis. Furthermore, characterization of lymphatic endothelial cells from different vascular beds including various tumor types would provide important novel targets for therapy, along with new information about normal and diseased lymphatic vascular function.
In human malignancies such as breast cancer, the lymph node status is the most important prognostic indicator for the clinical outcome of patients. It has long been denied that tumors induce lymphangiogenesis. An elaborated system of lymphatics has been observed in close proximity to invasive areas in breast cancer, and lymphangiosis carcinomatosis, the destruction of the lymphatic endothelial lining by tumor cells, is an unfavourable prognostic finding. Studies on the biological role of the lymphatic vasculature have been eased due to the discovery of specific growth factors and lymphatic markers.

The first short-term goal of our project is to define the role of the recently identified first lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) for tumor progression. LYVE-1, a CD44 homolog, is a specific cell surface protein of lymphatic endothelial cells; cells which fail to express CD44. LVYE-1 is widely used as a molecular marker to distinguish blood from lymphatic vessels. LYVE-1 has been shown to function as a specific high-affinity receptor for hyaluronan (HA) in lymphatic vessel endothelium by mediating the endocytosis of HA. However, the overall role of LYVE-1 in lymphatic vessels, in particular with regard to tumor progression, has remained unknown. So far, it could only be speculated that LYVE-1 might regulate the entry of tumor cells into the lumen of afferent lymphatic capillaries since interactions between HA and CD44 have been implicated in lymphocyte extravasation via the endothelium into inflamed tissue and HA has been shown to be a key mediator of tumor cell migration. In addition, increased amounts of HA in the tumor interstitium have been reported to predict poor survival of patients, possibly due to enhanced tumor progression. Here, we present evidence for an inhibitory role of soluble LYVE-1 on breast carcinoma growth and progression. Both overexpression of sLYVE-1 in transfected breast cancer cells and systemic application of recombinant sLYVE-1 protein resulted in a significant inhibition of tumor growth.

The lymphatic capillaries are thin-walled, relatively large vessels, composed of a single layer of endothelial cells. Unlike blood capillaries, a basement membrane is discontinuous or even absent in lymphatic capillaries which results in their intimate association to the adjacent interstitial areas. The anchoring filaments tether the outer portions of the endothelium to the extracellular matrix in the interstitium. The filaments are thought to maintain vessel patency in the presence of tissue edema (for review see Wilting et al, 2005). However, little is known as to the elements forming the basement membrane and as to the anchoring structures of lymphatics. Our second goal is to further study and identify extracellular matrix components, which are important for the structure and integrity of lymphatic vessels. Two different approaches are to be undertaken. Basically, lymphatic and blood endothelial cells isolated from various tissue types are cultured and their secreted and membrane bound proteins are to be analyzed and compared on 2D SDS-PAGE. The isolation of lymphatic as well as blood endothelial cells from human tissue is well established. However, the isolation of the equivalent cells in mouse has not yet been well established and the methods needed to be re-

adapted. In addition, so far there have been no commercially available products on the market such as specific antibodies coupled to Dynabeads for cell isolation procedures. Another more sophisticated approach will make use of transgenic mice expressing *hrGFP* under the control of the LYVE-1 promoter. At the start of the project, none of these mouse lines were available. In a large scale project to map the expression of all nerve specific genes at the Rockefeller University, BAC clones were chosen for this purpose. As it turned out, EGFP or other similar proteins are often too weak to be detected by microscopy and the transgenic DNA has to be integrated at least three to five times to obtain a detectable signal. Therefore, the BAC strategy was also adapted and to circumvent the problem of the EGFP level, the Cre recombinase was chosen instead of the hrGFP gene. If the mouse line works, the mice will then be crossed into an EGFP reporter mouse line to obtain a stronger EGFP signal.

2. METHODS

2.1. Cell culture and transfection

The human breast cell carcinoma cell line MDA-MB-435/GFP was maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 4.5 mg/ml glucose, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin. MDA-MB-435/GFP cells were stably transfected with the cDNA of the soluble ectodomain of the LYVE-1 receptor (sLYVE-1), incorporated into a pCEB expression vector, or vector alone. The sequences and correct orientation were verified by restriction mapping and by direct sequencing using the Sanger dideoxy method.

2.2. In vitro cell growth and apoptosis assays

To analyze anchorage-dependent cell growth, $5x10^4$ MDA-MB-435/GFP cells were plated in duplicate into 100 mm culture dishes, and the total cell numbers per dish were determined after one day (N_0) and after 7 days (N) at the end of the growth period (t) by using a hemocytometer. The cell-doubling time (T) was calculated by using the following formula: $T = \ln 2 \times t / \ln N/N_0$. Apoptosis induced by serum-withdrawal was studied in subconfluent MDA-MB-435/GFP cell clones after 72 hours of culture in serum-free medium and the percentage of apoptotic cells was determined as described (Hawighorst et al, 2002).

2.3. Tumorigenicity assay

Stably transfected MDA-MB-435/GFP cells ($1x10^6$ in 100 µl serum-free culture medium) were injected orthotopically into both second mammary fat pads of 6 weeks-old female nude mice (10 mice for each clone). Two vector transfected clones, three sLYVE-1 overexpressing clones, and the parental cell line were studied. The smallest and largest tumor diameter were measured weekly using a digital caliper, and tumor volumes were calculated using the following formula: Volume = $4/3 \times \pi \times (1/2 \times \text{smaller diameter})^2 \times 1/2 \times \text{larger diameter}$. Tumor data were analyzed using the two-sided unpaired t-test. Tumors were harvested after 11 weeks and were either embedded in OCT compound and frozen in liquid nitrogen for cryostat sectioning, or were fixed in formalin and embedded in paraffin for routine histology. For RNA extractions, tumors were snap frozen in liquid nitrogen. In an additional experiment recombinant sLYVE-1 protein was systemically applied to mice bearing a faster growing subclone of MDA-MB-435 breast cancer cells. All animal studies were approved by the Department of Research Animal Care of the "Bezirksregierung Braunschweig".

2.4. Immunohistochemistry

Immunohistochemistry was performed as described (Hawighorst et al, 2001)

2.5. Protein expression system, antibody production

Several recombinant expression systems are established in our laboratory. Beside bacterial standard expression systems, two different eukaryotic expression systems are in use. For large scale production, the pCEB vector system (Invitrogen) in combination with the kidney 293-EBNA cell line has been the most successful one. Several modified vectors containing different tags at either the 3' or 5' end of the coding sequence are in use. All recombinant proteins generated for this project utilizes this system. Recently, a stable expression system for insect cells has also been established and in some cases the expression level was higher as compared with the pCEB system. The later system might be easier for handling and large scale protein productions for *in vivo* studies. Highly purified proteins were also used for antibody production in guinea pigs and rabbits. Since most sera contain additional antibodies against other proteins, the sera were affinity purified against the fusion-proteins (without tags).

2.6. Isolation of lymphatic endothelial cells

These antibodies are being used for cell sorting experiments. The antibodies were coupled to Dynabeads (Invitrogen) and the cell isolations were done according to the protocols obtained from PromoCell (Heidelberg). The production of our own antibodies is crucial for the success of this project. The coupling of the beads require large amount of antibodies and our budget would not permit us to buy it from a company. One of the students attended a laboratory course at this company and is still in contact for technical questions. The adaptation is quite labour-intensive and it seems as if the protocol can not be transferred one to one.

2.7. Generation of transgenic mice

To generate Cre recombinase expressing lymphatic endothelial cells, a transgenic mouse model has been designed. To restrict the Cre expression to this cell type, the gene was placed under the mouse LYVE-1 promoter. The Cre recombinase was transferred into a BAC clone, which contains the LYVE-1 gene, via recombineering technique. This system was created in the laboratory of Neil Copeland at the NIH. Under a heat sensitive promoter, a recombination system is shortly activated during the transfer of the Cre recombinase DNA into these specially modified bacteria. At the end of the linear DNA, short homologous DNA pieces to the insertion place within the BAC clone are necessary for the integration. Since the linear DNA contains also a selection marker, only bacteria containing the insert survive.

3. RESULTS

3.1. Biological role of LYVE-1 for breast cancer tumor growth and progression.

3.1.1. Expression and secretion of soluble LYVE-1 in MDA-MB-435/GFP cells.

The soluble ectodomain of the mouse LYVE-1 (sLYVE-1) cDNA was cloned into the pCEB expression vector. To this end a 633 bp cDNA fragment (nucleotides 155 – 787, NM_053247) was amplified by PCR and was ligated into the Kpn I and Bgl sites of a modified pCEB4 expression vector (Koch et al, 2000) that contains a cytomegalovirus enhancer/promoter, the EBNA-1 gene, and a puromycin selection cassette. The nucleotide sequence was verified by sequencing. To investigate the distinct effects of sLYVE-1 on tumor growth and progression, MDA-MB-435/GFP breast cancer

cells were stably transfected with the expression vector containing sLYVE-1 or vector alone. We isolated and expanded 10 different clones each. Expression and secretion of sLYVE-1 was assessed at the protein level by Western analysis. pCEB vector-transfected MDA-MB-435/GFP cells did not express any detectable amounts sLYVE-1 protein, whereas three different pCEB-sLYVE-1 transfected clones strongly expressed and secreted the protein sLYVE-1 (data not shown).

3.1.2. Characterization of tumor cell growth and apoptosis in vitro.

Several in vitro assays were performed to detect potential phenotypic differences between control and sLYVE-1 transfected MDA-MB-435/GFP cell clones. Cell-doubling times under anchorage-dependent culture conditions in sLYVE-1 overexpressing clones were comparable to those observed in pCEB vector transfected or parental MDA-MB-435/GFP control cells (data not shown). By using the in situ terminal deoxynucleotidyl transferase and nick translation assay and a FACS-Scan no significant differences in induced apoptosis rates were observed between control transfected and sLYVE-1 overexpressing cell clones after serum withdrawal (Fig. 1). To analyze anchorage-independent growth a soft-agar assay is ongoing. No alterations in cellular morphology were detected (data not shown).

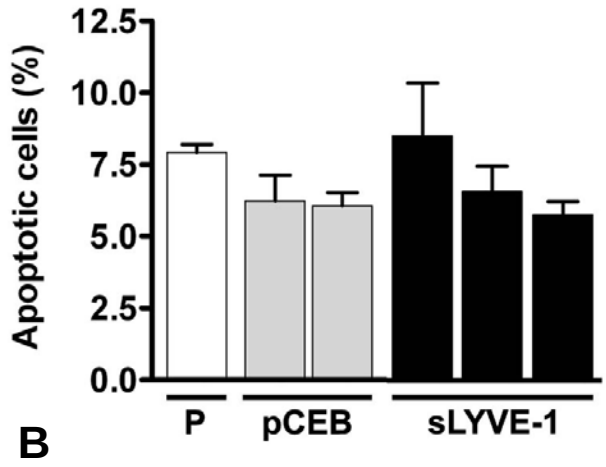


Figure 1: In vitro apoptosis assays did not detect potential differences. No significant differences in the percentage of apoptotic cells were found between parental (P), vector transfected (pCEB), and sLYVE-1 transfected MDA-MB-435/GFP cells.using a FACS scan. Apoptosis of the different MDA-MB-435/GFP clones was induced by serum withdrawal for 72 hours. Results are expressed as mean values ± SD of three independent experiments.

3.1.3. Production of different Fc-fusion proteins.

To investigate the effects of sLYVE-1 protein on in vitro and in vivo tumor growth as well as to produce specific antibodies, we produced soluble mouse LYVE-1 protein as a human Fc fusion protein. The same ectodomain as used in the in vivo studies was cloned into a pCEB expression vector, which contains the sequence for the human Fc protein at the 3' end of the multiple cloning sites. Puromycin-selected clones were verified for expression and secretion of Fc-sLYVE-1 by Western blot analysis. One clone with the highest expression level was chosen for large-scale preparation of the recombinant protein. Mouse sLYVE-1 hFc protein was purified from serum-free conditioned media (DMEM/F12 1:1, vol:vol) by Protein-G sepharose chromatography, yielding up to

4 mg of per litre of culture supernatant. For further studies, the human Fc tag can be removed from sLYVE-1 hFc protein through a Factor Xa cleavage site between the two proteins. For further studies, mouse CD31 as well as mouse CD34 hFc were also produced for antibody production. Several mg of affinity purified proteins were obtained. All fusion proteins have been sent out for antibody productions and the sera are in the process of affinity purification.

3.1.4. sLYVE-1 overexpression potently inhibits breast cell carcinoma growth and results in a decreased incidence of lung metastasis in vivo.

We compared in vivo tumorigenicity and metastatic spread of MDA-MB-435/GFP cells transfected with sLYVE-1 versus "vector only" versus parental cells. To this end, three distinct sLYVE-1 clones (1x 10^6 cells) and two vector-control clones as well as parental MDA-MB-435/GFP cells were injected into the second mammary fat pad of female immunodeficient nude mice (10 mice per group). Tumor growth was assessed weekly and after a maximum of 11 weeks the experiment needed to be completed for animal welfare reasons (some tumors of the control group showed merest hint for superficial skin ulceration).

At eleven weeks after tumor injection, overexpression of sLYVE-1 resulted in a significant inhibition of tumor growth by more than 45% (p<0.001; data not shown). No significant differences on tumor growth were detected between parental and pCEB vector transfected control cells.

We also harvested the regional lymph nodes and lungs to assess metastatic tumor spread. Macroscopically, regional axillary lymph nodes were enlarged in most cases and the size of lymph nodes was not indicative of the presence of metastases. To obtain accurate quantitative analysis of metastases, we used breast cancer cells genetically labelled with GFP. We performed immunohistochemistry on paraffin-embedded formalin-fixed serial lymph node sections using a commercially available antibody (polyclonal, rabbit, anti-GFP; Abcam plc, Cambridge, UK) against green fluorescent protein. By conducting this approach we could successfully detect metastasis in lymph nodes as well as in lungs (Fig. 2). Evaluation of serial stained paraffin sections of sentinel lymph nodes by microscopy revealed nearly comparable incidences of GFP-expressing metastases in mice bearing sLYVE-1 overexpressing tumors (89,7%) and mice bearing control tumors (86,7%).

We next investigated the effects of sLYVE-1 overexpression on the development of lung metastases. Immunohistochemical analysis of serial lung sections at 11 weeks after tumor implantation showed that the incidence of GFP-expressing metastases was decreased by more than 20% in mice bearing LYVE-1 overexpressing tumors (58,6%) as compared with mice bearing control tumors (73,3%).

In order to confirm the growth inhibitory effect of sLYVE-1 and to analyze the time course of metastasis formation in more detail another tumorigenicity and metastasis assay was conducted. To this end we built up two groups of mice. The observation period was 5 weeks in the first group and 10 weeks in the second group. Regional sentinel lymph nodes and lungs were harvested after 5 and 10 weeks, respectively. We injected one vector transfected control clone (pCEB#8) into the second mammary fat pad of nude mice and one sLYVE-1 overexpressing clone (sLYVE-1 #6) into the second mammary fat pad of nude mice (10 mice each). At 5 as well as at 10 weeks after tumor injection into the mammary fat pad overexpression of sLYVE-1 (clone#6) resulted in an significant inhibition of tumor growth by more than 60% (p<0.001) as compared to the vector transfected control (clone#8; data not shown).

To detect and quantify metastatic tumor cell dissemination in lymph nodes and lungs, semiquantitative PCR of human specific Alu sequences, which represent ~ 5% of human genomic DNA is currently being established in our laboratory. The evaluation of these data is ongoing.

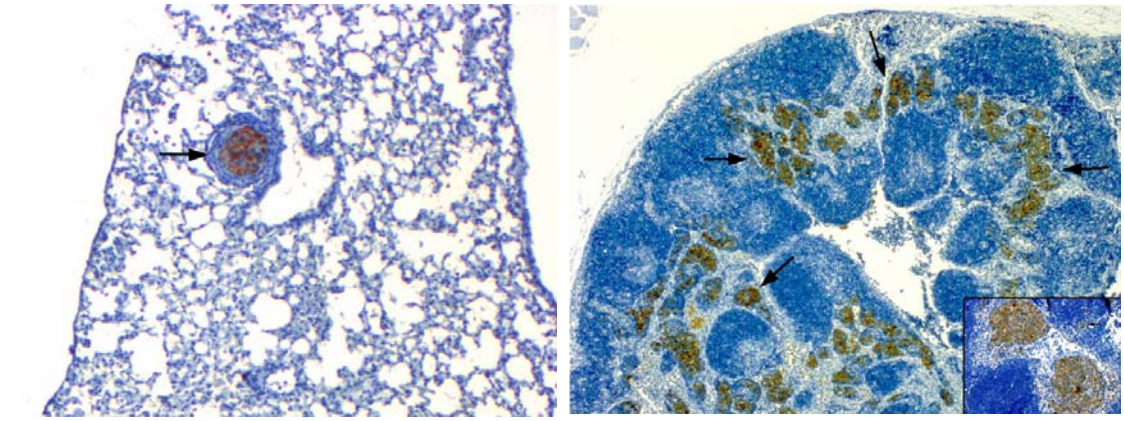


Fig. 2: Detection of breast cancer metastasis. Immunhistochemistry for GFP was applied to analyze metastatic tumor formation in lungs and lymph nodes. Immunhistochemistry of a mouse lung (right; arrow) and of a mouse lymph node (left; arrows) containing breast cancer metastases reveals strong GFP expression by dissiminated tumor cells.

To confirm the inhibitory effect of sLYVE-1 on tumor growth we performed another approach. Because local, intratumoral, or peritumoral injection of drugs will most probably not be feasible in most human patients with cancer, we initiated tumor studies with systemic delivery of sLYVE-1 by intraperitoneal injection into tumor bearing mice. Since it would take too much time and effort to produce such a huge amount of sLYVE-1 protein for a 12 week experiment, we decided to conduct this experiment over a time period of four weeks using a much faster growing subclone of original MDA-MB-435 breast cancer cells. To this end 1,5x 10^6 MDA-MB-435 cells were injected into the second mammary fat pad of nude mice. Beginning four days after tumor cell injection, mice were given intraperitoneally 1 mg per kg body weight of recombinant sLYVE-1 or PBS tertian for 22 d. First measurable tumor formation was seen at 9 days after tumor cell implantation. PBS-treated mice developed rapidly growing breast carcinomas at day 20 after tumor cell implantation (Fig. 3). In contrast, systemic treatment with sLYVE-1 markedly inhibited in vivo tumor growth (Fig. 3). After 21 days of treatment, tumor growth was inhibited by more than 30%. No apparent signs of toxicity were observed during the recombinant protein therapy.

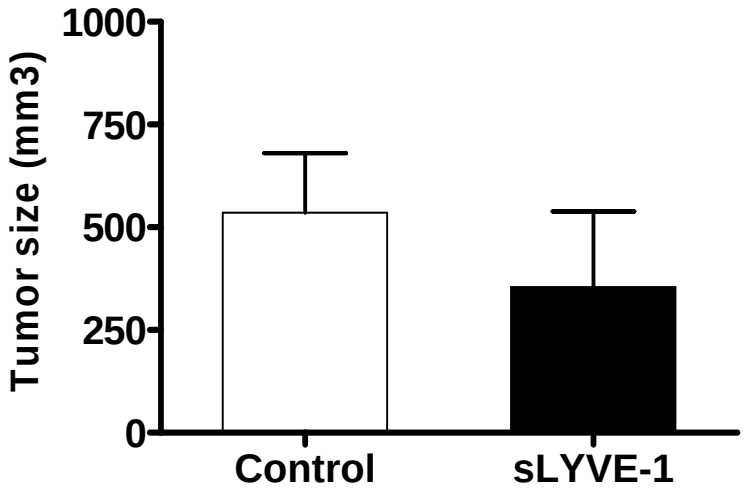


Figure 3: Systemic application of recombinant sLYVE-1 protein decreases breast carcinoma growth. Intraperitoneal injection of recombinant sLYVE-1 into mice bearing orthotopic breast carcinomas (sLYVE-1) resulted in an inhibition of tumor growth by more than 30% after 21 days of treatment (p<0.05) as compared to PBS treated control mice. Results are expressed as mean values ± SD.

