Mechanisms of Angiogenesis

Edited by M. Clauss and G. Breier

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Editors:
Matthias Clauss
Department of Cellular and Integrative Physiology
And Indiana Center for Vascular Biology and Medicine
975 Walnut Street IB 433
Indianapolis, IN 46202
USA

Georg Breier
University Clinic Carl Gustav Carus
TU Dresden, Institute of Pathology
Fetscherstr. 74
01307 Dresden
Germany

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Contents

List of contributors

Takayuki Asahara, Department of Regenerative Medicine, Institute of Biomedical Research and Innovation, 2-2 Minatojima-minamimachi Chuo-ku, Kobe 650-0047, Japan; e-mail: asa777@aol.com

Hellmut G. Augustin, Department of Vascular Biology and Angiogenesis Research, Tumor Biology Center, Breisacher Str. 117, 79106 Freiburg, Germany; e-mail: augustin@angiogenese.de

Christer Betsholtz, Laboratory of Vascular Biology, Division of Matrix Biology, House A3, Plan 4, Department of Medical Biochemistry and Biophysics, Scheeles vag 2, Karolinska Institutet, SE-17177 Stockholm, Sweden; e-mail: christer.betsholtz@mbb.ki.se

Christopher Daly, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA; e-mail: Christopher.Daly@Regeneron.com

Thomas Daly, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA; e-mail: Thomas.Daly@Regeneron.com

Robert M.W. de Waal, Department of Pathology 437, University Medical Centre Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands; e-mail: r.dewaal@pathol.umcn.nl

Valentin Djonov, Institute of Anatomy, University of Berne, Buehlstrasse 26, 3000 Berne 9, Switzerland; e-mail: valentin.djonov@ana.unibe.ch

Borja Fernández, Department of Animal Biology, Faculty of Science, University of Málaga, Campus de Teatinos s/n, 29071 Málaga, Spain; e-mail: borjafe@uma.es

Napoleone Ferrara, Department of Molecular Oncology, Genentech, Inc., 1 DNA Way, South San Francisco, CA 94080, USA; e-mail: nf@gene.com

Holger Gerhardt, Vascular Biology Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC 2A 3PX, UK; e-mail: holger.gerhardt @cancer.org.uk

Matthias Heil, Max-Planck-Institute for Physiological and Clinical Research, Department of Experimental Cardiology, Benekestrasse 2, 61231 Bad Nauheim, Germany; e-mail: m.heil@kerckhoff.mpg.de

Klaas Hoekman, Department of Clinical Oncology, VU University Medical Center, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands; e-mail: k.hoekman@vumc.ne

Ella Ioffe, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA; e-mail: Ella.loffe@Regeneron.com

Pieter Koolwijk, Department of Biomedical Research, Gaubius Laboratory TNO-PG, Leiden, The Netherlands; e-mail: p.koolwijk@pg.tno.nl

William P.J. Leenders, Department of Pathology 437, University Medical Centre Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
Bernard I. Lévy, INSERM U541, Hôpital Lariboisière, 41 bvd de la chapelle, 75475 Paris cedex 10, France; e-mail: bernard.levy@larib.inserm.fr
Per Lindblom, Vascular Development Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC 2A 3PX, UK; e-mail: per.lindblom @cancer.org.uk
Andrew N. Makanya, Department of Veterinary Anatomy, University of Nairobi, Riverside Drive, P.O. Box 30197, Nairobi, Kenya; e-mail: …
Keith L. March, Indiana Center for Vascular Biology and Medicine and Krannert Institute of Cardiology, 975 W. Walnut Street, IB 441, Indianapolis, IN 46202, USA; e-mail: kmarch@iupui.edu
Hugo H. Marti, University of Heidelberg, Institute of Physiology and Pathophysiology, Im Neuenheimer Feld 326, 69120 Heidelberg, Germany; e-mail: hugo.marti@pio1.uni-heidelberg.de
Kathy D. Miller, Indiana Cancer Pavilion, 535 Barnhill Drive RT-473, Indianapolis, IN 46202, USA; e-mail: kathmill@iupui.edu
Leni Moldovan, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, 473 W 12th Ave., Room 305A, Columbus, OH 43210, USA; e-mail: moldovan-2@medctr.osu.edu
Nicanor I. Moldovan, Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, 473 W 12th Ave., Room 305A, Columbus, OH 43210, USA; e-mail: moldovan-1@medctr.osu.edu
Hiromi Nishimura, Department of Regenerative Medicine, Institute of Biomedical Research and Innovation, 2-2 Minatojima-minamimachi Chuo-ku, Kobe 650-0047, Japan; e-mail: nsmr323@koto.kpu-m.ac.jp
Nicholas Papadopoulos, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA; e-mail: Nicholas.Papadopoulos@ Regeneron.com
Jalees Rehman, Indiana Center for Vascular Biology and Medicine, 975 W. Walnut Street, IB 441, Indianapolis, IN 46202, USA; e-mail: jrehman@iupui.edu
John Rudge, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA; e-mail: John.Rudge@Regeneron.com
Wolfgang Schaper, Max-Planck-Institute for Physiological and Clinical Research, Department of Experimental Cardiology, Benekestrasse 2, 61231 Bad Nauheim, Germany; e-mail: …
Jean-Sébastien Silvestre, INSERM U541, Hôpital Lariboisière, 41 bvd de la chapelle, 75475 Paris cedex 10, France; e-mail: jean-sebastien.silvestre @larib.inserm.fr
Jürgen R. Sindermann, Department of Cardiology and Angiology, and Institute for Arteriosclerosis Research, University of Münster, Albert-Schweitzer-Strasse 33, 48149 Münster, Germany; e-mail: sinderm@uni-muenster.de
George W. Sledge, Indiana Cancer Pavilion, 535 Barnhill Drive RT-473, Indianapolis, IN 46202, USA; e-mail: gsledge@iupui.edu

Christopher J. Sweeney, Indiana Cancer Pavilion, 535 Barnhill Drive RT-473, Indianapolis, IN 46202, USA; e-mail: chsweene@iupui.edu
Gavin Thurston, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA; e-mail: gavin.thurston@regeneron.com
Victor W.M. van Hinsbergh, Laboratory for Physiology, Institute for Cardiovascular Research, VU University Medical Center, Van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands; e-mail: v.vanhinsbergh@vumc.nl
Srilatha Vuthoori, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA; e-mail: Srilatha.Vuthoori@Regeneron.com
Stanley Wiegand, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA; e-mail: Stanley.Wiegand@Regeneron.com
George D. Yancopoulos, Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA; e-mail: George.Yancopoulos @Regeneron.com
Ian Zachary, Department of Medicine, University College London, 5 University Street, London WC1E 6JJ, UK; e-mail: I.Zachary@ucl.ac.uk

Preface

Is it advisable to go back from bedside to the bench?

During the last decade, few topics encountered such a broad interest in biology and medicine as angiogenesis. The amazing ability of the body to restore blood flow by induction of blood vessel growth as part of an adaptive process has alarmed physicians dealing with diseases in which angiogenesis is either exaggerated (as in tumors) or too slow (as in ischemic diseases of heart and brain). Not surprisingly, pro- and antiangiogenic strategies have found their way into clinical trials. For instance, for the USA, the NIH website in early 2004 displayed 38 clinical studies involving either pro- or antiangiogenic therapies. Given the expected overwhelming wealth of clinical data, the question may be asked whether further exploration of biological mechanisms is required or whether results from the bedside are instructive enough to proceed. This question depends also on the progress of pro- and antiangiogenic clinical trials. In the following, I give a short overview about some of the progress that has been made in this field.

Since Judah Folkman proposed antiangiogenic tumor therapy thirty years ago, it has become increasingly evident that agents which interfere with blood vessel formation also block tumor progression. Accordingly, antiangiogenic therapy has gained much attention as a potential adjunct to conventional cancer therapy. The polypeptides angiostatin and endostatin are the most prominent natural angiogenesis inhibitors and are in current clinical trials as well as many low molecular angiogenic inhibitors, including fumagillin (TNP-470, AGM1470), 2-methoxyestradiol or thalidomide. The exact antiangiogenic mechanism of these compounds, leading to a stop in tumor growth, are not entirely understood but most of them appear to act directly on the endothelium. Another group of antiangiogenic agents acts more specifically by inhibition of the key angiogenic receptor VEGF-receptor-2, for example PTK787A/ZK22254 and SU5416 [1]. From the clinical outcome (as of early 2004), the most advanced study uses a humanized a monoclonal antibody to vascular endothelial growth factor (VEGF) named bevacizumab. Phase II trial had investigated the safety and efficacy of two doses of bevacizumab, plus fluorouracil (FU)/leucovorin (LV) *versus* FU/LV alone in patients with metastatic colorectal cancer and using a different combination protocol in non-small cell lung cancer patients (NSCLC) [2–4]. Finally, a phase I trial has demonstrated direct evidence that the VEGF-specific antibody bevacizumab applied alone has antivascular effects in human rectal cancer [5].

The successful use of a defined specific tool (antibody) against an essential inducer of angiogenesis is encouraging. The background of this will be elucidated in several contributions of this book, including a chapter written by *Napoleone Ferrara*.

Despite the overall euphoria based on previous animal experiments and the first results from phase I and phase II studies, a cautious note should also be expressed. So far no antiangiogenic phase III trials have yet demonstrated increases in overall survival. Given the profound differences between man and mouse, species specificities of the tumor vasculature should be more addressed in the future. In addition, an improved understanding of the complex process of angiogenesis could be helpful to design more advanced therapies. A thorough reconsideration of our current knowledge on factors and principles involved in angiogenic processes may be helpful to avoid antiangiogenic therapy experiencing the same fate as tumor necrosis factor (TNF) based therapy during the last decades. TNF was first assumed to be directly tumor necrosis inducing before its dependence on EMAP II and its mode of action as a vascular affecting agent was discovered [6–8]. It is not surprising that the first clinical studies based on the direct tumor cell toxicity were not successful, however, later and improved approaches in light of the newer biological data were hampered by the earlier "bad press" of TNF. Therefore, exploration of mechanisms of angiogenesis, which is the subject of this book, is an appropriate step forward to improved clinical studies with antiangiogenic strategies.

One novel finding regarding the role of angiogenesis in malignancies is that endothelial progenitor cells (EPC) can home into the site of the tumor vasculature [9, 10]. This can account for angiogenic activities *in vivo* which cannot be explained by a direct action of angiogenic factors on endothelial cells. On the other hand, however, these endothelial precursor cells can also be employed for targeting tumors by injection of genetically modified EPC. EPC expressing cytotoxic suicide genes or prodrug-activating enzymes are expected to incorporate preferably into the angiogenic vessel wall within tumors. Background on endothelial precursor progenitor cells can be found in particular in the chapter by *Hiromi Nishimura and Takayuki Asahara*. Finally, an increasing number of anticancer agents turned out to act equally well or even preferably through the vasculature in comparison to their direct effects on cancer cells [11, 12]. This finding may be explained by the hyperproliferative condition of angiogenic vessels, which makes them more vulnerable in comparison to quiescent non-tumor endothelium. In consequence, some clinical trials currently employ chronic low-dose chemotherapy (so-called "metronomic therapy") in combination with antiangiogenic agents (for example vinblastine and celecoxib for treatment of Ewing's sarcoma). This is discussed more deeply in the article by *Kathy D. Miller, Christopher J. Sweeney and George W. Sledge*.

This book is subdivided into three main chapters: the first chapter elucidates principles of angiogenesis in physiological as well as pathological conditions. It aims to gather what is currently known on mechanisms leading to sprouting (*Holger Gerhardt and Christer Betsholtz*), intussusceptive growth of capillar-

ies (*Valentin Djonov and Andrew N. Makanya*), and to the formation of collateral vessels from preformed arterioles (*Borja Fernandez*). This is being reviewed with focus to the reproductive system (*Helmut Augustin*) and the growing heart (*Borja Fernandez*). Further clinically interesting topics are presented by three final contributions: *Robert M.W. de Waal and William P.J. Leenders* and *Kathy D. Miller, Christopher J. Sweeney and George W. Sledge* address several implications of tumor angiogenesis. Finally, *Jean-Sebastien Silvestre and Bernard Levy* shed some light on the role of pathological cofactors, especially angiotensin II, on angiogenesis.

In the second chapter the focus is on cellular and physical mechanisms leading to angiogenesis. In particular it addresses the concept that angiogenesis is a complex event involving the interactions of different cell types such as pericytes (*Christer Betsholtz, Per Lindblom and Holger Gerhardt*), monocytes (*Leni Moldovan and Nicanor I. Moldovan*) and endothelial progenitor cells (*Hiromi Nishimura and Takayuki Asahara*). The link to the influence of physical forces is being made by an article on the influence of exercise on angiogenesis, which interestingly also reveals a role of endothelial progenitor cells (*Jalees Rehman*). In general, major physical parameters include the regulatory role of either hypoxia (*Hugo Marti*) or increased shear stress (*Matthias Heil and Wolfgang Schaper*). Whereas hypoxia is considered to be the predominant principle driving sprouting angiogenesis, shear stress forces appear to be the most important mechanism leading to compensatory collateral blood vessel growth, which has been named arteriogenesis (*Matthias Heil and Wolfgang Schaper*). Shear stress forces may also be the reason for the surprising finding that the balance of luminal size is more the rule than the exception (*Jürgen R. Sindermann and Keith L. March*).