3.2. Isolation of lymphatic endothelial cells from new born mice

The isolation of human lymphatic endothelial cells is well established. In our project one aim was to establish the isolation of mouse lymphatic endothelial cells from new born skin. To avoid negative sorting steps, the LYVE-1 protein was chosen as target for the cell enrichment process. If this works, lymphatic endothelial cells could be isolated in one step. First, we tried to use commercial available LYVE-1 antibodies for coupling magnetic beads. However, the amount of antibodies was too low and the expenses much too high. Therefore, antibodies against LYVE-1 were generated in our own laboratory. In addition antibody against the extracellular domain of CD31 and CD34 were also produced. After affinity purification of the antibodies, about 2-3 mg specific antibodies per 10 ml of rabbit sera were obtained. The isolation of the lymphatic endothelial cells was very challenging and optimisation of this work is still ongoing. Briefly, skins from new born mice were treaded with dispase II over night and the following day the epidermis was removed. After incubation the dermis with collagenase I, singe cell suspension was obtained. The cell suspension was further incubated with Dynabeads carrying different antibodies. After enriching the cells by a magnet and several wash steps the cells were plated into cell culture dishes. For the survival of the cells the length as well as the source of the collagenase I turned out to be crucial factors. The isolation of lymphatic endothelial cells from skin works with this system; however the number of cells obtained was rather limited. The cells in culture still carry several beads (5 to 10 beads per cell). Further evaluations of the lymphatic endothelial cells are ongoing and the preliminary results are very promising. The isolation of the blood endothelial cells from new born mouse skin just started. In the near future, both cell populations will be analyzed on the mRNA level as well as on the protein level.

Since not much is known about the composition of the extracellular matrix around the lymphatic vessels, immunohistochemical studies are initiated. Different specific antibodies against several extracellular matrix proteins have been collected from our institute as well as from outside collaborators. We just started to stain skin samples with different antibodies. Beside different laminins and collagens, we are also investigating the composition of the microfibrills such as fibrillin and fibulin.

3.3. Generation of a lymph-endothelial cell specific reporter mouse

An alternative way for the purification of lymphatic endothelial cells is the introduction of a fluorescence protein into the cells. In a first attempt, the expression of the green fluorescence protein was placed under the control of a lymphatic endothelium specific promoter. For practical reason, we decided to amplify the promoter region on mouse genomic DNA (C57BL/6) using proof reading enzymes. 7 kb upstream of the first exon was amplified and cloned into the ILLN38 hrGFP. Since it is well established that there are often regulatory elements present in the first intron, we cloned the 2.7 kb intron into the vector as well. Several founder lines were obtained, but the hrGFP was too weak to be detected directly under the microscope. To circumvent this problem, the strategy was changed and a Cre recombinase was introduced into the second exon of a BAC clone (Fig. 4). The BAC clone contains the complete mouse LYVE-1 gene and is about 180 kb long. The cassette was introduced into the BAC clone with the recombineering system. After initial set up, the method works reliably and regularly. From one transfection experiment at least four positive integration events have been obtained. The same system can be used for introducing large deletions or point mutations into a BAC

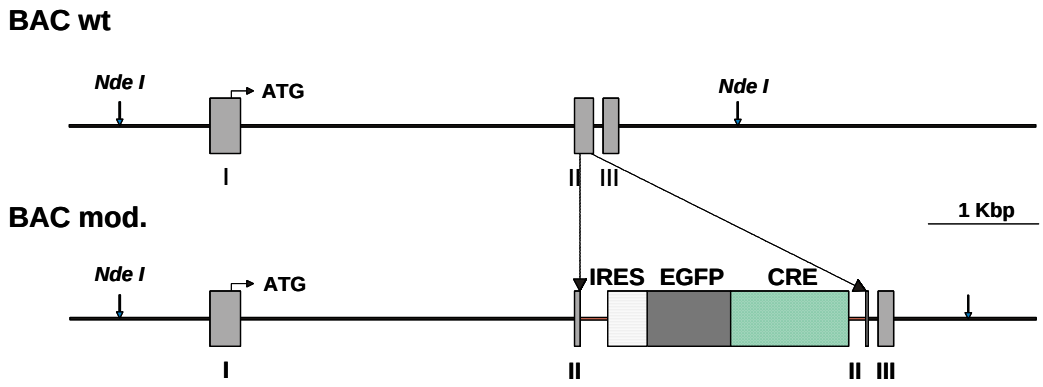


Fig. 4: Schematic drawing of the transgenic construct. Only a part of the BAC clone is depicted. The EGFP Cre recombinase cassette was inserted into the second exon of the BAC DNA. The BAC used for this construct is 180 kb long and contains the complete LYVE-1 gene.

clone. The positive clones were further checked by restriction mapping analysis. The DNA was injected into C57BL/6 by Dr. Naumann (MPI Dresden). Even though the pronuclear injection of BAC clones into the C57BL/6 mouse strain is extremely difficult, we still obtained three positive founder lines. The F1 generation was tested for the expression of Cre recombinase by RT-PCR and all were found to be positive (data not shown). To test the efficiency of the Cre recombinase, these mice are currently being crossed with floxed beta-1 integrin mice obtained from Dr. Reinhard Fässler (MPI München). In addition the mouse line will also be introduced into the Z/EG (lacZ/EGFP) mouse line (Novak et al, 2000). These mice express lacZ throughout embryonic development and adult stages. Upon excision of Cre, the lacZ gene will be removed and the expression of the second reporter, enhanced green fluorescent protein, will be activated. If this works, the lymphatic endothelial cells could also be isolated from tissues using a single step FACS.

3.4. LYVE-1 overexpression in the skin

Since the results from the LYVE-1 overexpression experiments in tumor cells are very promising, a second independent mouse model system has been introduced to further investigate this novel finding. To study the effect of soluble LYVE-1 on tumorigenicity *in vivo*, LYVE-1 has been overexpressed in the skin under the control of the keartin K14 promoter. The basic vector was kindly provided to us by Dr. Ingo Haase (Department of Dermatology, University of Cologne). Pro-nuclear injection of the linearized vector into fertilized oocytes was done in house and eight independent founders were obtained. The founders are all alive and no major alterations have been detected up to now. To reduce the number of lines, the expression pattern from all lines will be compared by immunohistochemistry as well as by semi-quantitative RT-PCR. Since the protein contains a flag-tag epitope at the C-terminus, the protein can be easily visualized with a specific flag antibody (Fig. 5). Most of the signal can be detected in the epidermis as well as in hair follicle. Several in vivo studies will be conducted with this new transgenic mouse line.

4. DISCUSSION

LYVE-1, the lymphatic receptor for the extracellular matrix mucopoysaccharide hyaluronan, has been a key component of many important studies on embryonic and tumor-induced lymphangiogenesis, and continues to be used for the detection and isolation of lymphatic endothelial cell. LYVE-1 is almost exclusively expressed on lymphatic vessels. The deduced amino acid sequence of LYVE-1 predicts a 322-residue transmembrane domain, a 63-residue cytoplasmatic tail and a 212-residue

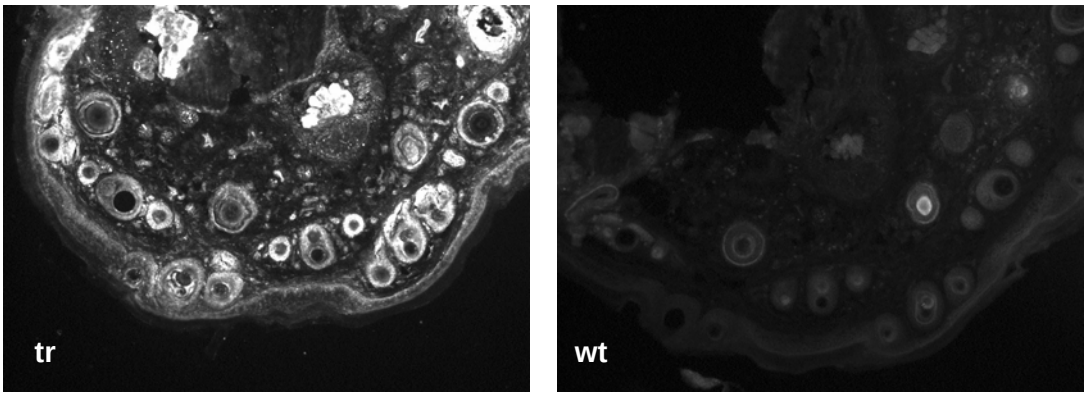


Fig. 5: Immunohistochemical studies of the transgenic mice. The overexpression of the tagged soluble LYVE-1 protein was detected with a Flag antibody on tail cross-sections. A strong signal can be detected in the basal keratinocytes of the epidermis as well as within the hair keratinocytes of a representative transgenic (tr; right figure) mouse whereas no staining was observed in the wild type (wt; left figure) control mouse.

extracellular domain containing a single Link module, the prototypic HA binding domain. There is evidence that LYVE-1 might play a role in transporting HA across the lymphatic vessel wall and might be implicated in the trafficking of cells within lymphatic vessels and lymph nodes (for review see Jackson, 2004). However, to determine the precise roles played by LYVE-1 in normal and diseased tissue is a current challenge. Studies in animal models using soluble LYVE-1 (sLYVE-1) may help to determine the role of LYVE-1 in vivo.

We studied the effects of induced sLYVE-1 gene expression in an established orthotopic nude mouse model of metastasising breast cancer, by using the human breast cancer cell line MDA-MB-453/GFP (Skobe at al, 2001) stably transfected with a modified sLYVE-1 expression vector or with vector alone. The MDA-MB-435/GFP cell line was chosen because of the constitutive expression of green fluorescent protein (GFP) which facilitates the detection of tumor micrometastases by immunhistochemistry. Our results demonstrate that tumor cell expression of sLYVE-1 potently inhibited the orthotopic growth of human breast carcinomas as compared with control tumors. In addition, we show that the incidence of lung metastasis was decreased in mice bearing breast carcinomas transfected with sLYVE-1.

The potential inhibitory role of sLYVE-1 is further supported by our findings that in vivo breast cancer formation by MDA-MB-435 cells could be suppressed by intraperitoneal injection of a recombinant soluble human sLYVE-1 immunoglobulin fusion protein. Our data strongly indicate that the observed effects of transfected sLYVE-1 on tumor growth were not because of direct effects on tumor cells since no differences in the anchorage-dependent growth rate or apoptosis rate were observed between the different transfected clones in vitro.

In summary, the experiments reported here establish sLYVE-1 as an inhibitor of tumor growth and progression in vivo. The molecular mechanisms of sLYVE-1 mediated inhibiton of tumor growth and progression still need to be established. LYVE-1 is structurally related to CD44 and other HA-binding proteins, the vast majority of which contain a consensus HA-binding domain termed the “Link” module (Kohda et al, 1996). The main difference between the two receptors is the presence of a shorter membrane-proximal domain in LYVE-1, and two sites for modification with sialidated N-glycans within the HA-binding Link module (Jackson, 2004). Most of the known effects of CD44 on cell adhesion, migration, tumor growth, and metastasis are intimately associated with its capacity to promote cell attachment to HA (Naor et al, 1997). The remarkable similarity between LYVE-1 and CD44 suggests that the two receptors might have very similar functional features. In common with CD44, the LYVE-1 molecule binds soluble and immobilised HA. The requirement for CD44-HA

binding in tumor development has been shown by the suppression of tumor formation in the presence of soluble human CD44s fusion proteins (Sy et al, 1992; Zawadzki et al, 1998).

Since MDA-MB-435 cells have been reported to express CD44 (Fichtner et al, 1997) it is conceivable that sLYVE-1 may also disrupt endogenous CD44-HA interactions and thereby promote apoptosis, decrease MMP-mediated invasion, and inhibit tumor cell proliferation as recently shown for soluble CD44 (Yu et al, 1997; Peterson et al, 2000; Yu et al, 2000; Ahrens et al, 2001).

CD44 can be proteolytically processed and shedded as soluble CD44 from the cell surface. The release of CD44 is likely to affect cellular behaviour through multiple mechanisms (for review see Cichy and Pure, 2003) Further research is needed to analyze the effects of sLYVE-1 on tumor growth and to evaluate a potential proteolytic processing of LYVE-1 which might also result in the generation of potentially biologically active fragments, and regulate cell-cell and cell-matrix interactions.

To this end we have generated a transgenic mouse model for the targeted overexpression of sLYVE-1 in the basal epidermal layer of the skin using a keratin K14 expression cassette. By immunohistochemistry of serial mouse-tail sections, we could detect sLYVE-1 protein in the keratinocytes layers of the skin. As shown from previous studies, this mouse model will allow us to study the influence of molecules during tumorigenesis. In addition to performing two step carcinogenesis (Hawighorst et al, 2001), the mice will be crossed into a HPV-8 expressing mouse line (Schaper et al, 2005). Upon UV irradiation these mice have been reported to develop a great number of tumors within a couple of weeks. This experiment will be done in collaboration with Dr. H. Pfister (Department of Virology, University of Cologne). It will be highly interesting to observe whether or not sLYVE-1 may also have a direct influence on tumor formation and progression in this mouse model.

Extracellular matrix proteins are important components for the integrity of tissues and provide the scaffold for epithelial as well as endothelial cells. However, the proteins from the extracellular matrix exhibit many additional functions such as sequestering growth factors or sending survival signals to cells just to mention some of their functions. Proteins of the basement membrane surrounding blood vessels are well characterized and several proteins have been shown to be important for the integrity of blood vessels, for example collagen IV. In contrast, not many extracellular proteins, which associate with lymphatic vessels, have been described. Reelin and Matrix Gla protein are two molecules, which have been identified to be exclusively expressed in the lymphatic system but not by blood endothelia (Saharinen et al, 2004). To further extend this group of proteins, we intended to obtain expression data through chip analysis as well as Proteonomics from isolated lymphatic endothelial cells. Obtaining these cells via cell sorting through the LYVE-1 membrane protein has been very recently described in the literature (Morisada et al, 2005). Lymphatic endothelial cells were isolated from mouse embryos. Therefore, the strategy we have chosen seems to be successful. In our hand, this method works as well for the isolation of lymphatic endothelial cells from newborn skin by using our own polyclonal LYVE-1 antibody coupled to Dynabeads. We are still in the process to optimize our isolation protocol.

One way to study the effects of extracellular matrix proteins on cellular events is to focus the investigations on their receptors. Integrins are the classical matrix protein receptors and many different alpha and beta chains haven been identified. Up to know the integrin $\alpha 1\beta 1$ and $\alpha 9\beta 1$ have been described in the literature to be important for the lymphatic system. The $\alpha 1\beta 1$ integrin belongs to the sub-family of the collagen binding integrins. We are currently studying the collagen components surrounding lymphatic endothelia.

Mice homozygous for a null mutation in the α9 gene have been reported to die soon after birth due to respiratory failure (Huang et al, 2000). Since these mice develop edema and accumulate pleural fluid in the lung, the authors speculate that the α9 integrin subunit might be important for the development of the lymphatic system. Recently it was further shown that α9 integrin binds to VEGF-C and D (Vlahakis et al, 2005). To further study the influence of integrins on the maintenance and development of the lymphatic system, a transgenic reporter mouse line expressing Cre recombinase under the LYVE-1 promoter has been generated. We have started to cross this new mouse line into an integrin β1 conditional knock-out mouse line. Since most of the integrins contain the β1 subunit, most of the α chains will be affected (Brakebusch et al, 2000). Mice lacking the β1 subunit in the lymphatic system will be carefully analysed in the future. Most likely, these mice will die after birth and we will try to dissect the underlying mechanism. The new reporter mouse line can then also be further crossed to different conditional integrin α subunit null mice. In addition, we will take advantage of a conditional EGFP mouse line. Since our reporter mice express the Cre recombinase in lymphatic endothelial cells, the EGFP would be activated and the sorting and isolation of the lymphatic cells would be simplified.

REFERENCES

Ahrens T, Sleeman JP, Schempp CM, Howells N, Hofmann M, Ponta H, Herrlich P, Simon JC: Soluble CD44 inhibits melanoma tumor growth by blocking cell surface CD44 binding to hyaluronic acid. *Oncogene* 20: 3399-408, 2001.

Alitalo K, Tammela T, Petrova TV: Lymphangiogenesis in development and human disease. *Nature* 438: 946-953, 2005.

Brakebusch C, Grose R, Quondamatteo F, Ramirez A, Jorcano JL, Pirro A, Svensson M, Herken R, Sasaki T, Timpl R, Werner S, Fässler R: Skin and hair follicle integrity is crucially dependent on b1 integrin expression on keratinocytes. *EMBO J* 19: 3990-4003, 2000.

Cichy J and Pure E: The liberation of CD44. *J Cell Biol* 161: 839-843, 2003.

Fichtner I, Dehmel A, Naundorf H, Finke LH: Expression of CD44 standard and isoforms in human breast cancer xenografts and shedding of soluble forms into serum of nude mice. *Anticancer Res* 17: 3633-45, 1997.

Hawighorst T, Velasco P, Streit M, Hong Y-W, Kyriakides TR, Brown LF, Bornstein P, Detmar M: Thrombospondin-2 plays a protective role in multistep carcinogenesis: a novel host anti-tumor defense mechanism. *EMBO J* 20: 2631-2640, 2001.

Hawighorst T, Skobe M, Streit M, Hong Y-W, Velasco P, Brown LF, Riccardi L, Lange-Asschenfeldt B, Detmar M: Activation of the Tie2 receptor by angiopoietin-1 enhances tumor vessel maturation and impairs squamous cell carcinoma growth. *Am J Pathol* 160: 1381-1392, 2002.

Huang XZ, Wu JF, Ferrando R, Lee JH, Wang YL, Farese RV, D Sheppard Jr.: Fatal bilateral chylothorax in mice lacking the integrin alpha9beta1. *Mol Cell Biol* 20:5208-15, 2000.

Jackson DG: Biology of the lymphatic marker LYVE-1 and applications in research into lymphatic trafficking and lymphangiogenesis. *APMIS* 112: 526-538, 2004.

Koch M, Murrell JR, Hunter DD, Olson PF, Jin W, Keene DR, Brunken WJ, Burgeson RE: A novel member of the netrin family, β-netrin, shares homology with β chain of laminin: identification, expression and functional characterization. *J Cell Biol* 151: 221-234, 2000.

Kohda D, Morton CJ, Parkar AA, Hatanaka H, Inagaki FM, Campbell ID, Day AJ: Solution structure of the link module: a hyaluronan-binding domain involved in extracellular matrix stability and cell migration. *Cell* 86: 767-775, 1996.

Morisada T, Oike Y, Yamada Y, Urano T, Akao M, Kubota Y, Maekawa H, Kimura Y, Ohmura M, Miyamoto T, Nozawa S, Koh GY, Alitalo K, Suda T: Angiopoietin-1 promotes LYVE-1-positive lymphatic vessel formation. *Blood* 105: 4649-56, 2005.

Naor D, Sionov RV, Ish-Shalom D: CD44: structure, function, and association with the malignant process. *Adv Cancer Res* 71: 241-319, 1997.

Novak A, Guo C, Yang W, Nagy A, Lobe CG: Z/EG, a double reporter mouse line that expresses enhanced green fluorescent protein upon Cre-mediated excision. *Genesis* 28: 147-55, 2000.

Peterson RM, Yu Q, Stamenkovic I, Toole BP: Perturbation of hyaluronan interactions by soluble CD44 inhibits growth of murine mammary carcinoma cells in ascites. *Am J Pathol* 156: 2159-2167, 2000.