The third and last chapter deals with molecules involved in angiogenesis. *Napoleone Ferrara* summarizes what is known about the role of the essential angiogenesis factor VEGF in the regulation of physiological and pathological angiogenesis. This chapter also addresses the concept that angiogenesis is a complex event which involves the orchestral setting of several growth factors and is connected to inflammation and coagulation. In this context, contributions on the anti-inflammatory actions of angiopoietin-1 (*Gavin Thurston, John Rudge, Ella Ioffe, Nicholas Papadopoulos, Christopher Daly, Srilatha Vuthoori, Thomas Daly, Stanley Wiegand, and George D. Yancopoulos*) and the role of the hemostatic system in angiogenesis (*Victor W.M. van Hinsbergh, Pieter Koolwijk and Klaas Hoekman*) provide some further insight into the complexity of angiogenesis. Finally, the identification of signaling steps in angiogenesis as potential important molecules for therapeutic intervention is reviewed by *Ian Zachary*. Altogether, there is hope that this collection of insights into mechanisms of angiogenesis stimulates new innovative research and some day will ease the doctors' way to the bedside.

Matthias Clauss
Georg Breier August 2004

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Basic principles in physiological and pathophysiological angiogenesis

Mechanisms of Angiogenesis
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How do endothelial cells orientate?

Holger Gerhardt[1] and Christer Betsholtz[2]

[1] *Vascular Biology Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC 2A 3PX, UK*
[2] *Laboratory of Vascular Biology, Division of Matrix Biology, House A3, Plan 4, Department of Medical Biochemistry and Biophysics, Scheeles vag 2, Karolinska Institutet, 17177 Stockholm, Sweden*

Introduction

The vasculature of vertebrates displays an intriguing variety of vascular patterns. Every organ not only has its specific set of supplying arteries and draining veins, but also shows an extension, orientation and density of capillaries that is intimately adapted to the anatomical boundaries and functional requirements. For many organs, these requirements go beyond the supply of nutrients and gas exchange. The capillary beds of the lung, liver, spleen and kidney are much more dense than would be necessary for oxygenation and nutrient supply. Conversely, certain tissues such as the lens and cartilage remain avascular. Yet other organs fall between these extremes, but nevertheless display characteristic vascular patterns. Aberrant, retarded or overshooting vascularization may severely impair organ function and is often associated with disease. The molecular and cellular basis for vascular patterning therefore receives increasing attention. For developmental biologists, the phenomenon of vascular patterning raises a number of fundamental questions, applicable also to the formation of other tubular or pervasive organ systems.

Theoretically, organ-specific vascular patterns may result from guided sprouting. A newly formed sprout that extends into the surrounding tissue may follow tracks, gradients or guide posts that provide attractive or repulsive signals. Alternatively, an initially random sprouting and vascular plexus assembly may be followed by organ-specific vessel remodeling and pruning. As of today, evidence indicates the existence of both of these mechanisms. Clearly, flow- and oxygen-dependent pruning of vessel branches is a major mechanism in the formation of the capillary-free spaces around arteries and arterioles [1, 2]. The formation of the earliest vascular structures through induction, differentiation and assembly of endothelial progenitors (vasculogenesis) [3] may initially be a random process that subsequently becomes refined by selective branch regression and expansion. Sprouting angiogenesis in the CNS, however, is characterized by precision guidance [4] and angiogenesis in other organs may follow similar rules. It is tempting to speculate

that the classic distinction between vasculogenesis and angiogenesis separates two processes that also differ in terms of endothelial guidance. This idea becomes clear when considering the implications of tubular sprouting as it occurs in angiogenesis.

Implications of guided sprouting – the problem of tubular sprouting

Tubular sprouting from an existing vascular system that already bears internal pressure and supports directional blood flow brings a number of problems that are very different from those of the vasculogenic assembly of progenitor cells. Whereas vasculogenesis may, at least in theory, occur from a homogeneous population of endothelial cells [5] the induction of an angiogenic sprout requires non-uniformity with regard to the functions of neighboring endothelial cells. If all endothelial cells lining a pre-existing vessel would respond equally to a chemoattractant, the outcome would be vessel disintegration with potentially catastrophic consequences. Uniform proliferation would likewise be insufficient for angiogenic sprouting. Instead selected endothelial cells are needed to pioneer the sprouting process, while their neighbors should follow, stay connected and shape the lumen-containing stalk of the sprout. Similar demands for functional specialization on a cell-to-cell basis apply to sprout fusion – a process that is likely also guided. Although these considerations may appear simplistic and self-evident, they are important for angiogenesis research in that they help in switching the focus from the study of general angiogenic responses in homogeneous endothelial cell populations to specific responses in distinct subpopulations of endothelial cells.

Lessons from *Drosophila* tracheal development

The study of *Drosophila* tracheal development has recently provided insight into basic mechanisms of tubular sprouting. Instead of blood vessels insects have an intricate and highly branched air-filled tubular epithelial network, called trachea, which delivers oxygen throughout the body. Mechanisms of guidance, specific cellular responses and underlying signaling pathways have been elucidated for tracheal development [6]. Recent studies have revealed structural, mechanistic and molecular analogies between angiogenic and tracheal sprouting [4, 7, 8], raising hopes that the continued survey of insect tracheal development may aid to our understanding of angiogenesis.

The *Drosophila* trachea develops from clusters of mitotically inactive ectodermal cells, the tracheal placodes. The *Drosophila* fibroblast growth factor (FGF) protein Branchless (Bnl/FGF) guides the outgrowth of tracheal branches from the tracheal placodes. Bnl/FGF is expressed outside the placodes in patterns that precede the cell migration events involved in tracheal sprouting and branching, and exerts its effect by binding to the FGF receptor Breathless

(Btl/FGFR) present on the tracheal cells [9]. Bnl/FGF expression is regulated by hypoxia, presumably in order to link tracheal development with the physiological need for oxygen [10]. Thus, the regulation and role of Bnl/FGF in *Drosophila* tracheal formation is highly analogous to the regulation and role of vascular endothelial growth factor (VEGF-A) in vascular formation in vertebrates.

Each growing tracheal sprout or branch bears specialized cells at its tip, which extend multiple long filopodia towards the cells expressing Bnl/FGF. The filopodia extension and migration of the tip cell is a result of Bnl/FGF mediated activation of Btl/FGFR [11]. In normal *Drosophila* larvae, this response is confined to the tip cell, and is suppressed in the neighboring stalk cells through tip cell expression of Sprouty [12]. Failure to suppress activation of the stalk cell is incompatible with normal patterning and results in ectopic hyper-sprouting. Thus, functional specialization of subpopulations is required for guided tubular sprouting in trachea formation. The filopodia of the tracheal tip cells are highly dynamic structures that seem to probe the environment for molecular cues, and guide the tip cell along a gradient of Bnl/FGF [11]. At certain locations, the tip cells make contact with guide-post cells that guide branch fusion or tubular navigation within certain organs, like the CNS [13]. Fusion of branches also involves functional specialization of the tracheal cells. A series of hierarchical genes are expressed in special fusion cells that are induced under the control of Dpp and Notch signaling [14]. Again, the fusion cell, which represents a tip cell with the additional specification to fuse, is guided utilizing filopodia [13].

Non-uniformity: the tip cell – stalk cell paradigm

At least two important lessons have been learned from the studies of *Drosophila* tracheal development: 1) Tubular morphogenesis relies on functional specialization of individual cells, and 2) guidance of a tubular sprout is mediated through directed migration of tip cells that utilize filopodia to sense guidance cues. By analogy, endothelial cells situated at the tip of the growing angiogenic sprout extend multiple long filopodia, and the following stalk cells are largely devoid of such structures. This morphological feature was described already in 1963 (see [15] and citations therein), and detailed observations on silver-stained endothelium in the CNS lead Marin-Padilla to propose that filopodia-like protrusions are used by the angiogenic sprouts to sense guidance cues [15]. Studies of endothelial cell proliferation in angiogenic sprouting provided additional evidence for functional specialization among the cells within an angiogenic sprout [16]. For example, in a model of cornea angiogenesis, proliferation was observed exclusively in the stalk of the sprouts [17]. Early attempts to live image blood vessel growth in salamander tails indicated dynamic protrusive activity at the tip of the elongating vessels and also contributed the first observations of fusion of sprouts [18]. More recently,

endothelial tip cell filopodia were visualized in the CNS in chicken embryos [19], in mouse embryonic hindbrain [20] and in postnatal mouse retinas [4, 21]. Thus, accumulating evidence suggests that functional specialization occurs between tip- and stalk cells in the angiogenic sprout, similar to what has been described for the developing *Drosophila* trachea. When asking "how do endothelial cells orientate?" one may therefore wish to rephrase the question and ask "how do endothelial tip-cells orientate?"

Growth factor gradients

Secreted growth factors are potent regulators of cell proliferation, migration and survival. Directed cell migration is achieved through localized production and paracrine action of growth factors. The range of action of these factors depends on their ability to diffuse in the tissue, which in turn depends on the amounts secreted, the extracellular stability and the propensity of the factor to adhere to other molecules situated at the cell surface or in the extracellular matrix (ECM). Obviously, the restricted diffusion of the growth factors in the extracellular space leads to non-uniform distribution and the formation of local gradients. Although this is generally believed to occur for all growth factors acting in a paracrine fashion, direct measurements of protein gradients in tissues are very difficult, and there are only a few examples where a graded distribution of extracellular growth factors has been visualized [4, 20, 22]. Directed cell migration can occur either up or down a gradient, depending on whether the factor constitutes an attractive or repulsive cue [23]. *In vitro*, a large number of secreted factors, such as VEGF-A, aFGF, bFGF, Angiopoietin-1, epidermal growth factor (EGF), and interleukin-8 (IL-8), have been reported to be chemoacttractants for endothelial cells. Tissue gradients of VEGF-A have long been postulated, and such gradients were recently also demonstrated by direct means [20]. In the CNS of midgestational mouse embryos, VEGF-A expression is only detectable in cells in the ventricular zone of the neural tube. The secreted VEGF-A protein, however, distributes in a graded fashion from the ventricular zone (high concentration) towards the neuroectoderm (low concentration). The first angiogenic sprouts that invade the neuroectoderm branch off from the vascular plexus surrounding the neural tube and extend towards the high VEGF-A concentration in the ventricular zone.

The diffusion range of VEGF-A depends on alternative mRNA splicing, which creates protein isoforms with different C-terminal sequencs [24, 25]. Cell culture experiments have shown that the short isoform, VEGF120, is freely diffusible, whereas the longer isoforms, VEGF164 and VEGF188, remain bound in the vicinity of the producing cell. The long isoforms carry stretches of basic amino acid residues that provide affinity for heparin, and probably also for heparan sulphate proteoglycans present on the cell surface and in the ECM (reviewed in [26]). Data from genetically modified mice

expressing only single VEGF isoforms recently demonstrated that extracellular gradients created by the heparin-binding isoforms are important for normal vascular patterning in the developing brain [20]. Similar gradients were also found in the early postnatal retina, where sprouting angiogenesis leads to circular expansion of a primitive vascular plexus beginning at the optic disc and proceeding towards the periphery [4]. (See schematic illustration in Fig. 1a). At both locations, endothelial tip cells extend long filopodia towards regions of high VEGF production. In mice expressing VEGF120 only, the extracellular VEGF-A gradients were altered. VEGF protein distribution was more widespread lowering VEGF-A concentration close to the VEGF-A source and increasing VEGF-A concentration at a distance. The flattening of the VEGF-A gradient correlated with decreased filopodia extension from the tip-cells and impaired migration of these cells [4, 20]. In addition, the stalk cells, which experience higher VEGF-A concentrations, showed ectopic protrusion of filopodia. Other means of disrupting the endogenous extracellular VEGF-A gradient, such as by intra-ocular injection of VEGF164, or by transgenic overexpression of any of the major VEGF-A isoforms from the lens, also led to decreased migration at the tip and increased proliferation in the stalk, demonstrating that VEGF-A gradients, not isoform-specific signaling, regulate tip-cell migration, and vessel patterning [4] (Fig. 1a, b). VEGFR2 is highly expressed by endothelial tip cells and the VEGFR2 protein distributes in a punctuated pattern along the tip-cell filopodia. Inhibition of VEGFR2 but not VEGFR1 activity disrupted tip-cell filopodia and tip-cell migration. Furthermore, local injection of VEGFR2 specific agonist VEGF-E led to disruption of tip-cell filopodia and migration, mimicking the result of local VEGF-A injection [4]. Together, these results demonstrate that induction and maintenance of tip-cell filopodia, as well as directed tip-cell migration is dependent on VEGF-A gradients that are sensed by VEGFR2 [4].