Saharinen P, Tammela T, Karkkainen MJ, Alitao K: Lymphatic vasculature: development, molecular regulation and role in tumor metastasis and inflammation. *Trends Immunol* 25: 387-395, 2004.

Schaper ID, Marcuzzi GP, Weissenborn SJ, Kasper HU, Dries V, Smyth N, Fuchs P, Pfister H: Development of skin tumors in mice transgenic for early genes of human papillomavirus type 8. *Cancer Res* 65: 1394-400, 2005.

Skobe M, Hawighorst T, Jackson DG Prevo R, Janes L, Velasco P, Riccardi L, Alitalo K, Claffey K and Detmar, M: Induction of tumor lymphangiogenesis by VEGF-C promotes breast cancer metastasis. *Nat Med* 7: 192-198, 2001.

Sy MS, Guo YJ, Stamenkovic I: Inhibition of tumor growth in vivo with a soluble CD44-immunoglobulin fusion protein. *J Exp Med* 176: 623-627, 1992.

Vlahakis NE, BA Young, A Atakilit, D Sheppard: The lymphangiogenic vascular endothelial growth factors VEGF-C and -D are ligands for the integrin alpha9beta1. *J Biol Chem* 280: 4544-52, 2005.

Wilting J, Hawighorst T, Hecht M, Christ B, Papoutsi M: Development of lymphatic vessels: tumour lymphangiogenesis and lymphatic invasion. *Curr Med Chem* 12: 3043-3053, 2005.

Yu Q, Toole BP, Stamenkovic I: Induction of apoptosis of metastatic mammary carcinoma cells in vivo by disruption of tumor cell surface CD44 function. *J Exp Med* 186: 1985-1996, 1997.

Yu Q, Stamenkovic I: Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* 14: 163-176, 2000.

Zawadzki V, Perschl A, Rosel M, Hekele A, Zoller M: Blockade of metastasis formation by CD44-receptor globulin. *Int J Cancer* 75: 919-24, 1998.

Analysis of Lymphangiogenesis during Tumorigenesis, Wound Healing, Lymphedema and Filariasis

PD Dr. Jonathan Paul Sleeman

Institute of Genetics, Universität Karlsruhe and
Institute of Toxicology and Genetics
Research Center Karlsruhe
Postfach 3640, 76021 Karlsruhe, Germany

Member of the SPP 1069 from 1999 until 2005

SUMMARY

The molecular regulation of lymphangiogenesis was only beginning to be deciphered at the conception of SPP1069, with the discovery of VEGFR-3 and its ligands VEGF-C and VEGF-D. The lymphatics play a role in a variety of diseases, most importantly in tumor metastasis. The vast majority of human cancers are carcinomas that have a predilection for metastasizing to regional lymph nodes via the lymphatics, and in many cases this is the first event in the onset of lethal dissemination of the cancer to vital organs. The relationship between tumors and the lymphatic vasculature is therefore of central importance to the pathology of human cancer, yet at the outset of SPP1069 it was poorly understood. An understanding of how tumors interact with the lymphatic vasculature would allow therapies to be designed that intervene effectively in metastatic disease. We therefore set out to investigate the possible role of lymphangiogenesis in tumor metastasis. We found that within tumors both the tumor cells themselves and host stromal cells can express VEGF-C and VEGF-D. Ectopic overexpression in tumor cells of a mutant form of VEGF-C that is only able to activate VEGFR-3 causes increased peri-tumoral lymphatic vessel density in vivo and promotes the formation of lymph node metastases. Conversely, expression in metastatic tumor cells of a soluble VEGFR-3 receptor-globulin fusion protein that neutralises VEGF-C and VEGF-D activity reduces peri-tumoral lymphatic vessel density and reduces the incidence of metastasis to regional lymph nodes. These data demonstrated that tumor-induced lymphangiogenesis driven by VEGFR-3 activation is able to promote metastasis, and suggested that inhibition of VEGFR-3 activation may be useful in the treatment of cancer. In view of this, we developed ways of inhibiting tumor-induced lymphangiogenesis, including the generation of a novel indolinone that inhibits VEGFR-3 activation and shows promise as an anti-cancer reagent. We also examined whether anti-VEGFR-3 therapy might have side effects and thereby discovered that VEGFR-3 plays a role in megakaryocyte differentiation, suggesting that platelet formation may be affected by anti-VEGFR-3 therapies. In addition to these studies we have also investigated some of the basic molecular biology of VEGFR-3 activation, and discovered, for example, a splice variant of VEGFR-3 that encodes a truncated protein with a possible function in fine tuning the cellular response to VEGF-C and VEGF-D. Aside from examining the role of lymphangiogenesis in tumor metastasis, we have also investigated the role of VEGFR-3 activation in aspects of wound healing, lymphedema and filariasis. Together, these findings have substantially advanced our knowledge of the role and regulation of lymphangiogenesis in pathology, particularly tumor metastasis. They also identify a number of new avenues to explore on the road towards therapeutic application.

INTRODUCTION

The lymphatic system plays a vital role in maintaining homeostasis, returning to the circulatory system around 10% of the volume of interstitial fluid escaping from tissue capillary beds (the physiology of the lymphatic system is thoroughly reviewed in Aukland and Reed, 1993, Guyton and Hall, 1996), ensuring that the colloid osmotic pressure of the interstitial fluid remains constant. Furthermore, the lymphatic system has a crucial function in the immune system, acting as a filter for pathogens by means of its lymph nodes. It also provides ready access to the circulatory system for cells of the immune system such as dendritic cells, which when activated in peripheral tissue in response to an immunological challenge migrate into and through lymphatic capillaries. The ultrastructure of the lymphatic endothelial cells (LEC) which form or line lymphatic vessels is specialized to accomodate its function (Aukland and Reed, 1993, Guyton and Hall, 1996), and shows significant differences to the ultratructure of capillaries formed by blood endothelial cells (BEC). For example, LEC have loose intercellular junctions which readily permit the passage of large biological macromolecules, pathogens and migrating cells, and the lymphatic capillaries have no or at best only an incomplete basement membrane.

The new growth of lymphatic capillaries is termed lymphangiogenesis. Lymphangiogenesis occurs after tissue injury, obstruction to or damage of lymphatic vessels (Strange et al., 1989; Junghans and Collin, 1989) and serves to reduce the increased tissue pressure associated with edema and inflammation (Anthony et al., 1997). However, prior to SPP1069 the molecular basis for the induction of lymphangiogenesis was only beginning to be understood.

At the outset of SPP1069, the fms-like tyrosine kinase receptor called VEGFR-3 (Flt4) was known to be specifically expressed on LEC in the adult. VEGFR-1, VEGFR-2 and VEGFR-3 comprise a subfamily of receptor protein tyrosine kinases which are characterised by an extracellular region containing seven immunoglobulin-related domains and an intracellular domain with homology to the PDGFR subfamily (reviewed in Alitalo et al., 2005). In contrast to VEGFR-1 and VEGFR-2 which are widely expressed on endothelial cells, the restricted expression of VEGFR-3 to LEC made it a prime candidate for transducing LEC-specific signals. VEGF-C and VEGF-D, the known ligands for VEGFR-3, are homologous to the VEGF/PDGF family. After binding to VEGF-C and VEGF-D, it had been shown that VEGFR-3 is capable of transducing signals which trigger proliferation in vivo and in vitro (Jeltsch et al., 1997; Oh et al., 1997, Achen et al., 1998), suggestive of a role for VEGFR-3 and its ligands in lymphangiogenesis.

It had been shown that VEGF-C and VEGF-D are progressively processed during their biosynthesis to remove the N- and C-terminal ends of the protein, leaving the central VEGF homology domain (Joukov et al, 1997; Achen et al., 1998). The fully processed forms of VEGF-C and VEGF-D had been demonstrated to have the ability to activate VEGFR-2, although they are bound with a lower affinity by VEGFR-2 compared to VEGFR-3 (Joukov et al., 1997; Achen et al., 1998), suggesting that fully processed VEGF-C/VEGF-D might also stimulate angiogenesis. However, transgenic expression of VEGF-C in the skin of mice stimulated proliferation of LEC in the dermis, but did not result in new blood vessel growth (Jeltsch et al., 1997). Moreover, recombinant VEGF-C only stimulated lymphangiogenesis on the chick chorioallantoic membrane (Oh et al., 1997). It was therefore unclear whether the activation of VEGFR-2 by fully processed VEGF-C in vitro is physiologically significant.

The lymphatic system is involved in several pathological events. Most significantly, metastasis via the lymphatic system plays a central role in the metastatic dissemination of most cancers (Sleeman, 2000). Even so, prior to SPP1069 most experimental work addressing systemic tumour dissemination had focused on haematogenic spread. Little was known about the mechanisms by which tumour cells enter, interact with and are transported within lymphatic vessels. The

mechanisms which regulate metastasis via the lymphatics were only poorly understood. The architecture of lymphatic vessels make the lymphatic system a comparatively easy target for tumor cell entry into the circulatory system. Passive transport of tumor cells in the interstitial fluid flow into the lymphatics was thought to play a role in lymphatic metastasis. However, the possibility that active mechanisms such as the induction of lymphangiogenesis by tumors might facilitate entry into the lymphatics had not been explored. Our preliminary experiments that supported the first SPP1069 application suggested, however, that tumor-induced lymphangiogenesis probably was involved in metastatic spread.

In addition to tumor metastasis, the lymphatics also play pathological roles in wound healing, lymphedema and filariasis. Lymphatic regeneration through collateral formation occurs after tissue injury or obstruction or damage of lymphatic vessels. However, the majority of new lymphatic vessels produced during wound healing are transient (Pullinger and Florey, 1937; Paavonen et al., 2000). Lymphedema results from a failure of the lymphatics to provide adequate interstitial fluid drainage, for example after surgical removal of lymphatic tissue. If lymphatic drainage is not correctly re-established, chronic lymphedema can ensue for which there is currently no adequate treatment. In these cases, it is unclear why lymphangiogenesis is apparently not induced or at least not sufficiently induced to generate enough lymphatic vessels to clear the excess interstitial fluid. Filarial parasites such as Wuchereria bancrofti and Brugia malayi cause lymphatic dilation due to LEC proliferation, lymphangitis and inflammation, immune response modulation, cytokine release, granuloma and fibrotic tissue formation, damage and blockage of lymphatic vessels and edema formation (reviewed in Rajan et al., 1997).

The main focus of our SPP1069 work was to analyse the role and regulation of lymphangiogenesis in pathological contexts. Particular emphasis was placed on VEGFR-3 as a key regulator of lymphangiogenesis. The role of lymphangiogenesis in tumor metastasis constituted the main pathological focus. Here we aimed not only to understand the regulation of tumor-induced lymphangiogenesis, but also how to control this therapeutically. As a corollary, we also investigated possible unwanted consequences of such intervention. Furthermore, we analysed some of the basic molecular biology of the regulation of VEGFR-3 activation. In addition to the major focus on tumor metastasis, we also examined certain aspects of lymphangiogenesis in other pathological situations.

METHODS

The following methods were used in the SPP1069 projects:

- Standard cell and molecular biology methods (PCR, Northern and Western blots, immunoprecipitation, cell culture of lines and primary cultures, transfection, etc)
- Endothelial cell tubule formation assays
- Histology and immunohistochemistry
- Transplantable tumor models (mainly rat)
- Lymphatic vessel visualisation in vivo using Evan’s blue
- Cell encapsulation in alginate polymers
- Synthetic organic chemistry
- Large scale protein synthesis and purification
- In vitro cultivation and differentiation of megakaryocyte precursors from bone marrow
- FACS analysis
- ELISA assays

RESULTS

VEGFR-3-driven tumor-induced lymphangiogenesis as a driving force behind metastasis to regional lymph nodes.

Both tumor cells and stromal cells can express VEGF-C and VEGF-D in vivo

First we set out to examine expression of VEGF-C and VEGF-D in a large panel of rat tumor cell lines with known metastatic proclivity and found that VEGF-C and VEGF-D are expressed in a variety of different tumor types. Some highly metastatic cell lines were found to be negative for these factors. However, further investigation revealed that metastatic tumor cells which are VEGF-C/D-negative in tissue culture can nevertheless give rise to tumors which express these factors in vivo. In situ hybridisation using VEGF-C and VEGF-D probes to stain these tumors suggested that both the tumor cells themselves as well as host stromal cells can be responsible for the VEGF-C and VEGF-D expression. These findings were substantiated by FACS sorting of disaggregated tumors to separate tumor cell from stromal cells, with subsequent RT-PCR analysis of VEGF-C and VEGF-D expression in both compartments (Krishnan et al., 2003). Together, these data show that expression of VEGF-C and –D in culture does not reflect expression in vivo, that expression of these factors can be induced in tumor cells in vivo, and that stromal cells contribute to VEGF-C and D expression in tumors.

Ectopic expression of VEGF-C and VEGF-D can be sufficient to trigger metastasis to regional lymph nodes

Initial studies in collaboration with Jörg Wilting (SPP participant) investigated the expression of endogenous VEGF-C and correlated it with effects on the lymphatic system in avian chorioallantoic membrane (CAM) assays. These data provided first correlative evidence that VEGF-C expression is associated with lymphangiogenesis in tumors (Papoutsi et al., 2001).

To see if VEGF-C/D expression is functionally important for tumor growth and metastasis, we overexpressed the fully processed forms of these factors in weakly metastatic NM-081 mammary carcinoma cells that express no VEGF-C or VEGF-D in vitro or in vivo. This ectopic expression resulted in slower growing tumors with enhanced metastatic potential.

To rule out the possibility that these factors might also activate angiogenesis, we developed a mutant form that only activates VEGFR-3, even as fully processed proteins. Mutation of a cysteine residue in the VEGF-C central homology domain blocks the ability of the fully processed form of VEGF-C to activate VEGFR-2, while VEGFR-3 can still be fully activated (Joukov et al., 1998). We therefore created recombinant rat VEGF-C and VEGF-D proteins containing similar point mutations and tested these for their ability to activate VEGFR-2 and VEGFR-3. The cys-mutated VEGF-C (VEGF-C $\Delta N \Delta C$ $C_{152}S$) is able to activate VEGFR-3 but not VEGFR-2, as expected. However, mutation of the corresponding cysteine in VEGF-D blocks its ability to activate both VEGFR-2 and VEGFR-3 (Kirkin et al., 2001).

We found that ectopic expression of VEGF-C $\Delta N \Delta C$ $C_{152}S$ in NM-081 cells promoted both peritumoral lymphangiogenesis and promotes metastasis to regional lymph nodes. Evan's blue imaging of lymphatic vessels impinging upon the tumors demonstrated that the VEGF-C $\Delta N \Delta C$ $C_{152}S$ expression induced lymphangiogenesis in the vicinity of the tumor. This was confirmed histologically using e.g. Prox-1 staining. These data are therefore consistent with the notion that enhanced VEGF-C/D expression in tumors stimulates lymphangiogenesis, which increases metastatic potential by increasing the number of entry sites for tumor cells to get into the lymphatic system (Krishnan et al,. 2003).

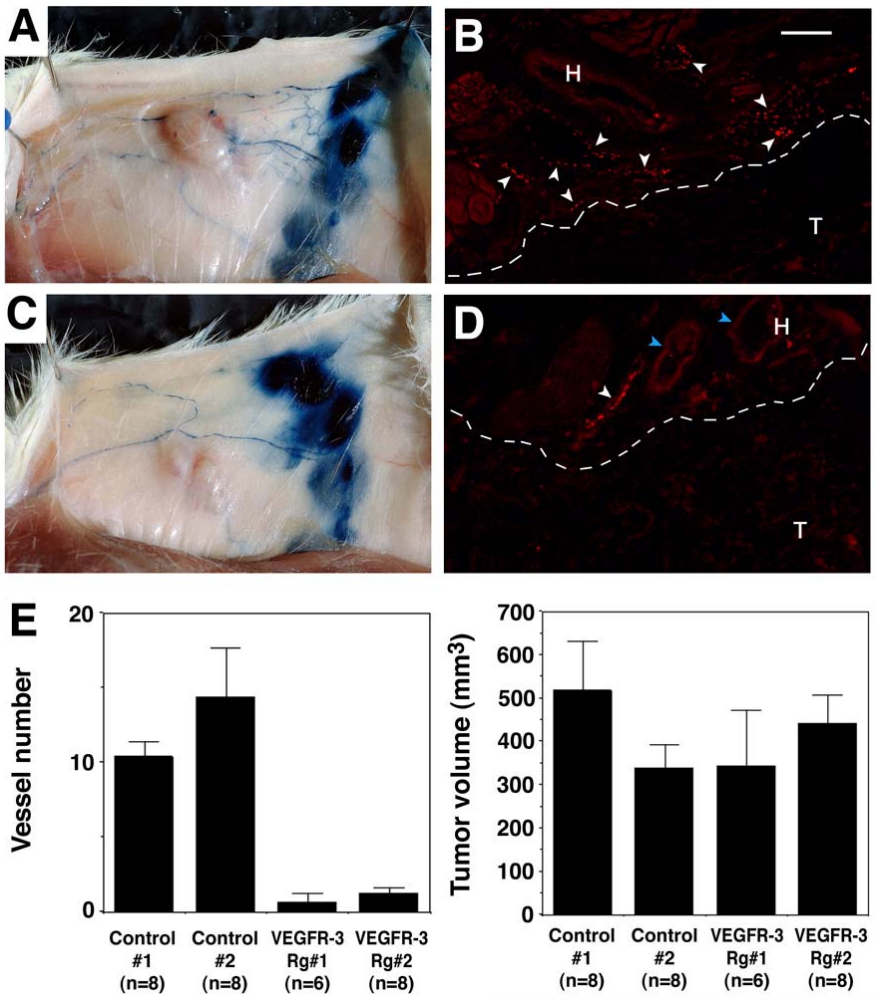


Fig. 1. Expression of VEGFR̃3̃-Rg decreases the number of lymphatic vessels impinging on MT-450 tumors. Clones of MT-450 cells stably transfected with either pcDNA3.1 (Control #1, Control #2) or pcDNA3.1 driving expression of rat VEGFR̃3̃-Rg (VEGFR-3-Rg #1, VEGFR-3-Rg #2) were injected subcutaneously into Wistar Furth rats. The resulting tumors were allowed to grow to 0.5-2 cm in diameter. They were then exposed and Evan's Blue was injected as described in the text to delineate lymphatic vessels. **A.** Example of an Evan's Blue injection experiment to identify lymphatic vessels impinging upon a tumor derived from MT-450 cells transfected with the empty pcDNA3.1 vector. **B.** Typical field of view of Prox1 staining of sections of MT-450 tumors at the tumor (T) – host (H) interface. White arrow heads indicate some of the lymphatic vessels with Prox1-positive nuclei. **C.** Example of an Evan's Blue injection experiment to identify lymphatic vessels impinging upon tumors derived from MT-450 cells ectopically expressing rat VEGFR̃3̃Rg. **D.** Typical field of view of Prox1 staining of sections of MT-450-VEGFR-3-Rg tumors at the tumor (T) – host (H) interface, demonstrating the much reduced lymphatic vessel density in these tumors. The white arrow head indicates a lymphatic vessels with Prox1-positive nuclei. The blue arrow heads point to Prox1-negative blood vessels. **E.** Quantification of lymphatic vessels impinging upon tumors derived from the different NM-081 clones (vessel number) compared to the volume of the tumors. The mean and standard error of the number of lymphatic vessels counted is shown. The number of tumors analysed is indicated. Bar B and D: 100μm

It could be argued that ectopic overexpression of VEGF-C and D in tumors might not be physiologically relevant. We therefore set out to determine what effect inhibiting enodgeneous VEGF-C and –D had on the metastatic potential of tumors. Fusion of the extracellular portion of growth factor receptors with IgG heavy chains enforces dimerisation of the receptor, resulting in a soluble protein which binds with high affinity to its cognate ligand. These receptor globulin (Rg) molecules can therefore be used to block activation of the corresponding cellular receptor by sequestering the ligand. Such an approach with VEGFR-3 had recently been published, and showed that blockade of VEGFR-3 activation inhibits lymphangiogenesis during embryogenesis (Makinen et al., 2001).

We took a similar approach to block VEGF-C/D-induced activation of VEGFR-3 in the context of tumors. We cloned the full-length rat VEGFR-3 cDNA and created a fusion of the extracellular portion with the rat IgG_{2a} heavy chain. The use of the rat IgG_{2a} heavy chain in the VEGFR-3-Rg is ideal for animal experiments as it is not bound by Fc receptors. Expression of the VEGFR-3-Rg construct in cells resulted in the secretion of a dimeric molecule which was very effectively able to block VEGF-C/D-induced activation of VEGFR-3 in cellular assays (Kirkin et al., 2001). This construct was ectopically expressed in highly metastatic MT-450 mammary carcinoma cells that produce tumors in vivo which express VEGF-C/VEGF-D. When the VEGFR-3-Rg-expressing MT-450 tumor cells were introduced into syngeneic rats, both peritumoral lymphangiogenesis (as assessed by Evan's blue imaging and immunohistochemistry – see Fig. 1) and metastasis to regional lymph nodes was substantially reduced (Fig. 2; Krishnan et al., 2003). These data substantiate the notion that specific activation of VEGFR-3 can be sufficient to induce lymphangiogenesis in the vicinity of tumors, and that this enhances metastasis to regional lymph nodes by increasing the number of entry sites into the lymphatics for invading tumor cells. It also suggests that VEGFR-3 might be a therapeutic target for the inhibition of metastasis.

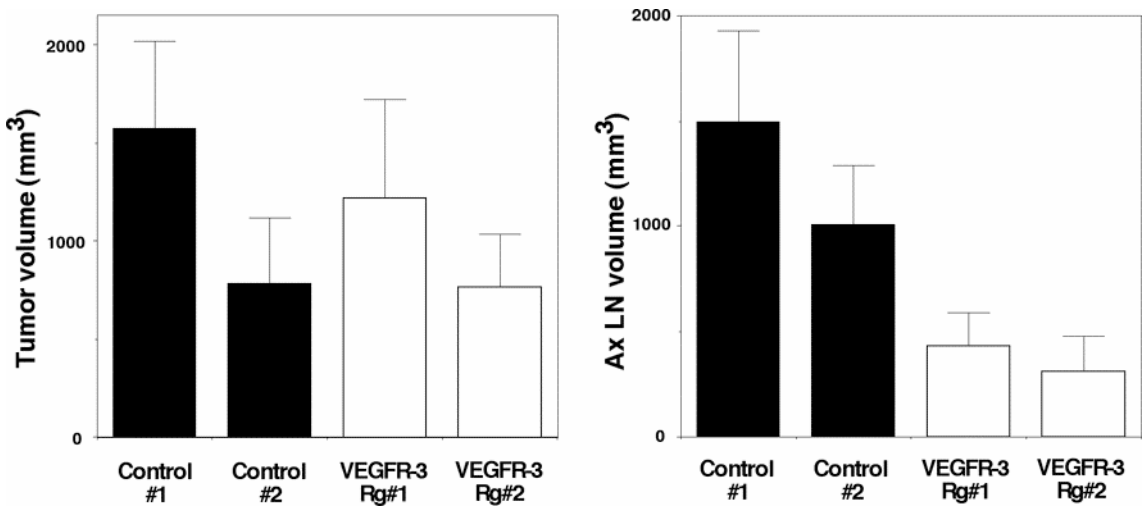


Fig. 2. Expression of VEGFR3-Rg decreases the metastatic potential of tumors derived from MT-450 cells. Clones of MT-450 cells stably transfected with either pcDNA3.1 (Control #1, Control #2) or pcDNA3.1 driving expression of rat VEGFR3-Rg (VEGFR-3-Rg #1, VEGFR-3-Rg #2) were injected subcutaneously into Wistar Furth rats (8 rats per clone). Tumors were allowed to grow for 37 days, then the animals were killed and an autopsy was performed. The mean volume of the primary tumor is shown, together with the standard error. The mean volume of the axillary lymph nodes (Ax LN) draining the tumors is presented as a measure of metastatic burden in the regional lymph nodes, together with the standard error.

VEGF-C and VEGF-D are upregulated in pre-invasive lesions in human cervical carcinomas

In collaboration with Philippe Van Trappen and Ian Jacobs (London, UK) and Herbert Weich (SPP1069 grant holder) we investigated VEGF-C and VEGF-D expression in human cervical carcinomas. Cervical carcinogenesis has well-defined stages of disease progression including three grades of pre-invasive lesions (cervical intraepithelial neoplasia grade 1-3 (CIN 1-3)) and invasive cervical cancer. However, the biological properties of CIN lesions prone to develop into invasive disease are not well defined. Recent observations suggest that early invasive disease may spread to regional lymph nodes and lymphangiogenesis may play a crucial role in this process. We found that VEGF-C and VEGF-D expression dramatically increases in CIN 3 lesions and remains high in carcinomas. VEGF-C expression correlated with lymph node involvement (Van Trappen et al., 2003). These results suggest that in cervical carcinogenesis a switch to a lymphangiogenic phenotype occurs at the stage of CIN 3. This switch may determine their further progression into invasive disease and ultimately to induce early metastatic spread to lymph nodes once they become invasive.

A VEGFR-3 ELISA for the detection of VEGFR-3 in human tumor samples

Together with Herbert Weich (SPP 1069 participant) we developed a highly sensitive VEGFR-3 ELISA assay and used this to analyse lysates from a variety of human tumor types. The levels of VEGFR-3 detected in the ELISA correlated directly with the number of VEGFR-3-positive vessels observed using immunohistology, suggesting that the ELISA can be used as a surrogate for measuring the density of VEGFR-3-positive vessels (Bando et al., 2004).

Approaches to the inhibition of tumor-induced lymphangiogenesis

The data above establish the importance of tumor-induced lymphangiogenesis for the formation of lymph node metastases, and suggest that by blocking the activity of key molecules such as VEGFR-3 it should be possible to suppress the onset of metastasis. Although it is unlikely that the growth of pre-existing metastases would be affected, the inhibition of tumor-induced lymphangiogenesis should reduce the onset of new metastases following diagnosis of cancer and its subsequent therapy. Furthermore, prophylatic suppression of metastasis would be useful for patients in remission who are at risk of recurrent tumor growth. Moreover, in the case of certain slow-growing benign cancers such as stage T1a prostate tumors, the risks and costs associated with surgical intervention are often judged to outweigh the benefit to the patient. In these cases a wait and watch approach is often adopted and the tumor is left in situ. However, the risk is that in a certain percentage of patients progression will occur and life-threatening metastatic spread will ensue. In the case of T1a prostate cancer patients, such progression occurs in 16-25% of cases within 8-10 years (Matzkin et al., 1994). In such situations, chronic administration of drugs that suppress metastasis formation would also be beneficial.

Small molecular weight inhibitors

Compounds based on the 3-substituted indolin-2-one scaffold have been identified as inhibitors of split receptor tyrosine kinases (RTKs) such as the receptors of VEGF, FGF and PDGF. Crystal structure analysis of different ligand protein complexes demonstrates that the indolin-2-one moiety serves as an anchor occupying the adenosine binding pocket of the kinase, whereas the substituents of the indolinone core are, due to specific protein-ligand interactions and individual spatial requirements, responsible for the selectivity and affinity for the individual receptor.

In collaboration with Athanassios Giannis (Universität Karlsruhe/Leipzig) we synthesized a panel of novel indolinones. In the first funding period these were screened for the ability to block VEGFR-3 activity using in vitro kinase assays. Those which showed inhibitory activity were further tested for their ability to block VEGF-C and VEGF-D-induced autophosphorylation of VEGFR-2 and VEGFR-3 in cellular assays. Thereby we identified indolinones which can be used to block ligand-induced VEGFR-3 activation (Fig. 3; Kirkin et al., 2001). During the second funding period we found that MAZ51 is able to block the proliferation of VEGFR-3-expressing primary human dermal microvascular endothelial cells consisting of mixed populations of BEC and LEC. It is also less potently able to induce their apoptosis. MAZ51 also inhibits the proliferation and induces the apoptosis of a variety of non-VEGFR-3-expressing tumor cell lines. These data suggest that MAZ51 blocks the activity of tyrosine kinases in addition to VEGFR-3. MAZ51 significantly inhibits the growth of rat mammary carcinomas in vivo (Kirkin et al., 2004). These data establish MAZ51 as a compound with anti-tumor properties that inhibits tumor growth both directly and also indirectly by interfering with tumor-host interactions such as angiogenesis and lymphangiogenesis. These studies were also performed in collaboration with Herbert Weich and Johannes Waltenberger (both SPP1069 grant holders) and Athanassios Giannis (University of Leipzig).

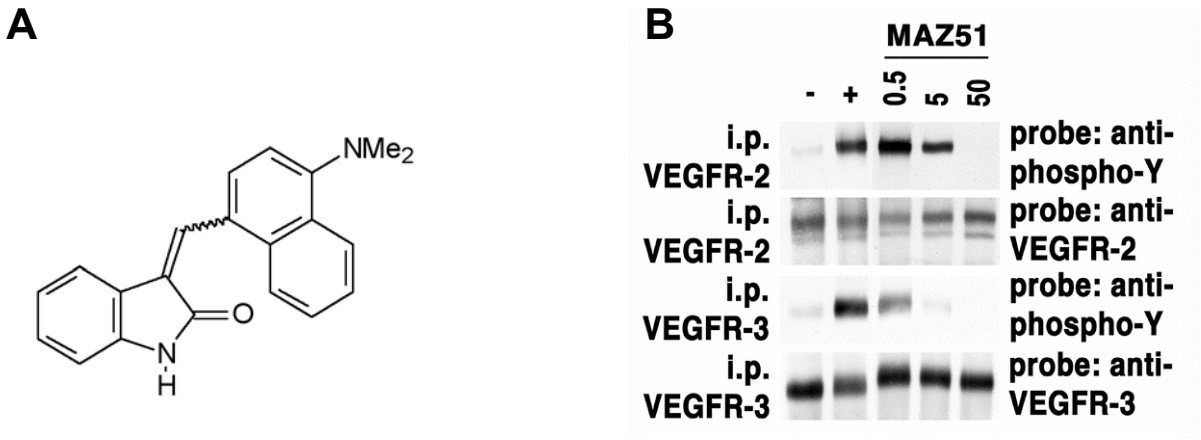


Fig. 3. VEGFR-3 inhibition by MAZ51. A. Structure of MAZ51. **B.** Inhibition of ligand induced autophosphorylation of VEGFR-3 by MAZ51. PAE cells expressing either VEGFR-2 or VEGFR-3 were incubated with 0,5μM, 5μM or 50μM of the indicated compounds. The cells were then mock stimulated (-) or stimulated with conditioned medium containing VEGF (PAE-VEGFR-2) or VEGF-C (PAE-VEGFR-3). After 15 minutes the cells were harvested and immunoprecipitated with VEGFR-2 or VEGFR-3 antibodies as appropriate. Immunoprecipitates were Western blotted and probed with anti-phosphotyrosine antibodies. Blots were then stripped and reprobed with VEGFR-2 or VEGFR-3 antibodies as appropriate for a loading control. At 5μM MAZ51 differentially inhibits VEGFR-3.

Myoblast-mediated gene transfer.

The VEGFR-3-Rg we created very efficiently blocks VEGF-C/D-induced activation of VEGFR-3, and therefore has the potential to be used therapeutically. One of the aims of the second funding period was therefore to express the VEGFR-3-Rg systemically using myoblast transfer, then to investigate the effect of neutralizing VEGF-C and VEGF-D in vivo on tumorigenesis, wound healing, lymphedema formation and filariasis. Myoblast transfer is a method whereby primary myoblasts are transduced to express a gene of interest using retroviral gene transfer. Transduced cells are introduced into the skeletal muscle masses of experimental animals. Here, the myoblasts fuse with skeletal muscle myofibrils, and the resulting syncytia express the gene of interest (reviewed in Ozawa et al., 2000).

While we could isolate and expand rat myoblasts, transduce them with retroviruses and fuse them with preexisting myofibrils (as assessed by lacZ expression), a highly frustrating aspect of the work in the second funding period was the low level of expression of the VEGFR-3-Rg we could achieve in the myoblasts. Based on the levels of VEGFR-3-Rg protein secreted into culture medium, we calculated that we would not be able to achieve sufficiently high levels of VEGFR-3-Rg systemically using myoblast transfer to be able to efficiently block the activity of endogenous VEGF-C and VEGF-D. Despite many attempts to improve the expression, we decided to switch to a different approach, namely cell encapsulation (see below).

Cell encapsulation

The encapsulation of biomolecule-producing cells in inert polymers with defined pore sizes is attracting increasing interest as a powerful way of delivering biomolecules in vivo (reviewed in Orive et al., 2003). Once the encapsulated cells are delivered in vivo, they are shielded from contact with immune cells of the host. This means that allo- and xenogenic cells can survive for up to several months in vivo. During this time they receive nutrients and growth-promoting signals through the polymer pores, and any biomolecules they produce are released into the host. For our purposes, encapsulation of VEGFR-3-Rg-expressing cells and their subsequent implantation in vivo has several advantages: (a) although purified recombinant Rg proteins could be injected regularly into experimental animals, cell encapsulation avoids the need for large scale protein purification. It also provides a continuous and constant systemic dosage of native protein compared to short intense bursts that are rapidly cleared; (b) Rg proteins could be expressed transgenically, but promoter expression during embryogenesis or chronic expression may cause problems. Cell encapsulation avoids these problems.

Alginate and cellulose sulfate polymers are the most widely characterised polymers used in cell encapsulation experiments. However, they have a relatively small pore size, which limits the size of biomolecules that are released into the host. To overcome this, it is possible to coat cell-containing alginate beads with poly-L-lysine, then depolymerise the alginate to leave a shell of poly-L-lysine surrounding the cells (Gugerli et al., 2002). The pore size of the poly-L-lysine shell is over 100 kDa, which is sufficient to maintain a barrier between the encapsulated cells and the host, but increases the range of biomolecules that can be released into the host. An additional advantage is that the mechanical stability of the beads is increased.

We have used 293 cells expressing high levels of VEGFR-3-Rg in cell encapsulation experiments using the poly-L-lysine coating method. The encapsulated cells survive for several weeks in tissue culture, and during this time they proliferate and grow to fill the whole of the capsule. More importantly, the VEGFR-3-Rg protein is efficiently released into the culture medium. In vivo, secretion of VEGFR-3-Rg by the encapsulated cells rapidly results in high levels of up to 2μg/ml of the

VEGFR-3-Rg protein in the serum of experimental rats. These levels are sustained for 4 weeks, then rapidly fall off. Subsequent analysis suggests that mechanical instability of the capsule material caused by sustained growth of the encapsulated cells results in breakdown of the capsule and thus elimination of the encapsulated cells from the body. Current work aims to (i) identify alternative cells that can be used in the capsules that exhibit contact inhibition upon confluency in the capsule (ii) develop improved biopolymers with enhanced stability. This project will continue beyond SPP 1069 with other sources of funding.

Hyperforin/Aristoforin

In collaboration with Jan Simon (Leipzig) and Athanassios Giannis we have shown as part of unrelated projects that hyperforin, a bioactive constituent of St John's Wort, has potent anti-tumor properties that it exerts by activating apoptosis via the intrinsic pathway (Schempp et al., 2002). We have developed hyperforin derivatives with enhanced solubility and improved pharmacological properties (Gartner et al., 2005). During the third funding period we examined whether hyperforin has anti-angiogenic properties. This proved to be the case (Schempp et al., 2005). Interestingly, hyperforin inhibits endothelial cell proliferation but does not induce their apoptosis. Currently we are investigating whether hyperforin/aristoforin also has any anti-lymphangiogenic properties. These projects are in addition to those proposed in the grant application.

Possible side effects of inhibiting VEGFR-3 activity

While developing ways of inhibiting VEGFR-3-regulated lymphangiogenesis for therapeutic purposes, we considered it also important to identify whether such therapies might have unwanted side effects. A number of studies suggest a role for VEGFR-3 in certain aspects of hematopoiesis, raising the possibility that anti-VEGFR-3 treatments may have hematological consequences. In VEGFR-3-deficient mouse embryos a 50% reduction in hematopoietic cell numbers was observed at E 9.5. While the number of hematopoietic stem cells did not differ from wild type controls, definitive hematopoiesis was affected in the knock-out embryos (Hamada et al., 2000). In the adult, VEGFR-3 expression is found on the surface of CD14+ monocytes derived from peripheral blood. Immature VEGFR-3-positive monocytic dendritic cells have also been detected in the eye. With this in mind during the third funding period we have investigated expression of VEGFR-3 in bone-marrow-derived hematopoietic lineages.

Initial experiments using cell lines derived from various hematopoietic lineages identified human erythroleukemic (HEL) cells as being weakly VEGFR-3 positive. TPA treatment of these cells causes them to differentiate down the megakaryocyte lineage, and we observed concomitant upregulation of VEGFR-3 expression. FACS analysis of bone marrow cells revealed populations of VEGFR-3-positive cells that were either CD38 single positive or CD38, CD41 double positive. These markers identify the VEGFR-3 positive cells as being early megakaryocyte precursor cells. In subsequent experiments bone marrow cells were cultivated under conditions that favour megakaryocyte precursor growth and differentiation. The bone marrow cultures were performed in the presence and absence of either VEGF-C $\Delta N \Delta C$ $C_{152}S$ to specifically activate VEGFR-3, or VEGFR-3-Rg or blocking anti-VEGFR-3 antibodies to inhibit VEGFR-3 activation. Specific activation of VEGFR-3 increased the number of CD41+ cells with normal ploidy, while reducing the number of CD41+ cells with a ploidy greater than 4N. The converse was true in the VEGFR-3-Rg/anti-VEGFR-3 blocking antibody experiments. These data therefore suggest that therapeutic manipulation of VEGFR-3 activity may interfere with megakaryopoiesis and consequently the production of platelets. These implications are currently being investigated in vivo.

Specificity of VEGFR-3 regulation

In addition to investigating the role of VEGFR-3 in regulating lymphangiogenesis in pathological contexts, we have also analysed some of the basic biology of the specificity of VEGFR-3 activation in response to its ligands. These approaches may be informative for further approaches towards the manipulation of VEGFR-3 activity. This work is of particular importance, because specific activation of VEGFR-3 by modified forms of VEGF-C has promise as a novel treatment of lymphedema (e.g. Saaristo et al., 2002).

VEGFR-3 has previously been shown to be alternatively spliced giving rise to two transcripts which encode proteins differing by 65 amino acids in the cytoplasmic tail ("VEGFR-3 long" and "VEGFR-3 short" forms). During the cloning of rat VEGFR-3 we identified a cDNA clone containing a 106 bp deletion in the portion of the cDNA encoding the cytoplasmic tail. Genomic mapping revealed that this cDNA represents a novel alternatively spliced form of VEGF-3 (VEGFR-3kt). We have detected the novel alternatively spliced VEGFR-3 at significant levels in several tissues, particularly the caecum and salivary gland. It is also expressed in murine and human tissues.

The alternative splicing results in truncation of the encoded protein within the tyrosine kinase domain due to a frame shift which also introduces a new peptide sequence at the C-terminus. Tyrosine residue 1337, which is crucial for signal transduction via VEGFR-3, is not present in VEGFR-3 kt protein. Upon stimulation with VEGF-C, VEGFR-3kt is not capable of autophosphorylation. When coexpressed with VEGFR-3kt, VEGFR-3 long is also not able to phosphorylate VEGFR-3kt upon VEGF-C stimulus. Together our data indicate that VEGFR-3kt is a kinase dead splice variant that functions to sequester VEGF-C/-D. Thus the purpose of VEGFR-3kt expression seems to be a fine tuning mechanism that endogenously regulates the level of VEGFR-3 long activation in response to ligand.