Integrated dual function of growth factor gradients – balanced control of guided tip migration and stalk proliferation

Manipulations of the extracellular gradient of VEGF demonstrate that tip cell guidance and migration depends on the shape of the gradient. Interestingly, the

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Figure 1. (see next page) (a) Graphical representation of retinas with the graded distribution of VEGF-A in red*. C represents the retinal center (optic nerve and vessel entrance) from which vessels sprout towards the periphery (p). The blue* circle and its radius (arrow) represent the distance of vessel spreading at approximately postnatal day 5 in the mouse retina in the various experimental situations indicated to the right (referred to as migration). The graphs in the middle illustrate how the VEGF gradients change across the retinal radius (central to peripheral) in the experimental situations indicated. Proliferation implies the increase in cell number in the sprout stalks. (b) Schematic illustration of sprout morphology in the various experimental conditions. Black dots represent VEGF-A molecules and their distribution. The VEGF-secreting cells are shown ahead of the sprout and the distribution and orientation of the filopodia at the tip and in the stalk is shown. For original data see [4] and [20]. (* For colored picture see color plate 1)

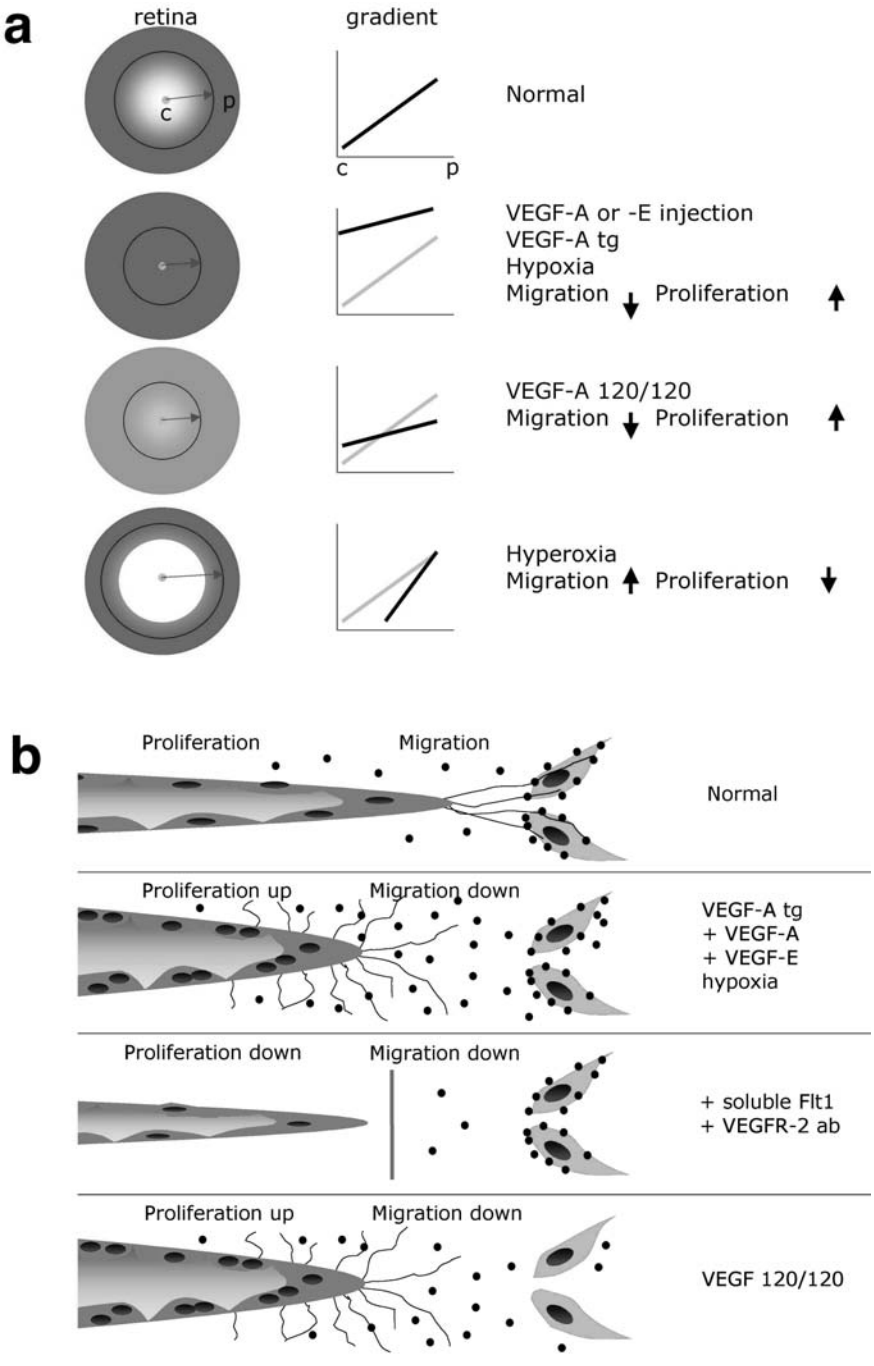

Figure 1. (Legend on previous page)

experimental manipulations led to a second observation of relevance for vessel patterning: Endothelial proliferation in the stalk correlates with the local levels of VEGF-A [4]. For example, while injection of VEGF-A into the vitreous inhibited filopodia formation and guided tip-cell migration in the retina by disrupting the endogenous VEGF-A gradient, the increased VEGF-A concentration stimulated stalk-cell proliferation (Fig. 1a, b). Similar results were observed in the VEGF120-only retinas. Here, the overall VEGF-A levels are unchanged but the increased diffusion leads to slower tip cell migration due to flattening of the VEGF-A gradient, but enhanced stalk cell proliferation due to increased VEGF-A concentration at the level of the stalks [4, 20]. Interestingly, the proliferative response is also dependent on VEGFR2 receptor activation, as specific VEGFR2 neutralizing antibodies inhibited both tip-cell migration and stalk-cell proliferation [4]. Furthermore, VEGF-E, which is a selective agonist for VEGFR2, stimulated stalk-cell proliferation, whereas the selective agonist for VEGFR1, placenta growth factor (PlGF), had little or no effect. Concomitantly decreased tip-cell migration and increased stalk-cell proliferation results in more endothelial cells per vessel length and increased vessel caliber. Conversely, situations with reduced proliferation but accelerated migration resulted in a sparse network with extremely thin vessels. VEGF-A gradients therefore appear to be necessary and sufficient to regulate vascular patterning – at least in the developing mouse CNS. Results from CNS-specific VEGF-A conditional knock-out in mice, accomplished by Nestin-Cre mediated recombination of a single loxP-flanked VEGF-A allele, support the role of VEGF-A in patterning in the CNS [27]. Here, reduction of the VEGF-A levels without affecting the isoform distribution led to reduced vessel density, however, without effects on vessel diameter or tip-cell migration. A lower VEGF level with intact relative isoform abundance does not change the steepness of the gradient, and hence does not affect the balance between migration and proliferation. Although additional studies will be needed to address whether this concept holds true for developmental vascular patterning throughout the vertebrate organism, it is already clear that altered VEGF-A gradients, as occurs in the VEGF120-only mice, affect vascular patterning in the same way in the limb, gut and inter-somitic regions, as in the CNS [20].

Studies of *Drosophila* tracheal air sac development indicate that integrated dual functions of growth factor gradients may provide a general mechanism in tubular sprouting. During air sac development, migrating tracheoblasts extend long Btl/FGFR-positive filopodia in the direction of migration and towards the source of Bnl/FGF [28]. Induced expression of dominant-negative Btl/FGFR caused filopodia retraction within 3–5 hr, which is comparable to effects of soluble Flt and VEGFR2 antibody injection experiments in the eye [4], suggesting similar dynamics of tracheal and endothelial tip-cell filopodia. Air sac tracheoblasts also respond to Bnl/FGF by either migration or proliferation. Uniform tracheal expression of a dominant active Btl/FGFR led to increased proliferation, but also led to widespread protrusion of short and studded filopodia, and to inhibition of migration. Hence, spatially restricted Btl/FGFR acti-

vation is most likely required for proper filopodial extension and migration of the tracheoblasts. Likewise, widespread endothelial VEGFR2 activation by direct VEGF-E injections into the eye led to increased proliferation and widespread protrusion of short filopodia, however the tip cell filopodial extensions were shortened and tip cell migration was inhibited. Thus, the balance between tip cell migration and stalk cell proliferation in air sac development, and the control of this balance by spatially regulated Bnl/FGF-Btl/FGFR interactions, is highly analogous to the balance between tip cell migration and stalk cell proliferation in retinal angiogenic sprouts controlled by spatially regulated VEGF-A/VEGFR2 interactions (Fig. 1a, b).

How is receptor activation translated into cell orientation?

It is intriguing that the tip cell, which senses the highest concentrations of VEGF-A and appears to express the highest levels of VEGFR2, responds by migration only. Apparently, VEGFR2 activation leads to a fundamentally different functional response in the tip cell compared to the stalk cells. How does VEGFR2 activation translate into directional tip cell movement? As of today, this question is unresolved. Studies on other systems in which cells orientate along growth factor gradients have addressed similar questions, and come up with interesting concepts. In growing axons, a specific tip-structure, the growth cone, has a central role in guiding the neurite extension towards the appropriate target [29–32]. The growth cones extend multiple dynamic filopodia that sense both attractive and repulsive cues presented by the environment in the form of diffusible or bound factors [33–35]. Theoretical modeling of how growth cones detect and read gradients suggests that they use a spatial rather than temporal sensing strategy [36]. Receptors present on the filopodia clearly have a role in gradient sensing in that they increase the area that can be probed by the growth cone. Interestingly, the dynamics and number of filopodia are increased and their length decreased in shallow gradients. This appears comparable to the observations on endothelial filopodia in situations of shallow VEGF gradients, such as in the VEGF120-only mouse.

Studies on the relation between filopodia dynamics, signal transduction and actin cytoskeleton reorganization during attractive or repulsive turning of the growth cone provides insight into the mechanisms of growth cone steering. Powerful axonal attractants like neurotrophins stimulate filopodia extension from the growth cone [37]. Neurotrophins activate trk receptor tyrosine kinases. Tyrosine phosphorylation can be detected at the tips of growth cone filopodia [38] and the dynamic behavior of the filopodia depends on the phosphorylation state of the growth cone [39]. Stimulation with the neurotrophin nerve growth factor (NGF) leads to rapid accumulation of β1-integrins at the tips of filopodia through an actin-myosin dependent transport mechanism [40]. Interaction with the proper ECM-ligands leads to clustering of the integrins and their coupling to the retrograde flow of actin [41]. The resulting retrograde

transport of clustered ligand-bound integrins on the filopodia is thought to create the traction force by which the growth cone moves forward on the matrix. Together, these observations provide a model for how growth factor gradients and the ECM cooperate in guiding axon extensions. Although our insight into endothelial tip cell function is far less detailed, it is tempting to speculate on analogous mechanisms. Endothelial tip cells, like axonal growth cones, extend filopodia in the direction of their migration [4]. VEGFR2 activation promotes filopodia extension from tip cells, similar to the neurotrophin/trk-induced extension of filopodia from growth cones. In the retina we find β1-integrins at the tips of endothelial filopodia and an appropriate ligand, fibronectin, on the network of astrocytes on which the tip-cells migrate. Based on these observations we speculate that VEGF-A gradients and VEGFR2 receptor activation are necessary for directional extension of endothelial tip cell filopodia, while the actual force leading to tip-cell migration depends on β1-integrin-fibronectin interactions between the filopodia and the ECM.

Attraction and repulsion in cell orientation

Other concepts that are well established and central to the understanding of axonal guidance are only beginning to surface in angiogenesis research. For example, guidance of axonal growth cones is regulated by both attractive and repulsive guidance cues. However, we currently lack firm evidence for actively repulsive cues in guidance of the endothelial tip-cells. In this context, it is noteworthy that the cell adhesion receptor Neuropilin-1 (Nrp-1), which is expressed both on neurons and endothelium, is involved in axon repulsion (reviewed in [42]), but is described to promote signaling through the VEGFR2 involved in attractive behavior in the endothelium ([43]; reviewed in [44]). Although the defective vascularization observed in many organs of mouse embryos deficient in Nrp-1 [45–47] would be compatible with the idea of Nrp-1 function in endothelial tip cell guidance, Nrp-1 expression in endothelial tip cells remains to be shown. Other repulsive cues first known from their function in axonal guidance, the Ephs and Ephrins, or Robo and Slit, are also expressed and functional in vascular patterning [48–50], however, their role in angiogenic guidance and vessel patterning is still largely unclear.

Does pathological angiogenesis involve endothelial misguidance?

As we begin to appreciate that developmental angiogenesis involves precision guidance and coordinated migration- and proliferation-control of the endothelial cells, we also see that angiogenesis in pathological situations, such as in tumor development, often results in highly aberrant vascular networks with little resemblance to any normal vascular beds. One may therefore ask if endothelial cells utilize different mechanisms to orientate in developmental and patho-

logical angiogenesis. Clearly, the outcome is very different: Tumor vessels are irregular and tortuous, lack hierarchical branching, display frequent arteriovenous shunts, show chaotic flow, stasis, increased leakage and hemorrhage. For any normal tissue, the benefits of such a vasculature would be limited by the tissue damage resulting from vascular leakage and hemorrhage. This is especially true for nervous tissues, the function of which relies on a tightly controlled extracellular milieu. In fact, in diabetic retinopathy, retinal vein occlusion and age-dependent macula degeneration, neoangiogenesis is held responsible for the loss of vision during the progression of these diseases.