Structural determinants of receptor specificity.

As described above, VEGF-C $\Delta N \Delta C$ $C_{152}S$ cannot activate VEGFR-2, while VEGFR-3 can still be fully activated (Kirkin et al., 2001). Similar findings were made with human VEGF-C mutated similarly (Joukov et al., 1998). However, the corresponding mutation in the VHD of rat VEGF-D rendered the protein inactive (see above). These data suggest that small structural changes can strongly influence the receptor specificity and activity of VEGF-C and VEGF-D. This notion is supported by the observation that the receptor specificity of VEGF-D is different between mouse and man (Baldwin et al., 2001).

During the second funding period we collaborated with Michael Pepper (Geneva) to further examine the receptor specificity of VEGF-C in bovine endothelial cells. We observed that rat VEGF-C $\Delta N \Delta C$ $C_{152}S$ is able to activate both bovine VEGFR-2 and bovine VEGFR-3, whereas the VHD of human VEGF-C with an identical cys mutation is only able to activate bovine VEGFR-3 (Tille et al., 2003). Again, these data suggest that small changes in structure can strongly influence receptor specificity.

During the third funding period we aimed to further investigate the structural basis for the specificity of VEGFR-2 and VEGFR-3 activation in response to different forms of VEGF-C using NMR. In collaboration with Dr. Matthaus Görlach (IMB, Jena) we set out to produce NMR solution structures of the VHD of VEGF-C and VEGF-C $\Delta N \Delta C$ $C_{152}S$, and also of the first and second Ig domains of VEGFR-2 and VEGFR-3. In comparison to the known structures for VEGF and its

complex with VEGFR-1, we aimed to determine how mutation of Cys_{152} alters the structure of VEGF-C such that its receptor specificity changes.

Using the insect cell system for the production and purification of the VHD of rat VEGF-C and the corresponding mutated VEGF-C ΔNΔC $C_{152}S$ protein developed in the first funding period, we produced VEGF-C VHD and VEGF-C ΔNΔC $C_{152}S$ proteins for NMR analysis. A similar system for the production of His-tagged recombinant rat VEGFR-2 and VEGFR-3 proteins containing the N-terminal first and second Ig domains was also established. Specifically, the appropriate domains of VEGFR-2 and VEGF-3 were amplified from rat spleen cDNA using RT-PCR, then cloned into the pAc5.1 vector (InVitrogen) to permit expression and secretion of the protein in Drosophila S2 cells.

The first structural NMR data obtained with these proteins confirmed ordered folding, but were insufficient for more detailed analysis. On the basis of these data, it was possible to define the ^{15}N and ^{13}C labelling quality required for subsequent analysis. Unfortunately, calculation of the costs for these labelling experiments indicated an outlay of more than 40.000€ for a single experiment due to the necessity of using the insect cell system. These costs were prohibitive to further NMR experimentation. We therefore decided to focus on protein crystallography as an alternative method

Tens of milligrams of protein have been produced and purified for each receptor and ligand recombinant protein. Progress has been slow due to an inability to concentrate the proteins high enough for crystal formation before precipitation occurs. Co-crystallisation of the recombinant VHD of the VEGF-C proteins with the recombinant VEGFR-2 and VEGFR-3 proteins has also proven difficult so far due to precipitation problems. We are currently seeking to overcome these problems in collaboration with Irmgard Sinning (Heidelberg) by modifying parameters such as pH, salt concentration and glycerol content, as well as using alternative methods of protein concentration. Once we have identified ways of concentrating the proteins sufficiently, we anticipate rapid progress towards crystal production and subsequent structural analysis.

Wound healing – ELISA for VEGF-C.

In collaboration with Herbert Weich (SPP 1069 participant), we have developed sandwich ELISA assays which allows the detection and quantification of VEGF-C. The ELISA has been used in the study of the role of VEGF-C in tumor growth and wound healing. We generated the VEGF-C/D antibodies needed for the ELISA assays and assessed the antibody specificity in ELISA assays using recombinant proteins as bait. Herbert Weich set up the ELISA assay using monomeric soluble VEGFR-3 protein. The ELISA has a maximum sensitivity of 100 pg/ml. During the second funding period we investigated how lymphangiogenesis is induced in wounds. Specifically, we used the ELISA assay to examine levels of VEGF-C in serum, plasma and wound fluid. We reasoned that if VEGF-C released from platelets was responsible for lymphangiogenesis induction in wounds, then serum should contain more VEGF-C than plasma. However, the results we obtained show that serum contains very low levels of VEGF-C (0.08 +/- 0.02 ng/ml), in contrast to plasma, which contains 0.28 +/- 0.03 ng/ml VEGF-C. We also expected that wound fluid should contain significant amounts of VEGF-C, and examined the levels of VEGF-C 3, 5, 7 and 10 days after the induction of wound fluid in sponges implanted subcutaneously into rats. These time points represent the time span in which lymphangiogenesis is detected in wounds. However, no detectable VEGF-C was found in the wound fluid (Weich et al., 2004). These data demonstrate that there is insufficient VEGF-C in wound fluid, plasma or serum to induce lymphangiogenesis. These data suggest that the induction of lymphangiogenesis in wounds is likely to be a highly local event, and not dependent on VEGF-C released from platelets.

The lymphatic pathology of filariasis.

During the second funding period we established a collaboration with Dr. Judith Allen (Edinburgh) to investigate the role of VEGFR-3, VEGF-C and VEGF-D in the lymphatic pathology of filariasis. Dr. Allen's lab has established an immunocompetent animal model (mongolian jirds) which represent the best available animal model for studying parasite-induced changes in the lymphatics. We investigated whether the antibodies we have developed in Karlsruhe can be used for studying expression of VEGF-C and VEGF-D in mongolian jirds. To this end, we have cloned and sequenced VEGF-C and VEGF-D from jirds. The data suggest that there is extensive homology in the amino acid sequence of jird VEGF-C and VEGF-D in comparison to mouse and rat VEGF-C and VEGF-D in the regions to which our anti-VEGF-C and VEGF-D antibodies bind and confirmed that the antibodies recognise the corresponding jird proteins. However, during the third funding period, it has emerged that a variety of factors have the capability to induce lympangiogenesis, many of which are produced during inflammation (Thiele and Sleeman, 2006). It is also became clear that inflammatory process are likely to be responsible for the lymphatic pathology of filariasis, suggesting that factors other than VEGF-C and –D may be responsible for the lymphatic pathology. In addition, developments in other parts of the project during the third funding period diverted efforts from this project, particularly the exciting findings with the megakaryocyte precursor cells (see above). We have therefore so far not had the capacity follow up on these observations, but plan to do so in the future with a differently designed experimental plan to take into account that factors other than VEGF-C and –D produced by inflammatory cells might play a role in the lymphatic pathology of filariasis.

DISCUSSION

At the time of the conception of SPP1069, the relationship between tumors and the lymphatic system was perceived to be rather passive. Since then, driven by the discovery of novel markers specific for lymphatic endothelial cells (reviewed in Sleeman et al., 2002), there have been rapid advances in understanding the biology of lymphangiogenesis. In turn, this has cast new light on the molecular and cellular basis of metastasis to regional lymph nodes (Alitalo et al., 2005). The majority of work has focussed on VEGFR-3 and its ligands VEGF-C and VEGF-D. Correlative studies with human tumors and functional studies using animal tumor models show that increased levels of VEGF-C or VEGF-D in tumors can lead to enhanced numbers of lymphatic vessels in the vicinity of tumors, which in turn promotes metastasis to regional lymph nodes by providing a greater number of entry sites into the lymphatic system for invading tumor cells (reviewed in Thiele and Sleeman, 2006). These findings identify tumor-induced lymphangiogenesis as a possible therapeutic target for the management of cancer, and have prompted studies to investigate whether inhibitors of VEGFR-3 activation might represent novel therapeutic agents for the suppression of metastasis (Kirkin et al., 2001; Krishnan et al., 2003; Kirkin et al., 2004; Achen et al., 2005). Our work in SPP 1069 has played a major contribution to these developments. However, as is often the case, the more that is discovered, the more open questions arise. There is still much to be learned about the relationship between tumors and the lymphatics that will have important ramifications for the design of clinical trials aimed at the application of anti-lymphangiogenesis therapies in the management of cancer.

Human tumors exhibit a variety of relationships with the lymphatics.

Early studies suggested a rather simple and comparatively uniform relationship between tumors and the lymphatics, in which VEGFR-3 activation resulted in peritumoral lymphangiogenesis and promoted metastasis to regional lymph nodes by increasing the probability of tumor cell entry into the lymphatics (Pepper, 2001). However, the subsequent explosion in the number of studies examining

VEGF-C and VEGF-D expression in human tumors, together with analysis of lymphatic vessel morphology, proliferation status and density in the tumor context has revealed a more complex picture. First, not all studies find a statistically significant correlation between VEGF-C and VEGF-D expression and lymphatic density, regional lymph node metastasis formation or poor prognosis (Thiele and Sleeman, 2006). The reasons for this are several-fold. First, if the primary tumor is located in a tissue that has a relatively high lymphatic vascular density, entry of invasive tumor cells into the lymphatics may occur efficiently in the absence of neo-lymphangiogenesis. Second, several studies provide evidence that tumors can co-opt pre-existing lymphatic vessels, again probably affording efficient entry of invasive tumor cells into the lymphatics (e.g. Agarwal et al., 2005). Third, it is now becoming clear that while VEGF-C and VEGF-D are major regulators of lymphangiogenesis, a variety of other factors are also able to induce lymphangiogenesis (Thiele and Sleeman, 2006). These factors include VEGF-A, hepatocyte growth factor (HGF) and members of the fibroblast growth factor, angiopoietin, platelet-derived growth factor and insulin-like growth factor families of secreted proteins. These potentially pro-lymphangiogenic factors have each been reported to be expressed in the context of tumors and therefore could contribute to tumor-induced lymphangiogenesis, although in most cases this remains to be demonstrated.

There is also now compelling evidence that lymphangiogenesis is not just a phenomenon that occurs in the stroma at the periphery of the tumor, but that is can also occur within the tumor (see Thiele and Sleeman, 2006). What regulates whether lymphangiogenesis occurs peritumorally, intratumorally or both is not clear. We also have no idea as the relative importance of the peritumoral lymphatics compared to intratumoral lymphatics in terms of entry and transport of tumor cells into the lymphatics and subsequent lymph node metastasis formation, and the current literature contains contradictory findings (e.g. compare Maula et al., 2003 and Franchi et al., 2004).

Taken together it is clear that we still have a lot to learn about the relationship between tumors and the lymphatics. These lessons will also be very important in the context of blocking tumor-induced lymphangiogenesis in a therapeutic context. If multiple factors produced by tumors are able to induce enlarged or increased numbers of lymphatic vessels, then targeting a single one of these factors is unlikely to be effective. Furthermore, understanding the regulation and relative importance of vessel cooption, peri- and intratumoral lymphangiogenesis will have a profound impact on approaches to inhibit the interaction of tumors with the lymphatics. Equally importantly, we still do not know whether tumor-induced effects on the lymphatics are reversible. If so, then therapies directed against the tumor-associated lymphatics will potentially have a broad application in trying to prevent the onset of metastasis. If not, then the onset of tumor-induced lymphangiogeneis in pre-invasive lesions (Van Trappen et al., 2003), in recurring tumors and in developing metastases (Hirakawa et al., 2005) may limit the efficacy of such approaches.

The intra- and extracellular signalling that regulates tumor-induced lymphangiogenesis is poorly understood.

To date, virtually all attempts to therapeutically inhibit tumor-induced lymphangiogenesis have focused on preventing the activation of VEGFR-3 expressed on LEC, either by blocking its interaction with its ligands, or by interfering with the function of its kinase domain. Approaches include the use of blocking antibodies or receptor globulins that inhibit the interaction of VEGFR-3 with its ligands, and low molecular weight chemicals such as indolinones that inhibit VEGFR-3 kinase activity (reviewed in Thiele and Sleeman, 2006). However, a fuller understanding of the intra- and extracellular signals that orchestrate lymphangiogenesis would likely identify additional targets and would also allow more efficient or precise inhibition of tumor-induced lymphangiogenesis.

The recent discoveries that growth factors in addition to those that activate VEGFR-3 can also act in a pro-lymphangiogenic manner (Thiele and Sleeman, 2006) suggest that a variety of cell surface receptor-ligand interactions may represent targets for blocking tumor-induced lymphangiogenesis. Furthermore, it may be necessary to block more than one such interaction in order to prevent tumor-induced lymphangiogenesis. Additional complexity comes from the observation that the pro-lymphangiogenic effects of a given receptor-ligand pair may be direct or indirect. For example, HGF has been reported to directly promote lymphangiogenesis, but also to have indirect effects through promoting activation of VEGFR-3 (Kajiya et al., 2005; Cao et al., 2006). VEGF-A can also have direct and indirect effects (Hirakawa et al., 2005; Cursiefen et al., 2004, Bjorndahl et al., 2005). Furthermore, in addition to the cognate receptors, other cell surface molecules may regulate the effects of pro-lymphangiogenic ligands. Alternative receptors have been reported. For example, VEGF-C and VEGF-D are able to bind to and activate $\alpha 9\beta 1$, an integrin that has been implicated in the development of the lymphatic vasculature (Huang et al., 2000; Vlahakis et al., 2005). Coreceptors may be also required. CD44, for example, has been shown to be required as a coreceptor for c-Met, the cognate receptor for HGF (Orian-Rousseau et al., 2002). In view of these findings it will be important to analyse in human tumors and animal models the spectrum of receptor-ligand interactions that can contribute to tumor-induced lymphangiogenesis, their relative importance to this process, and their mode of action. This will allow appropriate strategies for the inhibition of tumor-induced lymphangiogenesis to be developed.

With the recognition that a number of different growth factors have the capacity to induce lymphangiogenesis, it will be important to identify which intracellular signalling pathways these factors activate in order to exert their pro-lymphangiogenic effect. By studying the interaction and networking of the pathways that are activated by the different pro-lymphangiogenic factors, it may be possible to identify regulatory nodes that could be therapeutically targeted in order to block the effects of multiple pro-lymphangiogenic factors. Much remains to be discovered. For example, we are only just beginning to understand that signal transduction pathways that are activated and orchestrate lymphangiogenesis subsequent to the ligand-induced autophosphorylation of VEGFR-3. Recent work using blood vascular endothelial cells suggests that VEGFR-3 signals via the ERK, JNK and AKT pathways (Salameh et al., 2005). This may also be the case in LEC, but this remains to be shown. Furthermore, attention to date has been focused fairly exclusively on pro-lymphangiogenic signalling. Negative counter-regulation may exist, activation of which may also prove effective in blocking tumor-induced lymphangiogenesis.

The signal transduction pathways that are activated in response to pro-lymphangiogenic factors are likely to have a number of end-points, including transcriptional regulation. VEGF-C, for example, regulates expression of a number of genes (Yong et al., 2005). It is clear that the homeobox transcription factor Prox-1 plays an important role in regulating the expression of a profile of genes that determine aspects of LEC morphology and behaviour (Petrova et al., 2002; Hong et al., 2002). However, how Prox-1 is wired into the regulatory pathways that orchestrate lymphangiogenesis, what other transcriptional regulators play a role, and how different aspects of lymphangiogenesis (e.g. sprouting lymphangiogenesis compared to capillary enlargement) are regulated at the genetic level is not known. Other end-points of pro-lymphangiogenic signal transduction pathways are likely to include the cytoskeleton and adhesion complexes amongst others, but these remain to be identified.

A further approach that could conceivably inhibit tumor-induced lymphangiogeneis would be to block production of pro-lymphangiogenic factors in tumors. However, even for VEGF-C the picture is complicated, with tumor cells, stromal fibroblasts, tumor-associated macrophages and platelets all having been shown to act as sources of VEGF-C in the context of tumors (see Thiele and Sleeman, 2006). Given that a number of different factors may induce lymphangiogenesis in the context of

tumors, each of which may have more that one cellular source, it becomes clear that inhibiting tumor-induced lymphangiogenesis by preventing production of pro-lymphangiogenic factors is unlikely to be a viable approach.

Will the inhibition of tumor-induced lymphangiogenesis be effective in controlling metastatic cancer?

A major question remaining to be answered is the extent to which lymph node metastases contribute, if any, to metastasis formation in other organs. This question has exercised tumor biologists for decades (reviewed in Sleeman, 2000), and its answer will have a direct impact on the extent to which therapeutic inhibition of tumor-induced lymphangiogenesis is likely to be effective in the management of cancer. Lymph node metastases themselves are seldom life threatening and can in the main be removed surgically if necessary. Inhibition of metastasis formation in regional lymph nodes by suppressing tumor-induced lymphangigenesis will therefore only be effective if metastases in regional lymph nodes not only indicate that the primary tumor has gained metastatic competence, but also play a major contribution in tumor cell dissemination to vital organs, where subsequent impairment of function and destruction of these organs exerts the lethal effect of the cancer. A mechanism for the involvement of lymph node metastases in dissemination to vital organs is provided by the fact that lymph node metastases can shed tumors cells into the efferent lymphatics and thereby ultimately into the blood stream via the thoracic duct (Sleeman, 2000). The induction of lymphangiogenesis by metastatic tumors in lymph nodes that has been reported in some studies (Hirakawa et al., 2005) may also play a role in this regard. Analysis of the metastatic process in animal models provides some but as yet inconclusive evidence of a role for lymph node metastases in dissemination to vital organs, and there is a best only indirect evidence in this regard from the study of human tumor progression (reviewed in Thiele and Sleeman, 2006). Clearly more studies are required in this area.

A further consideration when considering the possible efficacy of inhibition of tumor-induced lymphangiogenesis in the management of cancer is the possibility that normal physiological processes might be affected, possibly leading to unwanted side effects. Studies in the adult organism suggest that lymphangiogenesis only occurs significantly during wound healing and tissue regeneration. Inhibition of lymphangiogenesis in regenerating tissues could conceivably result in edema. Blocking the activity of pro-lymphangiogenic factors may also affect the function of cells in addition to LEC. VEGFR-3, for example, has been implicated in certain hematopoietic processes (e.g Hamada et al., 2000), and it is also expressed in neuronal cells such as the neuropil of the spinal cord, neuronal cells of the retina and other non-vascular cells in the cerebral cortex (Le Bras et al., 2006). This raises the possibility that targeting VEGFR-3 may have hematological or neurological consequences, as we demonstrated in our SPP1069 work.

Conclusions

It is now clear that inhibition of tumor-induced lymphangiogenesis and suppression of the entry of tumor cells into the lymphatic vasculature represents a novel target for the management of cancer. Our work in SPP 1069 has made a major contribution to this conclusion. However, there are a number of issues regarding the therapeutic potential of anti-lymphangiogenic treatments in the context of cancer that remain to be resolved. The spectrum and relative importance of molecules that induce lymphangiogenesis and the signal transduction pathways that they activate in LEC need to be defined. Furthermore, the relative importance of the different ways in which tumors interact with the lymphatics, the reversibility of tumor-induced lymphangiogenesis, and possible side effects of anti-

lymphangiogenesis-based therapies all need to be investigated. Most importantly, the extent to which lymph node metastases contribute to the formation of metastases in other organs remains to be elucidated. These issues urgently need to be addressed so that clinical trials can be properly designed.

REFERENCES

Achen MG, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K and Stacker SA: Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA* 95: 548-553, 1998.

Achen MG, McColl BK and Stacker SA: Focus on lymphangiogenesis in tumor metastasis. *Cancer Cell* 7: 121-127, 2005.

Agarwal B, Saxena R, Morimiya A, Mehrotra S and Badve S: Lymphangiogenesis does not occur in breast cancer. *Am J Surg Pathol* 29: 1449-1455, 2005.