Similar to developmental angiogenesis, VEGF-A is a key regulator of pathological angiogenesis, including tumor angiogenesis and ischemia-induced neoangiogenesis [26]. If we adopt the concepts of VEGF-A-mediated endothelial guidance to pathological angiogenesis, the observed defects may argue for abnormal VEGF gradients. Since VEGF-A mRNA levels and protein levels are under the control of tissue hypoxia, both a growing tumor cell mass and focal tissue ischemia should be able to result in graded VEGF-A expression patterns similar to those seen during normal development. Considering the importance of heparin-binding moieties of the VEGF-A isoforms for developmental vascular patterning [4, 20, 51, 52], possible explanations for the vascular abnormalities might include a VEGF-A isoform switch, or changes in the ECM composition. VEGF-A isoform switches have been described in some pathological situations [53, 54] but not in others [55, 56]. Tumors would be expected to have different ECM composition than normal tissues, but the extensive variation between different types of tumors might argue against ECM composition as a general mechanism for VEGF-A gradient disruption in tumors. Among other possibilities increased protease activity should be considered. Many proteases have been implicated in pathological angiogenesis. For example, the production and activation of the matrix-metalloprotease 9 (MMP-9) appears to be able to promote angiogenesis by liberating VEGF from the matrix [56]. An inactive complex between VEGF-165 and connective tissue growth factor complex may be subject to proteolytic cleavage by a number of proteases, resulting in release of active VEGF [57]. The effects of inhibitors of matrix-metalloproteases and the results of gene knockout MMP-9 and MMP-2 are indeed suggestive of an important role for proteases in pathological angiogenesis. Since many of the molecular players in angiogenesis, growth factors, matrix and proteases, are changed in tumors, the reason why tumor vessels are abnormal may well turn out to be very complex. Clearly further studies are required to test the idea that disrupted VEGF-A gradients and defective endothelial tip cell guidance contributes to the abnormalities of the angiogenic response to pathological situations.

Summary

In sprouting angiogenesis, endothelial cells must orientate in the tissue environment in order to effectively invade tissues and form vascular patterns

according to the local needs. Here, we review recent data indicating that sprouting angiogenesis is a guided process resembling axonal guidance and insect trachea formation. Angiogenesis requires functional specialization of endothelial cells within the sprout. Cells situated at the tip of the sprouts sense and navigate the environment using long filopodia, whereas cells in the sprout stalks proliferate and form a vascular lumen. Migration of the tip cells depends on a graded distribution of VEGF-A and activation of VEGFR2 located on the tip-cell filopodia. Proliferation in the stalk is concomitantly regulated by the local VEGF-A levels. Thus, the shape of the VEGF-A gradient controls the balance between tip cell migration and stalk cell proliferation, which in turn determines the initial vascular pattern. An imbalance between the two processes may explain why abnormal vascular patterns develop in pathological angiogenesis.

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Mechanisms of Angiogenesis
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New insights into intussusceptive angiogenesis

Valentin Djonov[1] and Andrew N. Makanya[1, 2]

[1] *Institute of Anatomy, University of Berne, Buehlstrasse 26, CH-3000 Berne 9, Switzerland*
[2] *Department of Veterinary Anatomy, University of Nairobi, Riverside Drive, P. O. Box 30197, Nairobi, Kenya*

Angiogenesis is defined as the growth and development of new capillary blood vessels from pre-existing vasculature [1]. It is requisite in metazoans because the transfer of nutrients and wastes has to be accomplished by diffusion through the tissue and hence, the respiring cells need to be within 100–200 μm of the blood vessels, which is the diffusion limit for oxygen [2]. Angiogenesis is a normal process fundamental in wound healing, reproduction and development. Abnormal angiogenic activity occurs in non-neoplastic diseases such as arthritis, psoriasis, trachoma, and diabetic retinopathy and in the vascularisation of tumours (for details, see [3–8]).

Angiogenesis has two facets: sprouting angiogenesis and intussusceptive angiogenesis. This chapter concentrates on the basic mechanisms, facets and outcomes of intussusceptive angiogenesis.

Concept and definition of intussusceptive angiogenesis

Intussusception is defined in the Merriam-Webster dictionary as INVAGINATION, as may be manifested by the slipping of a length of intestine into an adjacent portion of the same, or the assimilation of new material and its dispersal among pre-existent matter. In 1986, Caduff and co-workers demonstrated tiny holes in the developing lung vasculature and postulated that these were due to intussusceptive (in-itself) microvascular growth a fact proved later by Burri and Tarek [9]. Intussusceptive angiogenesis refers to that process by which new blood vessels grow and develop from pre-existing vasculature through insertion of tissue pillars into the capillary lumina and expansion of the latter to form new capillary networks.

Mechanisms of intussusceptive angiogenesis

The quintessence of non-sprouting angiogenesis by intussusception is formation of a tissue pillar into the vascular lumen of a blood vessel. This phenom-

enon was first observed in the rapidly expanding pulmonary capillary bed of neonatal rats [10]. This was manifested as numerous tiny holes (1–2 μm in diameter) in vascular corrosion casts, which were shown to correspond to slender transcapillary (intraluminal) tissue pillars [9, 10]. Serial sectioning of tissue followed by transmission electron microscopy revealed that the pillars arose by invagination of the capillary wall into the vessel lumen [11].

Stages in pillar formation

Four consecutive steps in pillar formation have been described. During stage I, a zone of contact is established between opposite capillary walls. In stage II, there is reorganization of the inter-endothelial cell junctions and central perforation of the bilayer. In stage III, an interstitial pillar core is formed which is subsequently invaded by pericytes and myofibroblasts that then lay down collagen fibrils. By this stage, transluminal pillars have a diameter of ≈2.5 μm. During the fourth and final stage the pillars increase in girth without undergoing any further change in their basic structure. In addition to developing lung, intussusception has been demonstrated in the chick chorioallantoic membrane (CAM) by Patan et al. [12, 13] and has since been revealed to occur in many organs and species during both normal and pathological microvascular growth. Hence, it appears to be a general phenomenon as seen in the reports of Patan et al. [14–16], Djonov et al. [11, 17–19], Burri and Djonov [20] and Kurz et al. [21]. The concept of intussusception is schematically represented in Figure 1, while Figure 2 illustrates the various stages of intussusceptive microvascular growth (IMG) in the avian kidney.

Nascent reports indicate that non-sprouting angiogenesis is ubiquitous, occurring in tissues such as myocardium [22], skeletal muscle [23] and kidney [19]. The modes of vascular growth described have sometimes been referred to in different names, viz., “longitudinal splitting” [22] and “luminal division” [23], respectively, but both resemble intussusception during the initial stages of inception. In muscular tissue, transluminal pillars appear more elongated than in lung due to the longitudinal and parallel arrangement of the myofibres. The complex spatial structure of transluminal pillars and the inadequacy of older visualization techniques meant that the intussusceptive process eluded many contemporary investigators for a considerable duration of time. Vascular corrosion casting (Fig. 2) and serial sectioning for light or transmission electron microscopy followed by three-dimensional reconstruction (Fig. 3) [9, 11, 17–19], or confocal laser microscopy, are the definite techniques for unequivocal identification of pillars. Cognate methods for three-dimensional imaging, such as nuclear magnetic resonance, micro-computer tomography, angiography and ultrasonics do not have the resolution necessary (at least 1 μm) for the visualization of pillars. This circumstance may explain why intussusception was overlooked in the past.

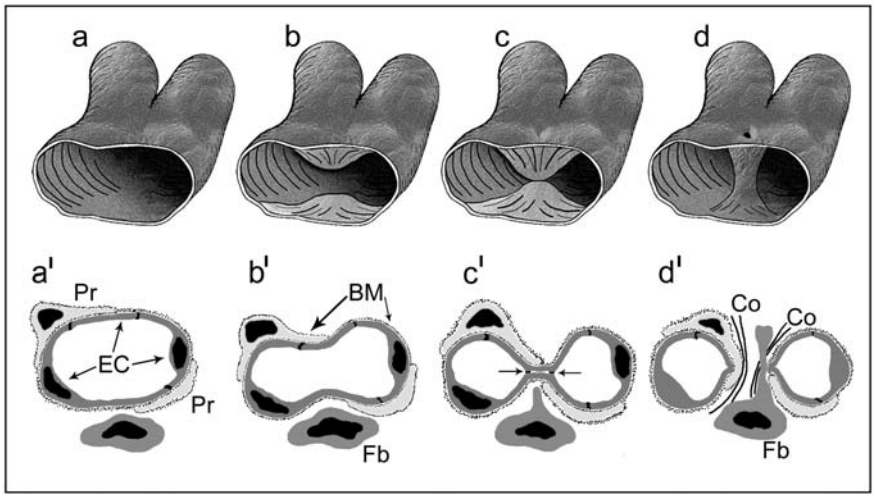

Figure 1. (a–d) Three-dimensional schematic illustrating the steps in the generation of new vascular segments by intussusceptive growth. The process begins with the protrusion of portions of the walls from opposite sides into the vessel lumen (a, b). After contact has been established and fortified (c), the endothelial bilayer becomes perforated centrally and a transluminal pillar is formed (d). (a'–d') Two-dimensional representation of the events depicted in a–d. Endothelial cells (EC) situated on opposite sides of a capillary protrude into its lumen until they contact each other (a'–c'). Once established, this contact is fortified by the formation of interendothelial junctions and then reorganized in such a manner that the endothelial bilayer is perforated centrally. The endothelial cells then retract, and the newly formed pillar increases in girth after being invaded by fibroblasts (Fb) and pericytes (Pr), which lay down collagen fibrils (Co in d'). After [30]. (For colored picture see color plate 2)

Dynamics of pillar formation

A remarkable characteristic of intussusceptive angiogenesis is that it is achieved at a relatively low rate of endothelial cell proliferation. In CAMs, this rate drops dramatically between days 10 and 11 of incubation, coinciding with the peak of intussusceptive pillar formation [11, 17, 24–26]. In the lung vasculature, the capillary volume and surface area were seen to increase 35-fold and 20-fold, respectively [27, 28], in the virtual absence of a change in endothelial cell number [29]. Comparative studies of various organs before and after the onset of intussusception have revealed the total endothelial cell volume to be redistributed during pillar formation by a thinning and spreading of the pre-existing cell population [30]. Endothelial cell attenuation during CAM growth was first documented as a serendipitous finding by Ausprunk et al. [24]. Subsequent morphometric analysis of chick CAMs revealed the thickness of endothelial cells to be reduced by more than 50% between days 10 and 14 of incubation [31].

Direct and definitive evidence for the existence of intussusceptive vascular growth has now been obtained [12] by using chick CAMs [32]. This is an