Alitalo K, Tammela T and Petrova TV: Lymphangiogenesis in development and human disease. *Nature* 438: 946-953, 2005.

Anthony JP, Foster RD, Price DC, Mahdavian M and Inoue Y: Lymphatic regeneration following microvascular limb replantation: a qualitative and quantitative animal study. *J Reconstr Microsurg* 13: 327-330, 1997.

Aukland K and Reed RK: Interstitial-lymphatic mechanisms in the control of extracellular fluid volume. *Physiol Rev* 73: 1-78, 1993.

Baldwin ME, Roufail S, Halford MM, Alitalo K, Stacker SA and Achen MG: Multiple forms of mouse vascular endothelial growth factor-D are generated by RNA splicing and proteolysis. *J Biol Chem* 276: 44307-44314, 2001.

Bando H, Brokelmann M, Toi M, Alitalo K, Sleeman JP, Sipos B, Grone HJ, Weich HA: Immunodetection and quantification of vascular endothelial growth factor receptor-3 in human malignant tumor tissues. *Int J Cancer* 111: 184-191, 2004.

Bjorndahl MA, Cao R, Burton JB, Brakenhielm E, Religa P, Galter D, Wu L and Cao Y: Vascular endothelial growth factor-a promotes peritumoral lymphangiogenesis and lymphatic metastasis. *Cancer Res* 65: 9261-9268, 2005.

Cao R, Bjorndahl MA, Gallego MI, Chen S, Religa P, Hansen AJ and Cao Y: Hepatocyte growth factor is a novel lymphangiogenic factor with an indirect mechamism of action. *Blood*, in press, 2006.

Cursiefen C, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, D'Amore PA, Dana MR, Wiegand SJ and Streilein JW: VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest* 113: 1040-1050, 2004.

Franchi A, Gallo O, Massi D, Baroni G and Santucci M: Tumor lymphangiogenesis in head and neck squamous cell carcinoma: a morphometric study with clinical correlations. *Cancer* 101: 973-978, 2004.

Gartner M, Muller T, Simon JC, Giannis A, Sleeman JP: Aristoforin, a novel stable derivative of hyperforin, is a potent anticancer agent. *Chembiochem* 6: 171-177, 2005.

Gugerli R, Cantana E, Heinzen C, von Stockar U and Marison IW: Quantitative study of the production and properties of alginate/poly-L-lysine microcapsules. *J Microencapsul* 19: 571-590, 2002.

Guyton A, Hall J: Textbook of medical physiology, ninth edition. Philadelphia: W. B. Saunders Company. 1996.

Hamada K, Oike Y, Takakura N, Ito Y, Jussila L, Dumont DJ, Alitalo K and Suda T: VEGF-C signaling pathways through VEGFR-2 and VEGFR-3 in vasculoangiogenesis and hematopoiesis. *Blood* 96: 3793-3800, 2000.

Hirakawa S, Kodama S, Kunstfeld R, Kajiya K, Brown LF and Detmar M: VEGF-A induces tumor and sentinel lymph node lymphangiogenesis and promotes lymphatic metastasis. *J Exp Med* 201: 1089-1099, 2005.

Hong YK, Harvey N, Noh YH, Schacht V, Hirakawa S, Detmar M and Oliver G: Prox1 is a master control gene in the program specifying lymphatic endothelial cell fate. *Dev Dyn* 225: 351-357, 2002.

Huang XZ, Wu JF, Ferrando R, Lee JH, Wang YL, Farese RV, Jr. and Sheppard D: Fatal bilateral chylothorax in mice lacking the integrin alpha9beta1. *Mol Cell Biol* 20: 5208-5215, 2000.

Jeltsch M, Kaipainen A, Joukov V, Meng X, Lakso M, Rauvala H, Swartz M, Fukumura D, Jain RK and Alitalo K: Hyperplasia of lymphatic vessels in VEGF-C transgenic mice [published erratum appears in Science 1997 Jul 25;277(5325):463]. *Science* 276: 1423-1425, 1997.

Joukov V, Kaipainen A, Jeltsch M, Pajusola K, Olofsson B, Kumar V, Eriksson U and Alitalo K: Vascular endothelial growth factors VEGF-B and VEGF-C. *J Cell Physiol* 173: 211-215, 1997.

Joukov V, Kumar V, Sorsa T, Arighi E, Weich H, Saksela O and Alitalo K: A recombinant mutant vascular endothelial growth factor-C that has lost vascular endothelial growth factor receptor-2 binding, activation, and vascular permeability activities. *J Biol Chem* 273: 6599-6602, 1998.

Junghans BM and Collin HB: Limbal lymphangiogenesis after corneal injury: an autoradiographic study. *Curr Eye Res* 8: 91-100, 1989.

Kajiya K, Hirakawa S, Ma B, Drinnenberg I and Detmar M: Hepatocyte growth factor promotes lymphatic vessel formation and function. *Embo J* 24: 2885-2895, 2005.

Kirkin V, Mazitschek R, Krishnan J, Steffen A, Waltenberger J, Pepper MS, Giannis A and Sleeman JP: Characterization of indolinones which preferentially inhibit VEGF-C- and VEGF-D-induced activation of VEGFR-3 rather than VEGFR-2. *Eur J Biochem* 268: 5530-5540, 2001.

Kirkin V, Thiele W, Baumann P, Mazitschek R, Rohde K, Fellbrich G, Weich H, Waltenberger J, Giannis A and Sleeman JP: MAZ51, an indolinone that inhibits endothelial cell and tumor cell growth in vitro, suppresses tumor growth in vivo. *Int J Cancer* 112: 986-993, 2004.

Krishnan J, Kirkin V, Steffen A, Hegen M, Weih D, Tomarev S, Wilting J and Sleeman JP: Differential in vivo and in vitro expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in tumors and its relationship to lymphatic metastasis in immunocompetent rats. *Cancer Res* 63: 713-722, 2003.

Le Bras B, Barallobre MJ, Homman-Ludiye J, Ny A, Wyns S, Tammela T, Haiko P, Karkkainen MJ, Yuan L, Muriel MP, Chatzopoulou E, Breant C, Zalc B, Carmeliet P, Alitalo K, Eichmann A and Thomas JL: VEGF-C is a trophic factor for neural progenitors in the vertebrate embryonic brain. *Nat Neurosci* 9: 340-348, 2006.

Makinen T, Jussila L, Veikkola T, Karpanen T, Kettunen MI, Pulkkanen KJ, Kauppinen R, Jackson DG, Kubo H, Nishikawa S, Yla-Herttuala S and Alitalo K: Inhibition of lymphangiogenesis with resulting lymphedema in transgenic mice expressing soluble VEGF receptor-3. *Nat Med* 7: 199-205, 2001.

Matzkin H, Patel JP, Altwein JE and Soloway MS: Stage T1A carcinoma of prostate. *Urology* 43: 11-21, 1994.

Maula SM, Luukkaa M, Grenman R, Jackson D, Jalkanen S and Ristamaki R: Intratumoral lymphatics are essential for the metastatic spread and prognosis in squamous cell carcinomas of the head and neck region. *Cancer Res* 63: 1920-1926, 2003.

Oh SJ, Jeltsch MM, Birkenhager R, McCarthy JE, Weich HA, Christ B, Alitalo K and Wilting J: VEGF and VEGF-C: specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane. *Dev Biol* 188: 96-109, 1997.

Orian-Rousseau V, Chen L, Sleeman JP, Herrlich P and Ponta H: CD44 is required for two consecutive steps in HGF/c-Met signaling. *Genes Dev* 16: 3074-3086, 2002.

Orive G, Hernandez RM, Gascon AR, Calafiore R, Chang TM, De Vos P, Hortelano G, Hunkeler D, Lacik I, Shapiro AM and Pedraz JL: Cell encapsulation: promise and progress. *Nat Med* 9: 104-107, 2003.

Ozawa CR, Springer ML and Blau HM: A novel means of drug delivery: myoblast-mediated gene therapy and regulatable retroviral vectors. *Annu Rev Pharmacol Toxicol* 40: 295-317, 2000.

Paavonen K, Puolakkainen P, Jussila L, Jahkola T and Alitalo K: Vascular endothelial growth factor receptor-3 in lymphangiogenesis in wound healing. *Am J Pathol* 156: 1499-1504, 2000.

Papoutsi M, Sleeman JP and Wilting J: Interaction of rat tumor cells with blood vessels and lymphatics of the avian chorioallantoic membrane. *Microsc Res Tech* 55: 100-107, 2001.

Petrova TV, Makinen T, Makela TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Yla-Herttuala S and Alitalo K: Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *Embo J* 21: 4593-4599, 2002.

Pullinger D and Florey H: Proliferation of lymphatics in inflammation. *J Pathol Bacteriol* 45: 157-170, 1937.

Rajan TV and Gundlapalli AV: Lymphatic filariasis. *Chem Immunol* 66: 125-158, 1997.

Saaristo A, Veikkola T, Tammela T, Enholm B, Karkkainen MJ, Pajusola K, Bueler H, Yla-Herttuala S and Alitalo K: Lymphangiogenic gene therapy with minimal blood vascular side effects. *J Exp Med* 196: 719-730, 2002.

Salameh A, Galvagni F, Bardelli M, Bussolino F and Oliviero S: Direct recruitment of CRK and GRB2 to VEGFR-3 induces proliferation, migration, and survival of endothelial cells through the activation of ERK, AKT, and JNK pathways. *Blood* 106: 3423-3431, 2005.

Schempp CM, Kirkin V, Simon-Haarhaus B, Kersten A, Kiss J, Termeer CC, Gilb B, Kaufmann T, Borner C, Sleeman JP and Simon JC: Inhibition of tumour cell growth by hyperforin, a novel anticancer drug from St. John's wort that acts by induction of apoptosis. *Oncogene* 21: 1242-1250, 2002.

Schempp CM, Kiss J, Kirkin V, Averbeck M, Simon-Haarhaus B, Kremer B, Termeer CC, Sleeman J and Simon JC: Hyperforin acts as an angiogenesis inhibitor. *Planta Med* 71: 999-1004, 2005.

Sleeman JP: The lymph node as a bridgehead in the metastatic dissemination of tumors. *Recent Results Cancer Res* 157: 55-81, 2000.

Sleeman JP, Krishnan J, Kirkin V and Baumann P: Markers for the lymphatic endothelium: in search of the holy grail? *Microsc Res Tech* 55: 61-69, 2001.

Strange C, Tomlinson JR, Wilson C, Harley R, Miller KS and Sahn SA: The histology of experimental pleural injury with tetracycline, empyema, and carrageenan. *Exp Mol Pathol* 51: 205-219, 1989.

Tille JC, Wang X, Lipson KE, McMahon G, Ferrara N, Zhu Z, Hicklin DJ, Sleeman JP, Eriksson U, Alitalo K and Pepper MS: Vascular endothelial growth factor (VEGF) receptor-2 signaling mediates VEGF-C(DeltaNDeltaC)- and VEGF-A-induced angiogenesis in vitro. *Exp Cell Res* 285: 286-298, 2003.

Thiele W, Sleeman JP: Tumor-induced lymphangiogenesis: a target for cancer therapy? *J Biotechnology,* in press, 2006.

Van Trappen PO, Steele D, Lowe DG, Baithun S, Beasley N, Thiele W, Weich H, Krishnan J, Shepherd JH, Pepper MS, Jackson DG, Sleeman JP and Jacobs IJ: Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D, and their receptor VEGFR-3, during different stages of cervical carcinogenesis. *J Pathol* 201: 544-554, 2003.

Vlahakis NE, Young BA, Atakilit A and Sheppard D: The lymphangiogenic vascular endothelial growth factors VEGF-C and -D are ligands for the integrin alpha9beta1. *J Biol Chem* 280: 4544-4552, 2005.

Weich HA, Bando H, Brokelmann M, Baumann P, Toi M, Barleon B, Alitalo K, Sipos B and Sleeman J: Quantification of vascular endothelial growth factor-C (VEGF-C) by a novel ELISA. *J Immunol Methods* 285: 145-155, 2004.

Yong C, Bridenbaugh EA, Zawieja DC and Swartz MA: Microarray analysis of VEGF-C responsive genes in human lymphatic endothelial cells. *Lymphat Res Biol* 3: 183-207, 2005.

Lymphangiogenesis and Hemangiogenesis: Common Molecular Determinants?

Prof. Dr. Jörg Wilting
Prof. Dr. Lothar Schweigerer

Pediatrics I, Children's Hospital, University of Goettingen,
Robert-Koch-Str. 40, 37075 Göttingen, Germany

Members of SPP 1069 from 2004 – 2006

SUMMARY

Since more than a hundred years, the discussion about the embryonic origin of the lymphatic vascular system has remained controversial. The development of lymphatic endothelial cells (LECs) from deep embryonic veins or mesenchymal lymphangioblasts has not been clarified.

The earliest signs of the lymphatic vascular system are the lymph sacs, which develop adjacent to specific embryonic veins. It has been suggested that sprouts from the lymph sacs form the complete lymphatic vascular system. We have studied the origin of the jugular lymph sacs (JLS), the dermal lymphatics and the lymph hearts of avian embryos. In day 6.5 embryos, the JLS is an endothelial-lined sinusoidal structure. The lymphatic endothelial cells (LECs) stain (in the quail) positive for QH1 antibody and soybean agglutinin. As early as day 4, the anlagen of the JLS can be recognized by their Prox1 expression. Prox1 is found in the jugular section of the cardinal veins, and in scattered cells located in the dermatomes along the cranio-caudal axis, and in the splanchnopleura. In the quail, such cells are positive for Prox1 and QH1. In the jugular region, the veins co-express the angiopoietin receptor Tie2. Our quail-chick-chimera studies show that the peripheral parts of the JLS form by integration of cells from the paraxial mesoderm. Intra-venous application of DiI-conjugated acetylated low-density-lipoprotein into day 4 embryos suggests a venous origin of the deep parts of the JLS. Superficial lymphatics are directly derived from the dermatomes, as shown by dermatome grafting. The lymph hearts in the lumbo-sacral region develop from a plexus of Prox1-positive lymphatic capillaries. Both LECs and muscle cells of the lymph hearts are of somitic origin. In sum, avian lymphatics are of dual origin. The deep parts of the lymph sacs are derived from adjacent veins, the superficial parts of the JLS and the dermal lymphatics develop from local lymphangioblasts.

We have also investigated the development of LECs in NMRI mice from embryonic day (ED) 9.5 to 13.5 with antibodies against the leukocyte marker CD45, the pan-endothelial marker CD31 and the lymphendothelial markers Prox1 and Lyve-1. Early signs of the development of lymphatics are the Lyve-1- and Prox1-positive segments of the jugular and vitelline veins. Then, lymph sacs, which are found in the jugular region of ED 11.5 mice, express Prox1, Lyve-1 and CD31. Furthermore, scattered cells positive for all of the four markers are present in the mesenchyme of the dermatomes and the mediastinum before lymphatic vessels are present in these regions. Their number increases during development. A gradient of increasing CD31 expression can be seen the closer the cells are located to the lymph sacs. Our studies provide evidence for the existence of scattered mesenchymal cells, which up-regulate lymphendothelial and down-regulate leukocyte characteristics when they integrate into growing murine lymphatics. Such stem cells may also be present in the human and may be the cell of origin in post-transplantation Kaposi sarcoma.

INTRODUCTION

The development of the lymphatic vascular system starts considerably later than the blood vascular system. In the chick, the first blood vessels can be seen after one day of incubation (Pardanaud et al., 1987), whereas morphological evidence for lymphatic endothelial cells (LECs) is present around day 5 (Clark and Clark, 1920). In the mouse, blood vessel development starts at embryonic day (ED) 7.5 (Breier et al., 1996); the anlagen of the lymphatic vessels (lymphatics) can be seen in the jugular region at ED 10 (Wigle and Oliver, 1999). In the human, jugular lymph sacs have been found in 6- to 7-week-old embryos of 10-14 mm total length. This is 3 to 4 weeks after the development of the first blood vessels. The first obvious morphological criteria of the developing lymphatics are the lymph sacs, which are located in close vicinity to deep embryonic veins. Studies on mammalian embryos have shown that there are eight lymph sacs; three paired and two unpaired. The paired ones are the jugular, subclavian and posterior lymph sacs, and the unpaired are the *Cisterna chyli* and the retroperitoneal (mesenterial) lymph sac. In the human, the subclavian lymph sac is an extension of the jugular lymph sac (Sabin 1909; van der Putte 1975). Except for the *Cisterna chyli*, the lymph sacs develop into primary lymph nodes.

The consecutive development of blood vascular endothelial cells (BECs) and lymphatic endothelial cells (LECs) has led to the hypothesis that LECs are derived from BECs, specifically from neighbouring veins (Sabin, 1909), and only very recently studies have supported this hypothesis on the basis of the expression pattern of the homeobox transcription factor Prox1. Prox1 deficient mice die at embryonic day 14.5. They possess a normal blood vascular system, but the development of the lymphatics is arrested at ED 10.5 (Wigle and Oliver, 1999). The first Prox1-positive endothelial cells (ECs) are located in the jugular region in the angle formed by the confluence of the anterior and posterior cardinal veins, and it has been postulated that these venous ECs are the precursors for LECs in the embryo. By budding and sprouting the cells seem to give rise to the lymphatic vascular system. However, scattered Prox1-positive cells have been observed in extra-embryonic parts of chick embryos, e.g. the allantoic mesoderm, and quail-chick grafting of the allantoic bud of ED 3 embryos has shown that there are mesodermal sources for LECs apart from the deep embryonic veins (Papoutsi et al., 2001). In fact, early descriptive studies by Huntington (1914) have suggested that the lymphatic vascular system develops without any contribution from embryonic veins; a hypothesis also supported by Kampmeier (1912). By now, the differentiation potency of early venous ECs has not been tested by means of cell lineage studies, which appears to be the only method to study the lymphangiogenic potency of these cells during normal development. We have employed this method in combination with grafting experiments to study the development of the **avian** jugular lymph sacs (JLS), the superficial dermal lymphatics and the lymph hearts. Our results provide evidence for a dual origin of the lymphatics from both the veins and scattered mesodermal precursor cells.

We then sought to investigate, if the mechanisms of embryonic lymphangiogenesis of avian embryos may also hold true for mammals. Therefore, we have studied the development of LECs in **murine** embryos. We have made use of antibodies against CD31, which detect both blood vascular endothelial cells (BECs) and LECs, against CD45, which is a marker of leukocytes, and against Prox1 and Lyve-1 (lymphatic vessel endothelial hyaluronan receptor-1), which are highly specific for LECs. We show that scattered, round cells with leukocyte and lymphendothelial characteristics are present in the dermatomes and the mediastinum, two regions that become rich in lymphatic vessels. These cells become ramified when they are located near the lymph sacs where they seem to integrate and down-regulate CD45 expression. This supports the existence of mesenchymal lymphendothelial precursor cells in mice. We assume that such cells are also present in the human,

where they can undergo malignant transformation and may represent the cell of origin in post-transplantation Kaposi sarcoma (Barozzi et al., 2003).

METHODS

Embryos

Fertilized chick and quail eggs were incubated in a humidified atmosphere at 37.8°C. Staging (HH stages) was performed according to Hamburger and Hamilton. The embryos were used for experimental studies and were also fixed at various stages of development for descriptive studies.

Intra-vascular injections

The lymphatics of 13-day-old chick embryos were perfused with 3% glutaraldehyde and 2% formaldehyde fixative. Two milliliters of Merkox-blue (Norwald, Hamburg, Germany) were mixed with 25µl of accelerator and injected into the lymphatics of the chorioallantoic membrane (CAM) or the umbilicus using fine glass needles and a micromanipulator, as shown previously. About 5µl DiI-conjugated acetylated low density lipoprotein (DiI-ac-LDL) (Paesel and Lorei, Hanau, Germany) at a concentration of 200µg/ml was injected into native lymphatics of the CAM of day 15 chick embryos. After 20 minutes of incubation the specimens were rinsed with physiological salt solution and studied with a fluorescence stereomicroscope after 2-3 h (Leica, Bensheim, Germany). DiI-ac-LDL (5µl) was also injected in ovo into the vitelline veins of day 4 quail embryos. The embryos were re-incubated until day 6.5, fixed in 4% paraformaldehyde (PFA), immersed in 5% and 15% sucrose and embedded in tissue freeze medium (Leica).

Grafting experiments

In order to study the origin of the jugular lymph sacs (JLS), we performed grafting experiments making use of the quail/chick chimera model. Two types of experiments were performed: **1**. Paraxial mesoderm was isolated from 2 day-old quail embryos and grafted homotopically into corresponding chick embryos. The implantation site was at the level of somites 8 – 12; (17 experiments) **2.** Lateral plate mesoderm at the level of somites 6 - 13 was isolated from 2 day-old quail embryos and grafted homotopically into corresponding chick embryos (6 experiments). In both experiments, the chick embryos were re-incubated until day 6.5, to study integration of endothelial cells into the lymph sacs. The specimens were fixed in Serra`s solution, embedded in paraffin, sectioned serially and studied with the QH1 and QCPN antibodies as described below.

In order to study the origin of the lymph hearts, somite 37 was grafted homotopically from stage 19 HH quail into chick embryos (9 experiments). After 6 days of reincubation, the specimens were fixed in Serra`s solution, embedded in paraffin, sectioned serially and studied with the QH1 and QCPN antibodies as described below. Additionally, the morphology of normal lymph sacs was studied in hematoxylin and eosin (HE) stained paraffin sections and in semi-thin sections, according to standard techniques.

The development of superficial, dermal lymphatics was studied by grafting of dermatomes of the thoracic region of stage 20 HH quail embryos into the wing bud region of day 3 chick embryos (12 experiments). After 5 days of re-incubation, the specimens were fixed in 4% PFA for 3 min., immersed in potassium phosphate buffer, 5% and 15% sucrose and embedded in tissue freeze medium. 20µm sections were double stained with Prox1 and QH1 antibodies as described below.

In situ hybridization

Normal and experimental embryos were fixed overnight at 4°C in Serra`s fixative. The samples were dehydrated, embedded in paraffin wax and 8 µm sections mounted onto silanized slides. The

sections were deparaffinized, post-fixed in 4% paraformaldehyde solution (PFA) and, in older specimens, treated with proteinase K and refixed in 4% PFA. For the detection of cProx1 mRNA, a chick probe was used that has been described. The 1880 bp probe was cloned into pBluescript SK- and corresponded to positions 1442 - 3322 of the coding region of the *Prox1* gene. Linearization was performed with *Eco*RI (T3) and *Sac*I (T7) for the preparation of sense and anti-sense probes, respectively. For the detection of cTie2 mRNA, a chick probe was used that has been described. The probe was cloned into pBluescript SK. Linearization was performed with *Xho*I (T7) and *Not*I (T3) for the preparation of sense and anti-sense probes, respectively. Probe labelling was performed with digoxigenin RNA labelling kit as recommended (Roche, Mannheim, Germany). Sixty microlitres of hybridization mixture was placed on each slide and the slides were incubated overnight at 65°C. After standard washing, the location of the digoxigenin was detected using a 1:4000 solution of an alkaline phosphatase-conjugated anti-digoxigenin antibody (Roche) in a blocking agent (1% bovine serum albumin in malate buffer) at 4°C overnight. The antibody was detected with 5-bromo-4-chloro-3-indolylphosphate (BCIP) and nitroblue-tetrazonium (NBT) (Roche) in alkaline phosphatase buffer, revealing a blue reaction product. The sections were counter-stained with nuclear fast red and the slides mounted. For hybridization of whole embryos, specimens were fixed in 4% PFA, rinsed, dehydrated in 100% methanol and frozen. They were then rehydrated**,** treated with proteinase K, rinsed and post-fixed with 4% PFA. Hybridization was performed at 70° C overnight. Specimens were rinsed and incubated with alkaline phosphatase-conjugated anti-digoxigenin antibody diluted 1:2000 in a blocking agent (Roche). The antibody was detected with BCIP/NBT in alkaline phosphatase buffer. The reaction was stopped with 1mM EDTA in PBT.

Immuno- and lectin-histochemistry on avian embryos

Endothelial cells of quail embryos or all quail cells were stained with the QH1 and QCPN antibodies, respectively (Developmental Studies Hybridoma Bank, Iowa City, IA). Staining was performed according to the indirect peroxidase method as described previously. The secondary antibody was peroxidase-conjugated goat-anti-mouse Ig (Sigma, Deisenhofen, Germany), diluted 1:300. Diaminobencidine (DAB) was used as a chromogen. For staining of the media of vessels, an anti-smooth muscle α-actin (αSMA) antibody (Sigma) was used, diluted 1:5000. Secondary antibody and chromogen were the same as described above. Peroxidase-conjugated soya-bean agglutinin (SBA; Sigma) was dissolved in distilled water (50 µg/ml) and applied as a 1:4 dilution.

Immunofluorescence on avian embryos

For immunofluorescence studies specimens were embedded in tissue freeze medium (Leica). Cryosections of 20 µm thickness were prepared. Non-specific binding of antibodies was blocked by incubation with 1 % bovine serum albumin (BSA) for 10 min. The QH1 antibody was diluted 1:100 and incubated with the sections for 1h, as described previously. After rinsing, the secondary Cy2- (green) or Cy3-conjugated (red) goat-anti-mouse antibody (Dianova, Hamburg, Germany) was applied at 1:100 for 1h. Staining with polyclonal Prox1 antibody was performed as described previously at a dilution of 1:1000. The secondary antibody was Cy3-conjugated (red) goat-anti-rabbit antibody (Dianova, Hamburg, Germany). In the controls the primary antibodies were omitted. After rinsing, the sections were mounted under cover slips.

Mouse embryos

NMRI wild type mouse embryos from embryonic day (ED) 9.5 to 13.5 were studied. Embryos were removed from the uterus and remained either unfixed or were fixed in 4% paraformaldehyde (PFA) or in absolute ethanol containing 3% acetic acid.

Immunohistochemistry on murine embryos

Paraffin sections of 8 μm thickness were stained with Lyve-1 antibodies. The secondary antibody was peroxidase-conjugated goat-anti-rabbit IgG (Sigma-Aldrich, Taufkirchen, Germany). Diaminobenzidine was used as chromogen. Sections were counter-stained with nuclear fast red.

Immunofluorescence on murine embryos

Embryos of each development stage remained unfixed or were fixed in 4% PFA for 20 minutes. They were rinsed in phosphate-buffered saline (PBS), transferred to 5% and 15% sucrose in PBS, and embedded in Tissue Freeze Medium (Sakura Finetek Europe, NL). Cryosections of 20 - 35 μm thickness were prepared. Non-specific binding of antibodies was blocked by incubation with 1% bovine serum albumin (BSA) for 1 hour (h) before incubation with the primary antiserum. The following primary antibodies were used in this study by double staining: rabbit-anti-mouse LYVE-1 (Regeneron, Tarrytown, NY) and rabbit-anti-human Lyve-1 (1:100; ReliaTech, Braunschweig, Germany), rat-anti-mouse CD31/PECAM-1 (1:50; BD Pharmingen, San Diego, US), rat-anti-mouse CD45 (1:50; BD Pharmingen, San Diego, US), mouse-anti-human CD45 (1:200, Dako, Hamburg, Germany), rabbit-anti-human Prox1 (1:750; ReliaTech, Braunschweig, Germany).The sections were incubated with the primary antibodies for 1 h, except for Prox1, which was incubated overnight. In the negative controls, the primary antibody was omitted. After rinsing, the secondary Alexa 488-conjugated goat-anti-rat IgG (Molecular Probes, Eugene, US) and Alexa 594-conjugated donkey-anti-rabbit IgG (Molecular Probes, Eugene, US) were applied at 1:200 dilution for 1 h. For double staining with polyclonal Prox1 and Lyve-1 antibodies, Prox1 was incubated overnight followed by incubation with the secondary antibody and intensive washing of the sections. Then, Lyve-1 staining was performed. After rinsing, the sections were mounted under coverslips with Fluoromount-G (Southern Biotechnology Associates, Birmingham, GB). They were then studied with a DM5000B epifluorescent Microscope (Leica, Bensheim, Germany), and a Zeiss Axioplan 2 LSM 510 (Zeiss, Göttingen, Germany).

RESULTS

Studies on avian embryos

We have performed descriptive and experimental studies on the development of the lymphatic vascular system of chick and quail embryos. In histological sections, the most prominent anlage of the lymphatic vascular system is the paired jugular lymph sac (JLS), which can be easily observed in day 6.5 embryos. The JLS is located in the deep cervical region, dorso-laterally of the jugular vein. In quail embryos, the endothelial lining of the JLS is positive for the QH1 antibody. Smooth muscle cells, which in later stages can be found in lymphatic trunks, are not present in the JLS. In the quail, but not in the chick, the endothelial cells (ECs) of the JLS can be stained with soybean agglutinin. Veins are weakly positive, whereas arteries are negative. It has been shown previously, that the transcription factor Prox1 is constitutively expressed in lymphatic endothelial cells (LECs) but absent from blood vascular endothelial cells (BECs) of murine, avian and human tissues (Wigle and Oliver, 1999; Rodriguez-Niedenführ et al., 2001; Wilting et al., 2002). In day 6.5 chick and quail embryos, the JLS are Prox1-positive, and, like in the mouse (Wigle and Oliver, 1999), the adjacent segment of the jugular vein expresses Prox. In contrast, the endothelial angiopoietin receptor, Tie2, is expressed in the jugular vein but not in the JLS. By means of Prox1 *in situ* hybridisation (ISH) it is possible to trace the anlagen of the lymphatics into earlier stages of development. In day 4 embryos, the jugular region is located around the level of somite 10 where the anterior and posterior cardinal veins fuse into the common cardinal vein. The vascular system of this region can be demonstrated with the endothelium-specific angiopoietin receptor Tie2. Prox1 is not expressed in the blood vascular

system, except for the jugular vessels. Prox1 positive cells can be seen in the lining of the anterior and posterior cardinal veins as demonstrated by ISH and immunofluorescence. In quail embryos, this expression pattern is better visible than in chick embryos. In accordance with previous studies we have observed Prox1 expression in the neural tube, lens, retina and other neural placodes, as well as in the liver, heart, spinal, trigeminal and sympathetic ganglia. Furthermore, scattered Prox1-positive cells are found superficially in all somitic segments along the cranio-caudal axis. Immunofluorescence studies show that the cells are located in the dermatomes. A subpopulation of them is positive for the endothelial marker QH1.

The origin of the JLS has not been studied in detail. Due to the expression pattern of Prox1 it has been suggested that in murine embryos the JLS develops by sprouting from the cardinal veins (Wigle and Oliver, 1999), but cell lineage studies have not been performed. Firstly, we have sought to determine the origin of the JLS from either the paraxial or the somatopleural mesoderm by grafting these mesodermal compartments homotopically from day 2 quail into chick embryos. Grafting of lateral plate shows that this tissue does not form any parts of the JLS. The somatopleura forms the scapula, body wall and limb structures, but not the endothelium of the JLS. Since we have shown previously that the lymphatics of the limbs are of somitic origin (Wilting et al., 2000), we determined if this also holds true for the JLS. Grafting of jugular somites shows integration of quail LECs into the superficial parts of the JLS, whereas the deep parts are formed by host (chick) LECs. In order to study the origin of the deep parts of the JLS we performed cell lineage studies with DiI-acLDL. Firstly we determined, if LECs possess functional LDL receptors by injecting DiI-acLDL directly into the lymphatics of the differentiated chorioallantoic membrane (CAM). The CAM possesses lymphatics, which accompany both arteries and veins. A few minutes after intra-lymphatic injection of DiI-acLDL a fine granular staining can be observed in the LECs, showing the existence of LDL receptors (data not shown). We then injected DiI-acLDL into a vitelline vein of day 4 quail embryos, before lymph sacs are present, and re-incubated the embryos until day 6.5. With the QH1 antibody the region of interest, i.e. aortic arch, jugular vein and JLS, can be identified. In the same section, the fine granular staining with DiI-acLDL can be seen in a number of LECs of the JLS. The data suggest a contribution of early venous ECs to the JLS.

We have observed Prox1/QH1 double positive cells in the dermatomes, which may represent lymphangioblasts. We tested their lymphangiogenic potential by grafting dermatomes of inter-limb-levels from day 4 (stage 20HH) quail embryos into the wing bud region of chick embryos. After 5 days of re-incubation, the lymphatics in the wing of the host embryos can be identified with the Prox1 antibody (**Fig. 1A**). The lymphatic vessels are located superficially in the dermis and are connected to the axillary region. Prox1 and QH1 double staining clearly shows integration of graft-derived quail LECs into the lymphatics of the chick host (**Fig. 1B-D**), demonstrating lymphangiogenic potential of the dermatomes.

A highly specialised part of the superficial lymphatics of birds are the lymph hearts (Budras et al., 1987). They are located dorsal to the transverse processes of the first free caudal vertebra, which can be demonstrated in chick and quail embryos by injecting Mekox-blue into the umbilical lymphatics. The embryonic lymph hearts are partially covered by the *M. coccygeus dorsalis*. They are made up of an endocardium and a myocardium. The origin of the cells of the lymph hearts has not been studied yet. In day 5.5 chick and quail embryos, the anlagen of the lymph hearts can be detected with Prox1 ISH. A dense plexus of lymphatic capillaries is visible in the dermatomes of somites 35 – 40. Grafting of somite 37, which is in the centre of the prospective lymph heart, homotopically from quail into chick embryos, shows that both LECs and muscle cells of the lymph heart are of somitic origin. This could be demonstrated by staining of quail cells with the QCPN antibody. Staining with the QH1 antibody provided further evidence for the quail origin of LECs in the

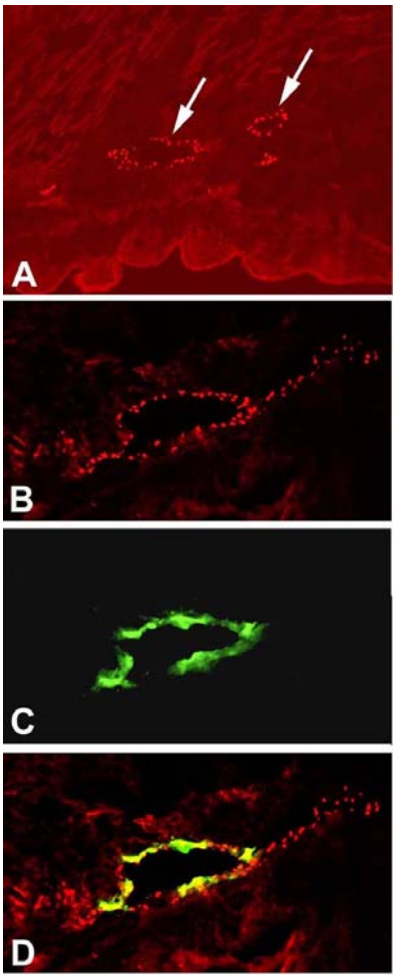


Fig. 1: Grafting of a single isolated dermatome of a stage 20 HH quail embryo into a day 3 chick embryo. Re-incubation period 5 days. **A**) Overview showing Prox1-positive lymphatic vessels (arrows) in the dermis of the wing of the host embryo. Double staining with anti-Prox1 (**B**) and QH1 antibodies **(C**). **D**) Merged picture. Note integration of QH1-positive quail LECs into the Prox1-positive and QH1-negative lymphatic vessels of the chick host.

chimeras. We counted the number of quail cells in the QCPN stained sections and found that approximately 18 – 20 % of the cells were of quail origin, suggesting that five somites contribute to the lymph hearts.

Studies on murine embryos

We have studied paraffin sections of out-bred mouse embryos (NMRI) at ED 10.5 – 13.5. At ED 13.5, the huge jugular lymph sacs (JLS) can be observed closely opposed to the jugular veins. The paired JLS accompany the jugular veins along the neck and are traversed by the brachial plexus. They form a dorsal extension towards the muscles of the back, a lateral extension towards the skin and a medial extension to the mediastinum. They can be followed caudally to the level of the thymus. The extensions of the JLS can more clearly be identified with the LEC marker Lyve-1. Sections of ED 13.5 embryos display pairs of JLS in direct proximity to the cardinal veins. Extensions of the lymphatic endothelium in dorsal, ventral, medial and lateral directions are visible. Additionally, there is a large number of scattered Lyve-1-positive cells in the loose connective tissue. The greatest numbers are found in the dermis, mediastinum and primitive meninx. At ED 12.5, Lyve-1 staining demarcates the JLS and scattered cells in the mediastinal and dorsal routes, and also in the dermatomes laterally from the neural tube. In ED 11.5 embryos, Lyve-1 is expressed in segments of the jugular vein, the JLS and some scattered cells in the jugular region. Additionally, some scattered Lyve-1-positive cells are found in the dermatomes, at a great distance from the jugular region. In ED 10.5, we observed Lyve-1 expression in specific compartments of the cardinal veins, the vitelline

vein, the hepatic sinusoids and parts of the midgut vascular plexus, and in a few scattered cells in the mesenchyme.

In order to investigate the nature of the scattered cells, we have studied the expression of the LEC markers Lyve-1 and Prox1 in combination with CD31 and CD45 in ED 10.5 – 13.5 mice. Double staining of Prox1 and Lyve-1 shows that there is a population of mesenchymal cells, which is positive for both markers. CD31 (PECAM-1 – platelet endothelial cell adhesion molecule-1) is a marker of both BECs and LECs, and is also present on leukocytes of the periphery. The leukocyte common antigen CD45, a transmembrane glycoprotein with a molecular weight of 180-220 kDa, is a marker for cells of hematopoietic origin except for erythrocytes. In 10.5 day-old murine embryos, Lyve-1/CD45 double positive cells can be observed in the mediastinal region, where single positive cells of both types are also present. With increasing age of the embryos, the number of Lyve-1/CD45 double positive cells increases. At ED 11.5 they can be observed in various regions, in the mediastinum, close to the JLS, and also in the dermatomes dorsal to the spinal ganglia. In the neural tube, which remains free from lymphatics, there are numerous CD45-positive cells, but no Lyve-1-positive cells. At ED 12.5, the number of Lyve-1/CD45 double positive cells further increases and they are found in the mediastinal region, the dermis and the primitive meninx. The cells usually have a round or oval shape. Those cells, which are located close to the JLS, often have long cytoplasmatic extensions. In day 13.5 mouse embryos, Lyve-1/CD45 double positive cells can be observed with a flat morphology. These cells seem to integrate into the lining of lymphatic vessels. To further investigate the lymphangioblastic nature of the Lyve-1/CD45 double positive cells, we have performed Prox1/CD45 double staining. The distribution of Prox1/CD45 double positive scattered cells is essentially the same as described for the Lyve-1/CD45. They are found e.g. in the dermatome and mediastinal region, and those cells that integrate into the lining of a lymphatic vessel express CD45 only weakly, indicating down-regulation of this protein. However, the basic differences reside in the fact that Prox1/CD45^{+}-cells are found at much lower numbers and they are never located in positions dorsal to the spinal ganglia in early embryos.