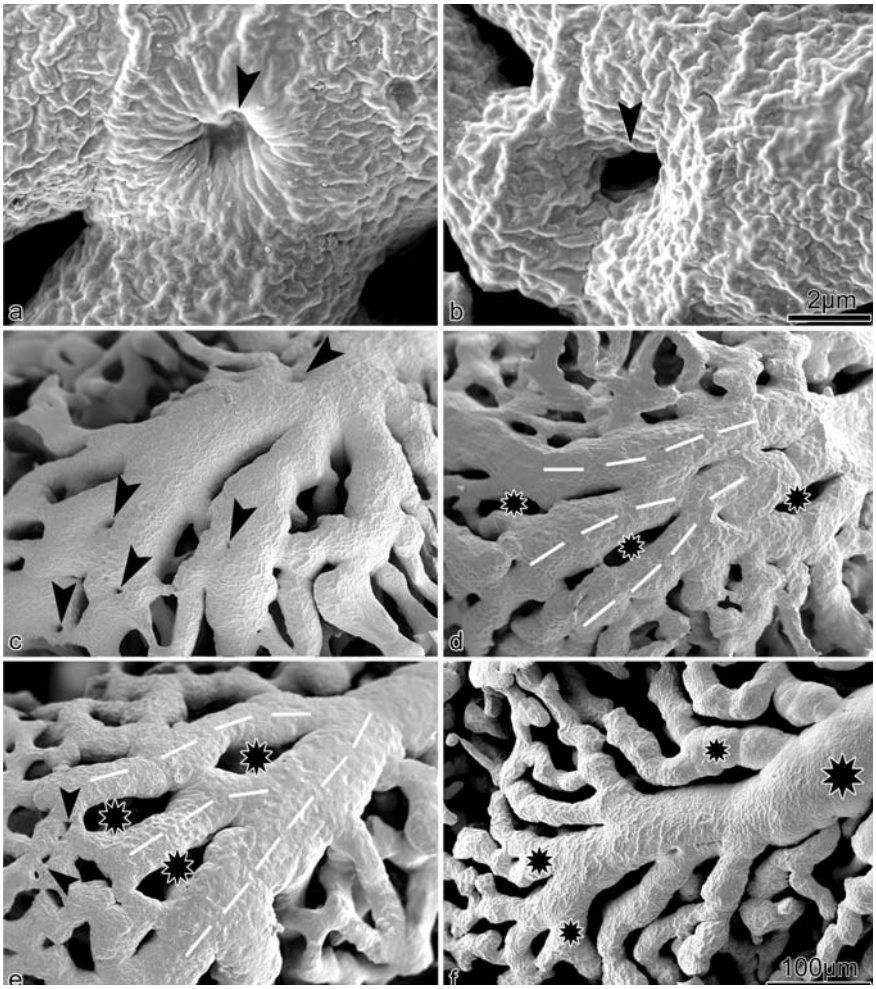

Figure 2. (a–f) Vascular corrosion casts from the avian metanephric kidney at day 15 of incubation illustrating the process of intussusceptive microvascular growth and intussusceptive arborisation. (a) Incipient transcapillary pillars appear as small depressions on the surface of the blood vessel cast (arrowhead) indicating the initial stages of pillar formation (see also Figs 1b, c, b' and c'). (b) As the two opposite components of the pillar approximate, there is fusion and subsequent perforation so that the pillar is now represented by a hole that pierces through the vascular lumen (arrowhead) (see also Figs 1 d, d'). Note that (a) and (b) are at the same magnification. (c–f) Low magnification microvascualar casts showing the various stages of pillar formation and vascular bed expansion. Incipient pillars are represented by depressions or small holes in the cast (arrowheads in c). Notice the irregular nature of the resultant vessels. Subsequently pillars increase in girth and fuse (asterisks in d and e) and in so doing delineate new vascular entities. Further expansion of pillars (asterisk in d and e) separates out the newly formed vessels (interrupted lines in d and e). Note that new pillars (arrowheads in e) now tend to form in the distal part of the vascular tree. Therefore, pillar initiation, augmentation and fusion, results in formation of complex vascular patterns, which include new capillary segments (small asterisks in f) and supplying and feeding vessels (large asterisks in f). Note the virtual absence of pillar holes and the "mature" and hierarchical appearance of the vasculature in (f). (c–f) are at same magnification.

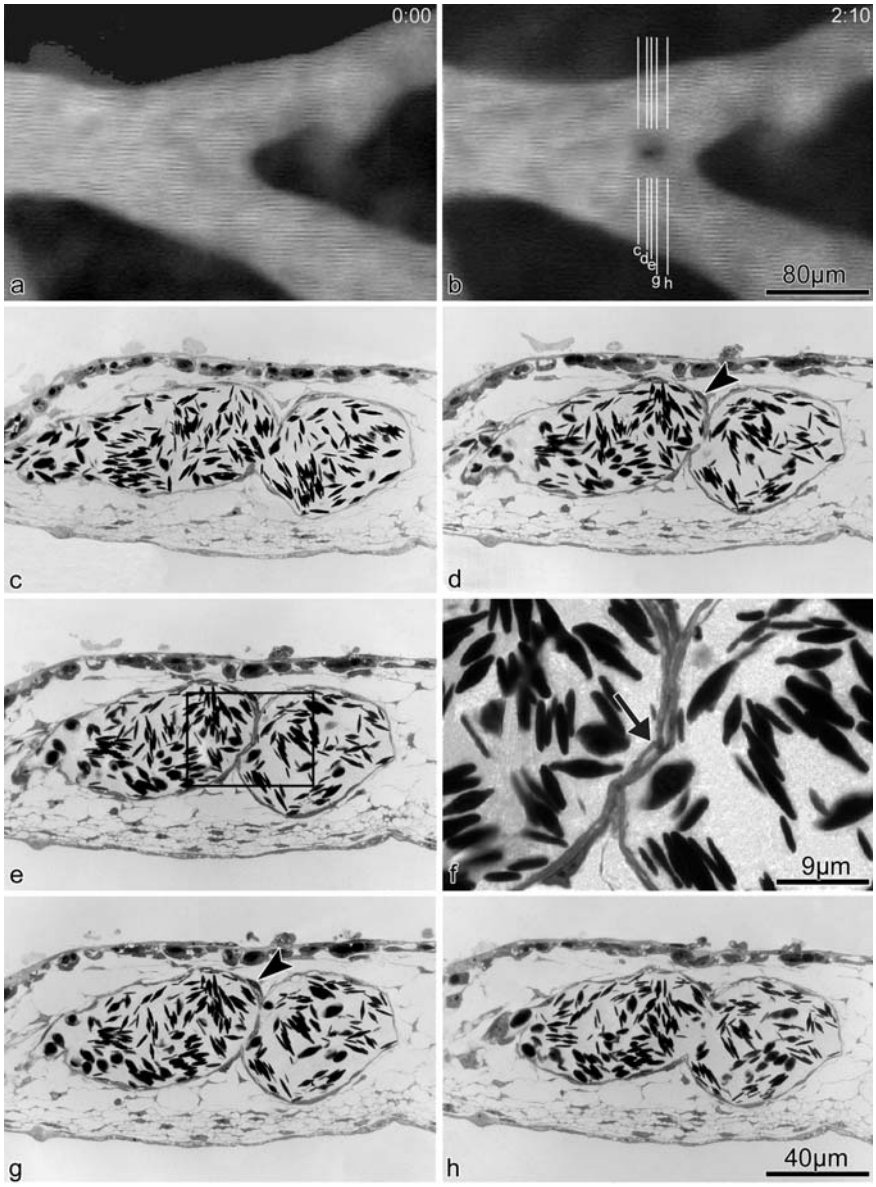

Figure 3. (a, b) *In vivo* video images illustrating pillar formation at a venous bifurcation. After 130 min of surveillance, a dark "spot" became visible, which was present on focusing through the entire breadth of the vessel lumen. (a) and (b) have been procured at the same magnification. (c–h): Semithin serial sections through the dark "spot" revealed the presence of an hour-glass-shaped pillar, created by the simultaneous protrusion of endothelial cells from opposite sides of the vascular wall into the lumen. The intensely stained zone (arrow in f; high-magnification view of the boxed region in e) represents an intercellular junction within the endothelial bilayer. Note mesenchymal cells in a pericytic position near intussusceptive branching remodeling site (arrowheads in d, g). Note that c-e, g, h at same magnification. After [19].

excellent tool for investigating normal vascular growth and remodeling processes, and for monitoring alterations induced by various pro- and anti-angiogenic factors [33]. The use of improved digital techniques in combination with fluorescein-isothiocyanate-dextran injection into the blood stream has enhanced the quality of earlier images. Pillar formation and remodeling have been indubitably observed in capillary plexuses [11, 17], as well as in small arteries and veins [19]. *In vivo* monitoring coupled with histological and ultrastructural analyses of serial tissue sections has demonstrated that pillar formation requires a period of 4–5 h for completion [19] (Fig. 3). This time is decreased to 1 h on doubling the blood flow rate [19], indicating the pivotal role of hemodynamics in control of intussusception. In contrast sprouting angiogenesis is a prolonged process characterized by extensive proliferation of endothelial cells, degradation of extracellular matrix and an increase in vascular permeability. Intussusception occurs in the virtual absence of endothelial cell proliferation, is achieved at low vascular permeability levels, and requires only a short duration for completion. It is a widespread phenomenon that occurs in the vascular systems of all species thus far investigated.

The phases of intussusceptive angiogenesis

The term intussusceptive angiogenesis circumscribes a host of processes that are involved in generation, growth, development and remodeling of vascular entities with diverse morphological and functional outcomes. Though chronologically sequential, the processes overlap both in space and time. Intussusceptive angiogenesis inaugurates with formation of pillars within the capillary bed, which subsequently expand leading to an increase in the complexity of the capillary network (Figs 2, 7), a process referred to as intussusceptive microvascular growth (IMG). In the distal parts of the supplying vessels, pillars may arise in series parallel to the long axis of the vessel and then merge to split the major vessel into small arteries and veins in distal parts of a vascular tree. This process has been referred to as intussusceptive arborization (IAR) and results in the formation of a vascular tree (Figs 4, 7). Thirdly, pillar formation occurring within small arteries and veins can lead to remodeling via an expansion or pruning of vessel branches and an optimization of the branching geometry and of the hemodynamic conditions of the vascular tree, (Figs 5, 7) a process referred to as intussusceptive branching remodeling (IBR).

Intussusceptive microvascular growth (IMG): expansion of capillary plexuses

Continuous pillar formation and growth lead to a rapid expansion of the capillary plexus, thereby affording a large surface area for the exchange of oxygen, carbon dioxide and nutrients. Consequently, new segments of the capil-

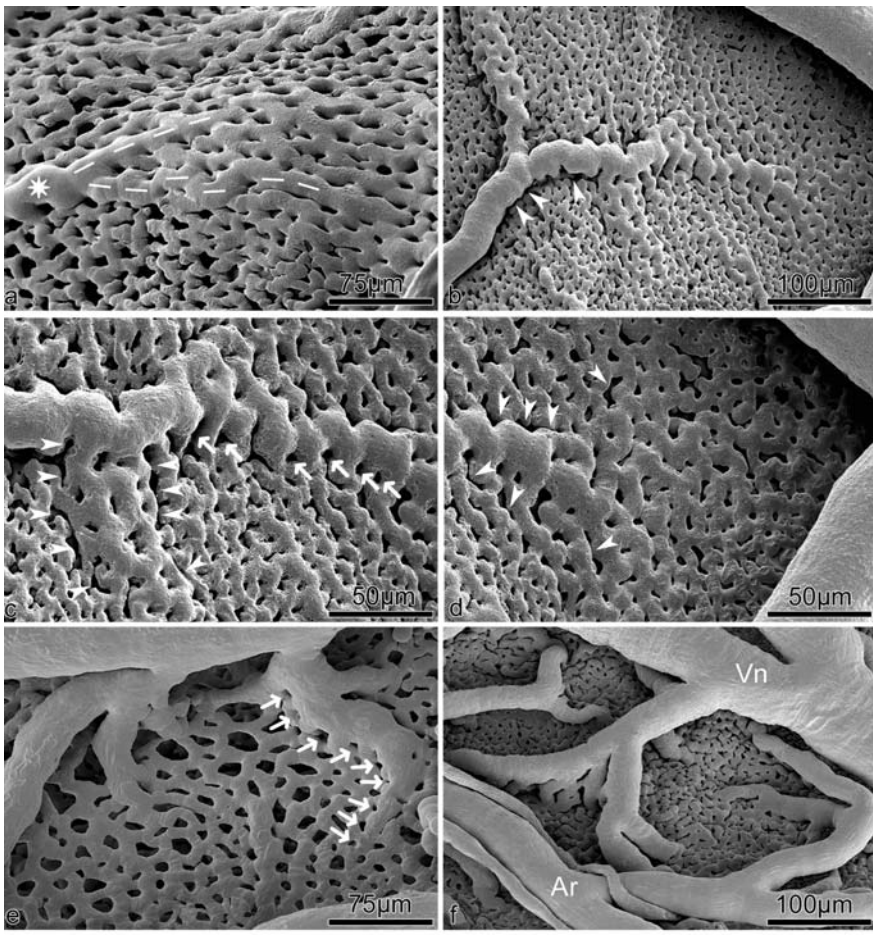

Figure 4. (a–f) Mercox casts of developing CAM vasculature demonstrating the process of IAR. (a) At day 9 of incubation a collecting venule (*asterisk*) with the adjacent triangle-like intensively perfused area is elevated and shifted out from the capillary plane. Rows of pillars demarcate the future blood vessels (hatched lines). (b) By day 10 the proximal part of the collecting vessel is completely separated from the capillary plexus by merging of horizontal tissue pillars (arrowheads). (c–f) IAR is initiated by a change in the pillar axes orientation (*arrows*) from perpendicular in the distal part to horizontal in the proximal part followed by merging into tissue septa. The areas of remodeling expand in lateral (c) and distal (d) directions giving rise to new branching generations by tissue septa formations (*arrowheads*). Note that c and d are taken from b at higher magnification. At day 12 (e), pillar axes (*arrows*) change orientation within a short distance, resulting in a size decrease of the remodelling areas, so that by day 14 (f) the latter disappear and the CAM vasculature consists of two layers: the capillary plexus and the layer of feed vessels, which remain connected by short abrupt vessel bridges. Vn = vein Ar = artery. After [17].

lary network arise with only little changes in the dimensions of its components (Figs 1, 2 and 7), viz., IMG. IMG was first observed in the growing postnatal lung [9, 10] and then in the microvasculature of many other tissues and organs