Double staining of embryonic tissues was also performed with CD31 in combination with Lyve-1 and Prox1. Lymphatic vessels are Lyve-1/CD31 double positive, but CD31 expression in LECs is always weaker than in BECs. In 11.5 day-old mouse embryos, the JLS are Lyve-1/CD31 double positive, whereas the scattered Lyve-1-positive cells in the dermatomes are CD31-negative. At ED 12.5, a gradient of CD31 expression can be observed in the scattered Lyve-1-positive cells both in the dermal and the mediastinal regions (**Fig. 2**). The closer the cells are located to the JLS the stronger the CD31 expression. Concomitantly, the shape of the cells changes from round to ramified,

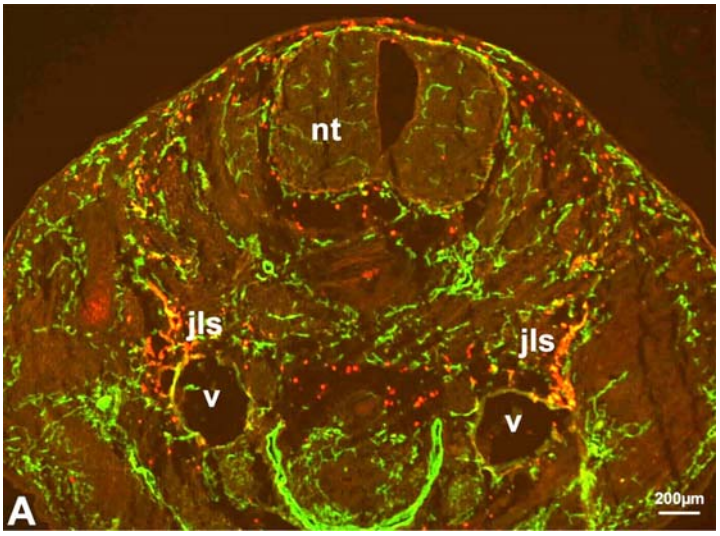


Fig. 2: Double staining of cryo-sections of ED 12.5 mice with antibodies against CD31 (green) and Lyve-1 (red). Overview showing the neural tube (nt), cardinal veins (v) and jugular lymph sacs (jls). Note the change in colour from red to yellow the closer the Lyve-1-positive cells are located to the jls.

indicating integration into the lining of the lymphatic endothelium. In the dermis and the mediastinum, scattered cell also co-express Prox1 and CD31.

DISCUSSION

Lymphangiogenesis in avian embryos

Development of lymphatics has been studied by injection methods, serial sections (Sabin 1909; Clark 1912; van der Putte 1975) and in living animals (Clark and Clark 1914). However, due to the lack of specific markers at that time, the origin of lymphatic endothelial cells had remained unknown. Are they derived from lymphangioblasts of the early mesenchyme (Huntington 1908; Kampmeier 1912), from veins by sprouting (Sabin 1909), or by both mechanisms? Recently, highly specific markers have been found, which allow for discrimination between BECs and LECs. Among these markers is the homeobox transcription factor Prox1 (Wigle and Oliver, 1999). Prox1 is a stable and specific marker of lymphatics in normal and diseased human tissues (Wilting et al., 2002), and its essential role for the developing lymphatic system has been demonstrated in Prox1 deficient mice (Wigle and Oliver, 1999). Heterozygous Prox1 null-mice mice show accumulation of chyle in the intestine. Homozygous null-mice have almost no signs of lymphatic vessel development, whereas the blood vessels appear to be completely normal. As determined by Prox1 expression, development of the lymphatics starts around embryonic day (ED) 9.5 in the jugular region of wild-type mice (Wigle et al., 2002). Prox1 positive endothelial cells are seen in the jugular section of the anterior cardinal vein, and in ED 10.5 embryos it appears that Prox1-positive cells are sprouting into the mesenchyme. However, movement of cells has not been studied and cell lineage studies have not been performed in mice. In their studies on Prox1 expression in the lymphatic system, Wigle and Oliver (1999) describe their observation that “during development scattered (Prox1-positive cells) along the embryonic axis increased. It could be argued that this increase is a consequence of an independent induction of Prox1-positive cells in the adjacent mesenchymal cell. However, our results favour the idea that this increase reflected an augmentation in the number of cells budding from the cardinal vein.” As a consequence, many original papers and reviews published in the recent years have concentrated on the idea that lymphatics develop from the venous system in a process of transdifferentiation of venous ECs into LECs (Wigle et al., 2002).

Our grafting experiments and cell lineage studies performed on avian embryos show that the lymphatics have a dual origin. The Prox1 expression pattern in chick and quail embryos is the same as in the mouse. Besides expression in a number of non-endothelial cell types, Prox1 is a highly specific marker of LECs; the only exception being the concave side of the semilunar valves (Rodriguez-Niedenführ et al., 2001). Prox1 also is a marker of prospective LECs. It is expressed in the jugular section of the cranial and caudal cardinal veins (the region around somite 10 in day 4 avian embryos). These veins are Prox1 and Tie2 double positive whereas later the lymph sacs and intestinal lymphatics (data not shown) are Tie2-negative. Like in the mouse, the jugular veins seem to be the anlagen of the JLS. Our cell lineage studies performed with DiI-acLDL on day 4 quail embryos show that labelled venous ECs end up in the JLS. However, only the deep parts of the JLS seem to be derived from the veins whereas the superficial parts develop by integration of lymphangioblasts from the local somites. This is supported by a previous observation. When quail paraxial mesoderm is inserted superficially into the jugular region of chick embryos, integration of LECs into the superficial parts of the JLS can be observed. When grafted more deeply into the host, both the jugular vein and the lymph sac are made up of grafted cells (Wilting et al., 2001). Our data suggest that the superficial lymphatics are derived from local lymphangioblasts, whereas the deep parts of the lymph sacs are formed by angioblasts of the paraxial mesoderm, which are incorporated into the jugular section of the cardinal vein, express Prox1 and sprout into the surrounding

mesenchyme. This raises the question about the origin of the jugular veins. They are obviously not derived from angioblasts of the local somites, although it has clearly been shown that somites possess angiogenic potential (Wilting et al., 1995). However, extensive migration of angioblasts and endothelial cells occurs during the formation of trunk vessels, and it is likely that the jugular veins are initially formed at a different level in the early embryo, and are shifted by allometric growth toward the jugular region. Most likely the jugular veins are formed at a more cranial level since the heart undergoes a marked descent during development.

Our studies show that the superficial/dermal lymphatics of avian embryos are derived from local lymphangioblasts. We have observed scattered Prox1-positive cells in the dermatomes of day 4 avian embryos. These cells represent a heterogeneous population. Some of them may represent sympathetic neurons since the sympathetic ganglia are also Prox1-positive, others are Prox1/QH1 double positive. QH1 is a marker of BECs, LECs and angioblasts. Our grafting experiments show development of LECs from dermatomal precursor cells, most likely from the Prox1/QH1 double positive cells. The dermatomes are derived from the early epithelial somites. These are Prox1-negative, showing that there is a Prox1-negative phase of lymphangioblast development. In accordance, our previous studies have demonstrated that lymphatics of extra-embryonic sites develop independently from the lymph sacs. Grafting of the allantoic mesoderm of day 3 quail embryos into corresponding chick embryos has shown that the lymphatics of the chorioallantoic membrane (CAM) develop from precursors within this mesoderm (Papoutsi et al., 2001). By embryonic day 4, but not day 3, the precursors can be identified by their Prox1 expression, which again shows that there is a Prox1-negative phase of LEC development. In fact, the very early stages of lymphatic development can also be observed in Prox1 null-mice. Lymphatic development in these mice is arrested around ED 11.5 and the vessels appear to have a blood vascular phenotype (Wigle et al., 2002).

Very recent data suggest that lymphangioblasts can not only be observed in avian embryos, but also in *Xenopus laevis* tadpoles and probably also in mice. Ny et al. (2005) report on clusters and cords of Prox1-positive cells in *Xenopus* tadpoles, which form the lumen of lymphatic vessels in later stages. During pathological, inflammation-induced lymphangiogenesis in the cornea of mice, CD11b-positive macrophages might transform into lymphangioblasts. In vitro, such cells are capable of expressing lymphatic endothelial markers and form tube-like structures (Maruyama et al., 2005).

The mechanisms of the formation of lymphatic vessels have not been studied in detail. One of these mechanisms seems to be sprouting (Karkkainen et al., 2004). In addition, formation of tubes by lymphangioblasts has been described very recently (Ny et al., 2005). We have shown that integration of lymphangioblasts into the lining of a lymphatic vessel is an additional mechanism, which is analogous to the growth of the blood vascular system. Another mechanism seems to be the fusion of lymphatic capillaries, which may take place during development of the lymph hearts. Lymph hearts are found in all avian embryos and are needed for the propulsion of lymph into the coccygeal veins. After hatching lymph hearts degenerate in some species, e.g. chicks, but remain active in birds that possess erectile copulatory organs, e.g. ducks (Budras et al., 1987). The lymph hearts are located superficially in the sacro-coccygeal region. In early embryos, a plexus of lymphatic capillaries can be observed in this region. This plexus spans from somite 35 – 40. The capillaries seem to fuse into a heart that possesses a single chamber, occasionally traversed by a trabeculum. After grafting of somite 37, only a small number (18 – 20%) of the lymph heart cells are of donor origin, suggesting that the other parts are derived from neighbouring segments. Both endocardium and myocardium of the lymph heart are of somitic origin. The myocardium is made up of striated muscle, which, however, is different from both cardiac and skeletal muscle (Budras et al., 1987). Nevertheless, the

data show that all striated muscles, except for the blood heart, are derived from the paraxial mesoderm.

Lymphangiogenesis in mammalian embryos

Recent studies on murine embryos have shown that the first cells committed to the lymphendothelial lineage are located in the endothelial lining of the anterior cardinal vein in the jugular region (Wigle and Oliver, 1999; Wigle et al., 2002). The ECs of the cardinal vein express Vascular Endothelial Growth Factor Receptor-3 (VEGFR-3), Lyve-1, a transmembrane glycoprotein (Banerji et al., 1999; Jackson et al., 2001) and CD31 (PECAM-1). Only a subset of them is Prox1-positive. These cells are located in the dorso-lateral part of the vein and seem to sprout into the dorso-lateral mesoderm, where they form the jugular lymph sacs (JLS). At ED 14.5 sprouts of the JLS grow toward the periphery and enter different organs (Wigle et al., 2002). Mice deficient for Vascular Endothelial Growth Factor-C (VEGF-C), the ligand of VEGFR-3, do not form lymph sacs, which can be rescued by the application of VEGF-C (Karkkainen et al., 2004). These studies are in line with the traditional view of the development of LECs from specific parts of the venous system, also called “centrifugal theory”. The main representative of this theory was Sabin (1909), who had performed India ink injections into the JLS of pig embryos. At about the same time, an opposing theory, the “centripetal theory”, was set up by Huntington and McClure (1908) and Kampmeier (1912a), who had studied cat and pig embryos. According to them, the lymphatics develop by confluence of mesenchymal cells, and only the lymph sacs might be of venous origin (Kampmeier, 1912). Our recent studies on avian embryos have supported the latter view. Intra-venous application of DiI-conjugated acetylated low-density-lipoprotein, which labels endothelial cells, into day 4 embryos, has revealed labelling of the JLS at day 6.5. This suggests a venous origin of the JLS. Additionally, quail-chick grafting of paraxial mesoderm has shown integration of graft-derived LECs into the superficial parts of the JLS. The lymphatics of the dermis are directly derived from the dermatomes. In avian embryos, there seems to be dual origin of LECs. The main parts of the lymph sacs are obviously derived from adjacent venous segments. Peripheral parts of the lymph sacs and the dermal lymphatics seem to be mainly derived from local lymphangioblasts. Lymphangioblasts have very recently also been observed in Xenopus tadpoles (Ny et al., 2006).

Here we have studied the development of lymphatic vessels with antibodies against CD31, CD45, Lyve-1 and Prox1 in NMRI mice during embryonic days (ED) 9.5 – 13.5. In accordance with previous studies we have observed the earliest signs of lymphendothelial commitment in specific parts of the venous system, i. e. the jugular segment of the cardinal veins and the vitelline vein. Additionally, Lyve-1 expression can be seen in the midgut vascular plexus, which may represent lymphatic anlagen, and in the liver sinusoids. It has been shown previously that the sinusoids of the liver and spleen are the only blood vascular compartments that express Lyve-1 (Banerji et al., 1999; Jackson et al., 2001). The second LEC marker we have used, Prox1, is also expressed in jugular segment of the cardinal veins. However, in contrast to previous studies we have observed scattered mesenchymal cells with lymphendothelial characteristics. An increasing number of Lyve-1/CD31 and Lyve-1/CD45-positive cells can be found in the mesenchyme of the dermis, the mediastinum and the primitive meninx, but not in the central nervous system (CNS), which is free of lymphatics. These cells may be macrophages, however, it is likely that a subpopulation of them represents lymphangioblasts. This is supported by the observation that a smaller number of Prox1/CD31 and Prox1/CD45 double positive cells are present in the same mesenchymal compartments. In the mesenchyme dorsal to the neural tube, Lyve-1-positive cells can be observed much earlier than Prox1-positive cells. We therefore suggest that Lyve-1 is an earlier marker of lymphangioblasts than Prox1. CD31 is a marker of both LECs and BECs and it is intriguing to see that a gradient of increasing CD31 expression can be seen in scattered Lyve-1-positive cells the closer these cells are

located to the JLS. It appears that the cells take up an endothelial phenotype before integrating into the lining of the lymph sacs. This view is supported by the observation that the shape of the cells changes from round to ramified when they approach the JLS. It may be surprising to see CD45 expression in the scattered Prox1- and Lyve-1-positive cells since LECs are CD45-negative. However, we have seen Prox1-positive LECs in the lining of early lymphatics with low CD45 expression, suggesting that CD45 is down-regulated after integration of lymphangioblasts into the embryonic lymphatics. In sum, our view of the development of lymphatic vessels in mice is different from those, who have used the same markers recently. We suggest that some part of the lymphatic vascular system may be derived from veins, however, there also is integration of lymphangioblasts into the growing lymphatic vessels. One basic difference between our study and the previous ones resides in the fact that we have used outbreed mice, whereas other groups have studied inbreed strains (Wigle et al., 2002; Karkkainen et al., 2004). However, in such inbreed mice, Wigle and Oliver (1999) describe that they have observed a growing number of scattered Prox1-positive cells in the paraxial mesoderm. But they suggest that these cells are derived from the lymph sacs. We have shown that such cells arise in the mesenchyme at a great distance from the lymph sacs and therefore represent lymphangioblasts. Lymphangioblasts seem to be highly mobile cells, and they express both lymphendothelial and leukocyte markers. The principles of embryonic lymphangiogenesis seem to be the same in murine, avian and amphibian embryos. Our experimental studies on quail-chick chimeras (Wilting et al., 2000; Papoutsi et al., 2001) and studies on Xenopus larvae (Ny et al., 2005) have shown evidence for the existence of lymphangioblasts in these species.

REFERENCES

Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG: LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 144: 789-801, 1999.

Barozzi P, Luppi M, Facchetti F, Mecucci C, Alu M, Sarid R, Rasini V, Ravazzini L, Rossi E, Festa S, Crescenzi B, Wolf DG, Schulz TF, Torelli G: Post-transplant Kaposi sarcoma originates from the seeding of donor-derived progenitors. *Nat Med* 9: 554-561, 2003.

Breier G, Breviario F, Caveda L, Berthier R, Schnurch H, Gotsch U, Vestweber D, Risau W, Dejana E: Molecular cloning and expression of murine vascular endothelial-cadherin in early stage development of cardiovascular system. *Blood* 87: 630-641, 2003.

Budras KD, Hullinger RL, Berens von Rautenfeld D: Lymph heart musculature in birds. *J Morphol* 191: 77-87, 1987.

Clark EL: Injection and reconstruction of the jugular lymph ”sac” in the chick. *Anat Rec* 6: 261-264, 1912.

Clark EL, Clark ER: On the early contractions of the posterior lymph hearts in chick embryos - their relation to the body movements. *Anat Rec* 8: 80-81, 1914.

Clark ER, Clark EL: On the origin and early development of the lymphatic system of the chick. *Contr Embryol* 9: 447-482, 1920.

Huntington GS: The genetic interpretation of the development of the mammalian lymphatic system. *Anat Rec* 2: 19-46, 1908.

Huntington GS, Mac Clure CFW: The anatomy and development of the jugular lymph sacs in the domestic cat (Felis domestica). *Anat Rec* 2: 1-18, 1908.

Jackson DG, Prevo R, Clasper S, Banerji S: LYVE-1, the lymphatic system and tumor lymphangiogenesis. *Trends Immunol* 22: 317-321, 2001.

Kampmeier OF: The value of the injection method in the study of lymphatic development. *Anat Rec* 6: 223-233, 1912.

Karkkainen MJ, Haiko P, Sainio K, Partanen J, Taipale J, Petrova TV, Jeltsch M, Jackson DG, Talikka M, Rauvala H, Betsholtz C, Alitalo K: Vascular endothelial growth factor C is required for sprouting of the first lymphatic vessels from embryonic veins. *Nat Immunol* 5: 74-80, 2004.

Maruyama K, Ii M, Cursiefen C, Jackson DG, Keino H, Tomita M, Van Rooijen N, Takenaka H, D'Amore PA, Stein-Streilein J, Losordo DW, Streilein JW: Inflammation-induced lymphangiogenesis in the cornea arises from CD11b-positive macrophages. *J Clin Invest* 115: 2363-2372, 2005.

Ny A, Koch M, Schneider M, Neven E, Tong RT, Maity S, Fischer C, Plaisance S, Lambrechts D, Heligon C,

Terclavers S, Ciesiolka M, Kalin R, Man WY, Senn I, Wyns S, Lupu F, Brandli A, Vleminckx K, Collen D, Dewerchin M, Conway EM, Moons L, Jain RK, Carmeliet P: A genetic Xenopus laevis tadpole model to study lymphangiogenesis. *Nat Med* 11: 998-1004, 2005.

Papoutsi M, Tomarev SI, Eichmann A, Pröls F, Christ B, Wilting J: Endogenous origin of the lymphatics in the avian chorioallantoic membrane. *Dev Dyn* 222: 238-251, 2001.

Pardanaud L, Altmann C, Kitos P, Dieterlen-Lièvre F: Vasculogenesis in the early quail blastodisc as studied with a monoclonal antibody recognizing endothelial cells. *Development* 100: 339-349, 1987.

Rodriguez-Niedenführ M, Papoutsi M, Christ B, Nicolaides KH, von Kaisenberg CS, Tomarev SI, Wilting J: Prox1 is a marker of ectodermal placodes, endodermal compartments, lymphatic endothelium and lymphangioblasts. *Anat Embryol* 204: 399-406, 2001.

Sabin FR: The lymphatic system in human embryos, with a consideration of the system as a whole. *Am J Anat* 9: 43-91, 1909.

Van der Putte SCJ: The development of the lymphatic system in man. *Adv Anat Embryol Cell Biol* 51: 1-60, 1975.

Wigle JT, Oliver G: Prox1 function is required for the development of the murine lymphatic system. *Cell* 98: 769-778, 1999.

Wigle JT, Harvey N, Detmar M, Lagutina I, Grosveld G, Gunn MD, Jackson DG, Oliver G: An essential role for Prox1 in the induction of the lymphatic endothelial cell phenotype. *EMBO J* 21: 1505-13, 2002.

Wilting J, Brand-Saberi B, Huang R, Zhi Q, Köntges G, Ordahl CP, Christ B: Angiogenic potential of the avian somite. *Dev Dyn* 202: 165-171, 1995.

Wilting J, Papoutsi M, Schneider M, Christ B: The lymphatic endothelium of the avian wing is of somitic origin. *Dev Dyn* 217: 271-278, 2000.

Wilting J, Papoutsi M, Othman-Hassan K, Rodriguez-Niedenführ M, Pröls F, Tomarev SI, Eichmann A: Development of the avian lymphatic system. *Microsc Res Tech* 55: 81-91, 2001.

Wilting J, Papoutsi M, Christ B, Nicolaides KH, von Kaisenberg CS, Borges J, Stark GB, Alitalo K, Tomarev SI, Niemeyer C, Rössler J: The transcription factor Prox1 is a marker for lymphatic endothelial cells in normal and diseased tissues. *FASEB J* 16: 1271-1273, 2002.