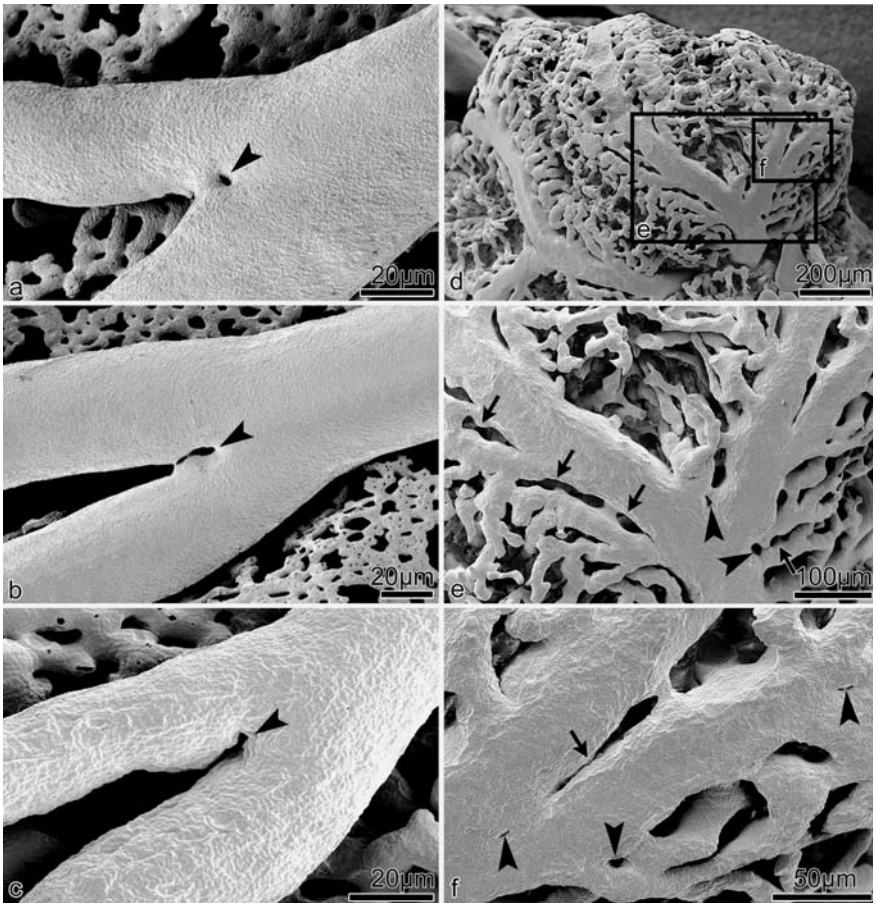

Figure 5. (a–f) Vascular casts illustrating the process of intussusceptive branching remodeling in the chick CAM (a, b,c) and avian metanephric kidney vessels (d, e, f). (a–c) Illustration of the branching angle modification by IBR. The process initiates with a small pillar (small hole indicated by arrowhead in a) which expands (b) until the distal connection between the vessels is severed (c) with a resultant replacement of the bifurcation point to proximal and alteration in the size of branching vessels. (d–f) Vascular casts of a metanephric kidney glomerulus at day 20 of incubation. Series of tissue pillars (arrowheads) and longitudinal folds of the endothelial wall (arrows) are apparent at the bifurcations, indicating ongoing intussusceptive branching remodeling. After [19].

of several species, including the rat [14], the chick CAM [11, 12, 17], retina [17, 19] and kidney [19], in a mouse model of tissue repair [15], in heart development [22], in the human endometrium [34, 35], in cerebral vascularization after stroke [36] and in tumor angiogenesis [15, 18]. It is now evident that IMG represents a general and ubiquitous mechanism of capillary growth. This phenomenon explains the way in which the capillary beds of organs, which arise initially by sprouting and/or vasculogenesis, can undergo rapid expansion

without any compromise in vascular physiology or function, as is reflected by the low vascular permeability conditions and the low rate of endothelial cell proliferation associated with IMG.

Intussusceptive arborization (IAR): formation of a feeding vascular tree

The hierarchical organization of the vasculature generally resembles the branching pattern of a tree and hence the name vascular tree [1]. Major supplying vessels give way to feeding vessels, which break into even smaller arterioles and venules. The latter groups give way to numerous capillaries. At each level, the sizes of the vessels decrease but the number of individual vascular entities increases. Notably the original pattern of vasculature formed from vasculogenesis or aniogenesis hardly resembles a tree. The culmination of the latter pattern is thoroughly and adroitly crafted through the process of intussusceptive arborization (IAR). As a capillary plexus grows, the perfusion distance between arteries and veins increases, which necessitates an adaptation in the system of supplying and draining vessels. Intussusceptive pillar formation has been shown to be involved in the differentiation of parts of the capillary plexus into immediate pre- and postcapillary feeding vessels, viz., IAR [11, 17]. IAR furnishes a mechanism whereby preferentially perfused segments of a capillary plexus can be transformed into terminal arterioles and collecting venules by changing their size and position, the number of sprays in a bunch of feeding or collecting vessels being thereby increased. IAR is initiated by the formation of serried "vertical" pillars, which demarcate future feeding vessels. These pillars undergo reshaping into narrow tissue septa that progressively fuse to delineate a new vascular entity. The remaining connecting bridges are "severed" by the formation of "horizontal" folds, the feeding vessels being thereby definitively separated from the capillary plexus. As a result of this process, a complex arterial and venous vascular tree arises to form a second layer of draining and feeding vessels (Fig. 4).

Intussusceptive branching remodeling (IBR): optimization of branching geometry

The concept of symmorphosis postulates a quantitative match of design and function so that an organism does not invest in superfluous structures. This has been demonstrated to be the case for the circulatory system [37]. In development and maturation of vasculature, structural-functional optimization is achieved by the process of intussusceptive branching remodeling (IBR). The branching geometry of supplying vessels is adapted to optimize the pre- and postcapillary flow properties. IBR can also lead to the removal of putative supernumerary branches (vascular pruning), thereby optimizing the efficiency of the blood supply and the hierarchy of the vascular tree.

Implementation of IBR is accomplished via transluminal pillars and folds, which occur close to the bifurcation sites of arteries and veins of up to 120 um in diameter. These structures appear *de novo*, and are capable of rapidly changing the vascular geometry and the hemodynamic properties at the affected branching points [19, 21, 30]. The pillars located close to bifurcation points enlarge (pillar augmentation) until their distal ends approximate, contacts and merge with connective tissue in the branching angle (Figs 3, 5). Pillars located more than 8–10 μm from the bifurcation point tend to elongate into flat longitudinal folds that protrude progressively into the lumen until this is subdivided into two distinct channels (for more details see [19]). Thus, IBR narrows the branching angle by relocating the branching point more proximally. This may represent an important adaptive response to the continually increasing blood flow and blood pressure during embryogenesis and growth. Direct experimental evidence for this hypothesis has been furnished by Frame and Sarelius [38] who reported the bifurcation angle of golden hamster cremaster muscle vessels to be modified in response to blood flow alterations. A 12%–14% reduction in the branching angle of retinal arteries has also been reported to occur in hypertensive human subjects [39]. Secondly, IBR optimizes the hemodynamic conditions at bifurcation sites by remodeling the diameter of one or both branches (mainly by "pillar augmentation"). Consequently, IBR yields a branching pattern that approximates to the ideal predicted by "Murray's Law" of minimal power consumption and constant shear stress [19, 21], a case of symmorphosis [37].

Pruning as a result of IBR

Intussusceptive vascular pruning (IPR) may be considered to be a facet of IBR. Presumably, in normal growing tissue, IPR severs the vascular branches that are no longer required. It is implemented by the successive asymmetric formation of pillars, which occasion the subtotal lumen obstruction of one of the daughter branches. The reduction in blood flow associated with the narrowed bore probably contributes to the regression, retraction, and ultimate atrophy of the affected branch (Figs 6, 7). The pruning phenomenon was first described for retinal vessels by Ashton [40] and is thought to be stimulated by growth factors and by oxygen tension [1, 41]. The thinning, retraction, and atrophy of vessel branches have been well described by Clark and Clark [42]. The latter authors demonstrated the complete separation of a side branch from the main vessel within 3 days and its disappearance by the fourth day. All previous reports dealing with vascular pruning described the phenomen, but did not recognize the crucial role played by eccentric pillar formation and fusion i.e., IPR.

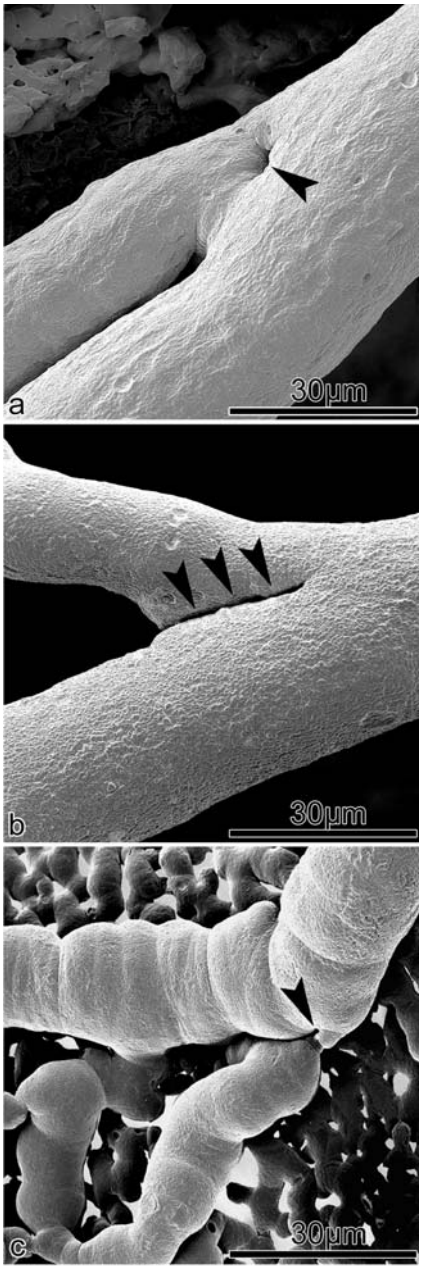

Figure 6. (a–c) Vascular casts of feed vessels in 12- and 13-day-old chorioallantoic membranes illustrating the putative role of intussusceptive branching remodeling in vascular pruning. Initially, a single (arrowhead in a) and later multiple (arrowheads in b) eccentrically located pillars arise. This mode of vessel splitting results in complete luminal obstruction (arrowhead in c) and severance of one of the daughter branches. Adapted from [19].

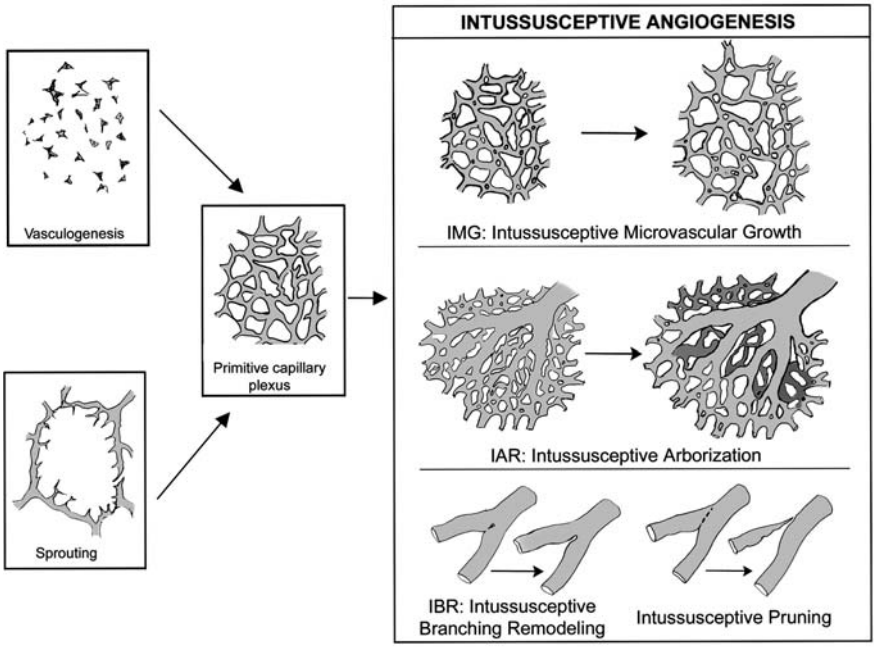

Figure 7. Diagramatic synopsis of intussusceptive angiogenesis (IA). When a primitive capillary plexus is generated by vasculogenesis or sprouting, intussusception is triggered and is responsible for rapid vascular growth and remodeling. IMG is responsible for rapid capillary expansion; IAR for subsequently segregation of feeding vessels from the capillary plexus and IBR optimizes branching geometry and is responsible for vascular pruning. After [30].

Sprouting and intussusception: two complementary angiogenic mechanisms

The various techniques used for *in vitro* and *in vivo* study of angiogenesis, such as three-dimensional collagen gels, corneal implants, tumor implantation, wound healing and embryonic grafting, elicit only capillary sprouting during tissue neovascularization. Blood flow in the aforementioned models is limited and intussusception would not be expected to occur. Consequently, and perhaps also because of the visualization difficulties alluded to above, recognition of intussusception has eluded many investigators of angiogenesis. In the studies conducted this far, it has been shown that the vascular system inaugurates by vasculogenesis [1] followed by an early "sprouting phase" characterized by appearance of multiple capillary sprouts that invade the mesenchyme and, after fusion, form the primary capillary plexus. During the second "intussusceptive phase", capillary sprouting is supervened and perhaps also superseded by transcapillary pillar formation. Further vascular growth and remodeling thus occurs primarily by intussusception. Ultimately, the rapid expansion of the

capillary network (IMG) coupled with vascular tree formation (IAR) and the dynamic adaptation of the latter by branching remodeling (IBR) results in functionally efficient vascular networks (Fig. 7). Three factors are thoutght to stimulate the switch from sprouting to intussusceptive angiogenesis. These include the relatively short duration required for intussusceptive angiogenesis, the low cost metabolically and energetically and finally the non interference with the local physiological conditions. This process does not depend upon extensive endothelial cell proliferation, basal membrane degradation or the invasion of surrounding tissue and "physiological" levels of transpermeability that permit vascular growth and remodeling to occur within a functionally uncompromised organ are maintained. In summary, we emphasize the fact that in new embryonic capillary networks intussusceptive angiogenesis (IA) initiates with IMG, which starts with the formation of the first pillars. This is followed by IAR, which establishes a vascular tree. IBR finally remodels the maturing vascular network. However, the three mechanisms may be contemporaneous in the same organ since as some vascular entities are maturing, others are in formative stages.

Control and regulation of intussusceptive angiogenesis

Pillar formation is quintessential in intussusceptive angiogenesis. Through pillar formation new vascular networks are formed, augmented, expanded and remodeled to accommodate changes in functional needs. Based on the method of implementation and morphofunctional outcomes, IA can be considered to comprise three cognate processes, namely IMG, IAR and IBR. Presumably, each process is controlled by a specific program that is initiated and regulated by definite molecules, cells and hemodynamics. Information concerning the identity of these is gradually emerging.

Hemodynamics

Hemodynamic forces are obvious and important determinants of vascular architecture. Djonov et al. [19] demonstrated that clamping of one of the dichotomous branches of an artery in the CAM microvasculature increases blood flow and/or pressure in its counterpart with an almost immediate effect on branching morphology. IBR is initiated within a few minutes, pillars are detected after 15–30 min, and the branching angles are decreased by about 20% after 40 min. Shear stress, which acts tangentially on capillary walls and is known to be modified by experimental increases in blood flow, may be responsible for these changes. Shear stress is related to the diameter of a vessel. Hence, insertion of a pillar into the blood stream near a branching point will reduce this force in post-pillar vessel segments [19, 21].

Molecular control of intussusception

Endothelial cells are known to sense changes in shear stress. Such changes are transduced by molecules such as PECAM/CD31 [43] into the interior of the cell. This mechanotransduction system then leads to changes in the transcription rate of many proteins, such as eNOS, adhesion molecules and angiogenic factors [44, 45]. Physiological or pathophysiological adaptations to changes in shear stress involve interactions between pericytes, macrophages and endothelial cells [46]. It has been demonstrated morphologically that pericytes and/or periendothelial cells are recruited during the initial and final phases of vascular pillar formation in several organs [11, 19] and are thought to contribute either to the synthesis and mechanical stabilization of the transcapillary pillar core or to the maintenance of a low vascular permeability during intussusception.

The putative inducers of sprouting angiogenesis include angiopoetins and their Tie-receptors [47], PDGF-B [48] and ephrins and their Eph-B receptors [49, 50] and probably also influence vascular remodeling. Angiopoietin-2 and PDGF-B are both essential for pericyte recruitment in the retina [51], brain [48] and placenta [52] and therefore may be important in formation and maturation of the tissue pillars during intussusceptive angiogenesis. The injection of PDGF-B into fully developed CAMs, for example, leads to formation of abundant large pre- and postcapillary microvessels but not to the expansion of capillary meshes [53]. On the other hand, knockout mice lacking angiopoietin-1 and Tie-2 show abnormal vascular development in which the vessel growth is arrested at a primitive stage and further remodeling does not occur [54]. Conversely, over-expression of angiopoietin-1 or of angiopoietin-2 simultaneously with VEGF is associated with the formation of "large" vessels, and numerous small holes in the capillary plexus [55], a finding reminiscent of intussusception. Ashton [40] has shown that when VEGF is maintained at moderate but constant levels, the vessels remain at an immature state whereas down-regulation of this factor is associated with pruning. VEGF appears to be an early promoter of angiogenesis while the angiopoietins and their receptor Tie-2 as well as the ephrins and their corresponding Eph receptors appear to act at a somewhat later stage of angiogenesis [3] and are probably associated with regulation of intussusception. VEGF promotes formation of new capillary segments and vascular maturation through pericyte and smooth muscle recruitment [56, 57]. Such periendothelial cells are important for vascular integrity and maturation. Newly formed vessels that are denuded of such cells become VEGF-independent and fail to mature [51]. Treatment of CAM [58] and the retinal vasculatre [59] with VEGF results in both sprouting and intussusceptive angiogenesis. Whether the latter process occurs consequent to sprouting is unclear, but evidently VEGF plays an important role in its initiation. VEGF is a highly potent and universal regulator of vascular responses to tissue oxygenation levels [41, 57], and probably responds to variation in oxygen concentration with the appropriate angiogenic responses [41]. Though many molecules have been implicated in angiogenesis, their precise role in initiation and

progression of intussusceptive angiogenesis remains to be investigated. The interplay between such molecules, local hemodynamic conditions and oxygen tension in regulation and control of intussusceptive angiogenesis are currently fertile areas for investigation.

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Mechanisms of Angiogenesis
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Angiogenesis in the female reproductive system

Hellmut G. Augustin

Dept. of Vascular Biology & Angiogenesis Research, Tumor Biology Center, Breisacher Str. 117, D-79106 Freiburg, Germany

Introduction

Angiogenesis and vasculogenesis are widely considered as oncofetal mechanisms. They are operative during embryonic development and are largely downregulated in the adult, being almost exclusively associated with pathological conditions such as the growth of tumors, hence the notion as an oncofetal mechanism [1]. The recognition of angiogenesis as an oncofetal mechanism also defines a unique therapeutic window allowing the targeting of tumor angiogenesis without eliciting major side effects as it targets a physiological mechanism that is largely downregulated in the healthy adult.

There is one major exemption from the general statement that angiogenesis is largely not operative in the healthy adult and that is the female reproductive system. The organs of the female reproductive system undergo cyclic changes that are associated with intense growth of new blood vessels [2]. These occur in the ovary, the endometrium and the mammary gland. Likewise, pregnancy-associated growth does not just lead to blood vessel morphogenic processes in the fetus, but also to maternal angiogenesis in the placenta and the mammary gland. In fact, the study of reproductive angiogenic processes has had major impact on the field of angiogenesis research in general. The first angiogenic factor, later identified as the heparin-binding growth factor bFGF, was identified in the ovary [3]. The ovary was among the first organs in which the most important angiogenic molecule, vascular endothelial growth factor (VEGF), was first characterized [4, 5]. More recently, the first endogenous regulator of blood vessel regression, Angiopoietin-2 (Ang-2), was demonstrated to be abundantly expressed in the ovarian corpus luteum during luteolysis [6]. This chapter will provide an overview about the dynamics of angiogenic processes in the organs of the female reproductive system including the discussion of the major molecular regulators of reproductive angiogenesis and the intricate interplay between the endocrine and the vascular system. It will primarily focus on the angiogenic processes in the ovary as the cyclic ovarian corpus luteum provides a unique physiological model of the life cycle of a transient neovasculature consisting of an angiogenic phase followed by a phase of vessel maturation and a period of physiological vessel regression during luteoly-

sis. Angiogenic processes in the endometrium, the mammary gland and the placenta are summarized more briefly at the end of the chapter focusing on selected aspects of vascular morphogenesis that are unique to these organs. The reader is referred to the specialized literature for a more detailed account of the molecular mechanisms and the endocrine regulation of angiogenesis in these organs [2].

Angiogenesis in the ovary

The cyclic angiogenic processes in the ovary are among the best studied reproductive angiogenic processes [7–10]. Development of the dominant follicle is associated with a distinct wave of angiogenesis. Likewise, post-ovulatory growth of the corpus luteum (CL) requires intense angiogenesis. In fact, the cyclic growth, maturation and luteolytic regression of the CL provides a unique system of a natural life cycle of a growing neovasculature with sprouting angiogenesis, maturation of the blood vessels and regression of the neovascular network. As such, angiogenesis in the ovary is not just of reproductive biological interest, but has also been studied paradigmatically as a model for the aberrant angiogenesis that occurs in tumors.

Follicular angiogenesis

Small primordial follicles do not have a vascular network of their own. As follicles develop in the post-pupertal female, they develop an antrum. It is at this time that a first capillary plexus ensheathing the growing follicle becomes detectable. This vascular sheath forms two concentric networks of vessels in the theca externa and interna. The vessels do not penetrate the membrana granulosa of the unruptured follicle. Particularly the growth of the inner network of capillaries in the theca interna coincides with a period of rapid growth and differentiation of the follicle [11]. This observation has led to speculations that establishment of the vascular network around developing follicles may be rate limiting for the selection of the dominant follicle(s), i.e., the follicle(s) that is/are destined to ovulate. Extracts of the follicular wall were found to contain endothelial cell migratory and proliferative activity [12]. Similarly, conditioned media from granulosa cells obtained from pregnant mare serum gonadotropin (PMSG)-treated rats stimulated endothelial cell proliferation [13]. More recently, experimental evidence has been provided demonstrating that an antiangiogenic regimen with a neutralizing VEGFR-2 antibody at the pre-ovulatory stage is capable to inhibit follicular development. This has unambiguously demonstrated that the intra-ovarian VEGF/VEGFR-2 pathway is critical for gonadotropin-dependent angiogenesis and follicular development [14, 15]. In turn, insufficient vascular supply could act as the trigger that leads to follicular atresia. Consistent with these hypotheses, pre-ovulatory fol-

licles of monkeys were found to have similar concentrations of gonadotropin binding sites; however, only the follicle that was destined to ovulate became heavily labelled after intravenous injection of labelled gonadotropin [16].

Post-ovulatory growth of the corpus luteum

Following ovulation, blood and plasma extravasate into the ovulatory cavity where they form a fibrin-rich clot [17]. The following angiogenic phase lasts for approximately one third of the ovarian cycle. The corpus luteum in larger monovulatory species such as bovine and humans grows during this time from approximately 200 mg of ovulatory tissue towards a solid tissue mass of several grams. The early CL is characterized by its intense red coloration reflecting the immature nature of the growing vasculature with leaky and blind ending vascular sprouts. Due to its red coloration, the growing CL is also called corpus rubrum (CR).

It is obvious that intense growth of tissues such as the formation of the CL must be accompanied by a correspondingly intense wave of growth of blood vessels. Early luteal proliferation indices have been determined in the rat [18], sheep [19], bovine [20, 21], mare [22], marmoset monkey [23, 24], rhesus monkey [25] and human [26, 27]. A systematic comparative analysis of endothelial cell proliferation in the growing CL and in different malignant human tumors revealed that reproductive angiogenesis may be four to twenty fold more intense than the angiogenesis in some of the most malignant human tumors including glioblastomas [21]. As many as 40% of CL microvessels were found to contain proliferating endothelial cells. In contrast, glioblastoma multiforme have on average a compartment of proliferating endothelial cells of close to 10%. Prostate and lung carcinomas have on average a proliferating endothelial cell compartment of only around 2% [21]. These studies have collectively shown that proliferation indices are highest in the early luteal phase and that the endothelial cell compartment is by far the most abundant and intensely proliferating cell population accounting for more than 50% of all cells in the growing corpus luteum [20, 25, 27, 28]. Yet, there is considerable variation between different species. Functional experiments to manipulate reproductive angiogenesis have been performed in laboratory animals including rats and mice [29, 30]. The four day reproductive cycle of these species is associated with an intense angiogenic burst following ovulation. The short duration of the cycle, however, makes it difficult to discern discrete phases of vessel maturation and vessel regression that follow the angiogenic phase of the ovarian cycle. More recently, manipulatory experiments in primates with VEGF neutralizing agents (VEGF antibody, VEGF trap, VEGF receptor antibody) have been performed in non-human primates [23, 31–34]. These have greatly contributed to discerning distinct functional stages of CL angiogenesis. Direct injection of a soluble VEGFR-1 fusion protein (sFlt-1) into the pre-ovulatory follicle of rhesus monkeys on the day of the mid-cycle luteinizing hor-

mone (LH) surge or one day earlier was shown to decrease subsequent luteal progesterone in the serum. However, the length of the luteal phase was of normal duration and treatment did not affect luteal angiogenesis [35]. Several experimental approaches have been taken to manipulate post-ovulatory CL angiogenesis in marmoset monkeys. Suppression of endogenous LH by gonadotropin-releasing hormone (GnRH) antagonists one day post-ovulation inhibits early luteal angiogenesis [23]. This confirms the importance of LH in stimulating VEGF production in the growing CL, even though LH withdrawal also elicits a number of inhibitory functions in the ovary that may indirectly control CL angiogenesis. VEGF inhibitors such as the VEGF trap have been delivered at various stages of the marmoset reproductive cycle to specifically study the role of VEGF at different stages of the ovarian cycle. Early post-ovulatory VEGF inhibition (days 0–3 or days 0–10) strongly suppresses CL angiogenesis as evidenced by reduced numbers of proliferating endothelial cells and a reduction in the concentrations of plasma progesterone [34]. Intriguingly, even later VEGF inhibition (days 3–4 post ovulation [31] or 7–10 day following ovulation [33]) similarly resulted in a marked decrease in angiogenesis and reduced plasma progesterone levels suggesting that VEGF is also required for the maintenance of the post-angiogenic CL. In fact, the very potent VEGF blockade with the VEGF trap R1R2 on days 7, 14 or 19 post ovulation is capable of inducing functional luteolysis. This finding is in line with the observation that physiological luteolysis is associated with a sharp drop in VEGF production in the CL [36].

Collectively, these studies indicate that VEGF is the most important and rate-limiting growth factor controlling angiogenesis in the ovary. Recently, a functionally VEGF-related, yet structurally different molecule has been described that may play a major role as a regulator of ovarian angiogenesis. Endocrine gland VEGF (EG-VEGF) was identified as the first organ-selective angiogenic growth factor [37]. EG-VEGF is expressed at highest levels in the adrenals, the ovary and the testis. EG-VEGF induces endothelial cell proliferation, migration and fenestration of endothelial cells. Yet, *in vivo* it only acts on endothelial cells in select organs such as the adrenals [38], hence its name endocrine gland VEGF. Functional experiments have confirmed the angiogenesis-regulating functions of EG-VEGF in the testis [37]. The term EG-VEGF is somewhat of a misnomer as it has no structural relationship with VEGF. In fact, EG-VEGF has also been identified as Prokineticin-1 which is involved in regulating the contraction of gastrointestinal smooth muscle cells [39]. Clearly, much needs to be learned about this novel cytokine, but the abundant expression in the ovary suggests important functions of this cytokine which may be involved in ovarian cyst formation [40].

Other angiogenic cytokines have also been characterized in the ovary and some of them have regulated expression patterns corresponding to highest levels during the angiogenic phase of the corpus luteum cycle. Among these, the pleiotropically acting heparin-binding growth factor basic FGF (bFGF) has been studied most intensely in the context of ovarian angiogenesis [41, 42].

Basic FGF was identified as the first angiogenic cytokine in the ovary [3]. Yet, bFGF protein is expressed in the corpus luteum with little temporal variations during the ovarian cycle [43]. Likewise, the lack of an overt reproductive phenotype in bFGF-deficient mice argues for an at least dispensable function of bFGF during ovarian angiogenesis [44]. Nevertheless, the endothelial cell mitogenic activity isolated from bovine corpora lutea has been demonstrated to be neutralized by more than 80% with antibodies against bFGF [45, 46]. Future work will show the functional relevance of the FGFs for reproductive angiogenesis as well as the molecular interplay between the FGFs and the VEGFs.

Mid-luteal maturation of the corpus luteum

The mature corpus luteum is characterized by an intense yellow coloration reflecting its endocrine function as well as the maturation of the transient CL vasculature. Maturation of a neovascular bed is functionally characterized by the recruitment of mural cells. Microvessels recruit pericytes which ensheath the capillaries and control the quiescent phenotype of the endothelial cells. Larger vessels recruit smooth muscle cells which similarly stabilize the blood vessels and control their vasotonus. Approximately 60% of the microvessels in the mature mid-stage CL are covered by pericytes [47]. This indicates that the CL vasculature acquires only partially a mature phenotype. Surprisingly, even the earliest neovasculature in the growing corpus rubrum has a pericyte coverage index of close to 60% [47]. This illustrates that physiological angiogenesis follows a well orchestrated program during which endothelial cell proliferation and subsequent pericyte recruitment are intimately linked. This is very much in contrast to the disorganized angiogenic processes in human tumors. Most tumor types have a lower degree of microvessel maturation with pericyte recruitment indices around 15% (glioblastomas, renal cell carcinomas) and 40% (colon carcinomas, prostate carcinomas) [21].

Functionally, the vasculature in the mature mid-stage corpus luteum is characterized by an intense degree of capillarization. Each luteal cell is in direct contact with two to three neighbouring capillaries. Surprisingly, there is very little arteriolization of the vascular tree in the corpus luteum. Microsphere injection experiments have demonstrated that their distribution in the corpus luteum largely depends on the systemic blood pressure indicating that CL perfusion is primarily controlled systemically and not through local regulation of the vasotonus [48].

Maturation of the corpus luteum vasculature is likely being controlled by an intricate interplay between VEGF and the angiopoietins. VEGF does not just act as an angiogenesis inducing growth factor in the early post-ovulatory CL, but is also a survival factor of endothelial cells in immature microvessels [49, 50]. The spatiotemporal changes of VEGF expression in the CL in relation to the morphological changes of the transient vasculature in the cyclic CL sup-

port a role of VEGF as a vascular survival factor in the mid-luteal CL. A comparative analysis of VEGF expression in the different phases of the ovarian cycle revealed that VEGF is expressed at similar intensity in the angiogenic corpus rubrum as well as in the non-angiogenic mature corpus luteum. Both VEGF receptors can be detected throughout the ovarian cycle albeit with some quantitative changes [47]. These findings correspond to the observation that the vasculature in the mature CL matures only partially through the recruitment of pericytes.

The angiopoietins (Ang-1 and Ang-2) have been identified as ligands of the endothelial receptor tyrosine kinase Tie-2 [6, 51, 52]. Ang-1-mediated activation of Tie-2 regulates endothelial cell survival and blood vessel maturation [53] and exerts a vessel sealing effect [54]. In turn, Ang-2 acts in blood vessels primarily as functional antagonist of Ang-1/Tie-2 by binding the receptor without inducing signal transduction [6, 55, 56]. 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 [57] and Ang-2 acting as dynamically regulated antagonizing cytokine [55, 58]. The spatiotemporal pattern of Ang-1 and Ang-2 expression in the cyclic corpus luteum CL appears to correspond to this model. Messenger RNA for both molecules is expressed in the angiogenic corpus rubrum as well as in the mature non-angiogenic corpus luteum at approximately equimolar concentrations [47]. In contrast, luteolysis is associated with a rapid and dramatic shift of the ratio of Ang-2 *versus* Ang-1 towards Ang-2 (at least 10-fold stronger expression of Ang-2 compared to Ang-1 [47]). Functionally, this overexpression of Ang-2 over Ang-1 can be interpreted as a destabilization of microvessels that are covered by pericytes.

Blood vessel regression during luteolysis

Luteolysis is initiated at the end of the ovarian cycle. This leads to the dissolution of the parenchymal CL tissue as well as all of its associated vasculature. Physiological regression of blood vessels reflects a remarkable biological phenomenon which occurs as rarely in the adult as physiological angiogenic processes. Regression of blood vessels during luteolysis has first been analyzed in the bovine corpus luteum [36] which is characterized by a particularly pronounced temporal dynamic of tissue dissolution and vessel regression. Following the induction of luteolysis, the volume of the CL is reduced to less than 50% within 2 to 3 days (regressing CL). This phase of rapid tissue dissolution is followed by a slower phase of tissue disintegration which may last several weeks (residual CL) to eventually leave a small hyaline scar called the corpus albicans.

The earliest angioregressive signs in the luteolytic CL are degenerative changes in the capillary endothelium of microvessels that are not lined by pericytes. Endothelial cells loose their intimate contact to the underlying basement membrane and detach to eventually float off into the vessel lumen. Luteal

cells undergo massive apoptotic cell death during this period. Ultrastructural analyses revealed that endothelial cells in regressing microvessels exhibit early signs of apoptotic cell death including membrane blebbing and a nuclear condensation. Still, when applying apoptosis detection techniques that are based on the detection of nucleosomal fragmentation products (TUNEL, ISEL), very few apoptotic endothelial cells can be identified in the regressing CL [36, 59]. This has led to the conclusion that endothelial cells from regressing blood vessels are most likely shed into the circulation from where they are cleared in the liver and spleen through similar mechanisms as hematopoietic cells.

The analysis of the molecular mechanisms of luteolysis have been the focus of intense research for many years (for review see [60]). Luteolysis leads to a dramatic downregulation of VEGF expression in the ovary [36]. As outlined above, the sudden downregulation of VEGF is functionally interpreted as the removal of its survival factor function leading to the rapid disintegration of immature microvessels that are not covered by pericytes. Ultrastructurally, this microvessel dissociation process can be traced within the first few days following the onset of luteolysis. Endothelial cells detach from their basement membrane to eventually float off into the circulation. At the same time, a gradual arteriolization of small arteries leads to a contractive occlusion of the vasculature and consequently a shut off of the circulation.

Endocrine regulation of ovarian angiogenesis

Ovarian angiogenesis is primarily regulated by the same vascular specific angiogenic cytokines that control the growth of blood vessels in other organs. Yet, the cyclic processes in the ovary are under endocrine control. It thus appears obvious to imply a functional relationship between the endocrine and the vascular system. Estrogens have been most extensively studied for their angiogenesis inducing capacity. Estrogens are capable to induce angiogenesis *in vivo* [61, 62]. Correspondingly, anti-estrogens have been shown to exert angioinhibitory activity [62]. Causal evidence for the role of estrogens for angiogenesis could be generated in estrogen receptor deficient mice in which angiogenesis can not be induced by estrogens [63].

The angiostimulatory activities of estrogens may be mediated by direct endotheliotropic functions of estrogens or indirectly by modulating the expression of angiogenic cytokines in other cell types. Both mechanisms appear to be operative. Endothelial cells express estrogen receptors [64]. 17β-estradiol stimulates endothelial cell migration and proliferation and promotes the formation of capillary-like tubes *in vitro* [62]. Anti-estrogens inhibit bFGF as well as VEGF induced endothelial cell proliferation [62]. The indirect angiogenesis inducing capacity of estrogens manifests itself in their ability to control the expression of angiogenic cytokines in other cell types. Estrogens have been shown to regulate the expression of VEGF in a number of cell types (endometrial cells, macrophages) [65, 66].

A number of other steroid hormones has also been tested for their angiomodulatory activity. The endogenous estrogen metabolite 2-methoxy estradiol (2-ME) has been shown to exert angioinhibitory activity [67, 68]. Likewise, medroxy progesterone acetate (MPA) has antiangiogenic activity as was demonstrated in the rabbit cornea assay as well as in tumor experiments [69].

Angiogenesis in the endometrium

Similar to the cyclic processes in the ovary, the cyclic processes in the endometrium are characterized by distinct phases of growth, maturation and regression [70]. The uterine vasculature has some unique structural properties. Yet, the fine structural properties of uterine blood vessels are indistinguishable from the vasculature in other organs. The arcuate arteries within the myometrium give rise to the radial arteries which supply the endometrium. They pass through the myometrial-endometrial junction and split to form smaller arteries that supply the basal layer of the endometrium and the spiral arteries that continue up to the endometrial surface. The spiral arteries are unique resistance arteries with a distinct coiled appearance that becomes more pronounced during the secretory phase of the menstrual cycle. Functionally, the maternal spiral arteries would eventually connect the endometrium to the fetal placenta. The cyclic changes in the vasculature have consequently to be conceptualized as part of the cyclic changes that would prepare the endometrium for eventual nidation of a conceptus. The spiral arteries lack significant amounts of elastin [71] and each of them supplies an endometrial surface area of approximately 4–9 mm^2 [72].

Despite the intense turnover of cells during the menstrual cycle, the majority of endometrial vessels, with the exception of the spiral arteries during the secretory phase, undergo little changes during the menstrual cycle. Microvascular density and endothelial cell proliferation remain relatively constant throughout the menstrual cycle [73, 74]. This has in fact challenged the question when and how angiogenesis occurs during the menstrual cycle if no distinct cyclic pattern of endothelial cell proliferation can be observed. Likewise, proliferating endothelial cells in the endometrium always appear within vessel profiles and are never associated with vascular sprouts. It would be expected that intense growth and turnover of blood vessels should occur during the proliferative phase of the ovarian cycle and following menstruation. Recent systematic experiments have demonstrated that vessel growth during the proliferative phase occurs mainly by elongation [75]. Vessel elongation may be complemented by branching of vessels through intussusceptive mechanisms and arteriogenic mechanisms through the proliferation of smooth muscle cells. Yet, classical sprouting angiogenic processes does not occur to a greater extend in the endometrium.

The secretory phase is associated with distinct vessel remodelling processes. Proliferation of smooth muscle cells is highest during the mid-secretory

and late-secretory stages of the cycle [76] which has led to the notion that smooth muscle cell proliferation in the endometrium is likely to be under the control of progesterone and it has been reported that endometrial smooth muscle cells express estrogen and progesterone receptors [77].

Menstruation leads to rapid and dramatic vascular changes. Venous and arterial stasis occurs 1–4 days prior to the onset of bleeding which is associated with an infiltration of leukocytes [78]. Menstrual bleeding occurs from the wall of an arteriole or capillary once a previously constricted spiral artery relaxes to allow the flow of blood. Some blood is also leaving the capillaries by diapedesis as well as by reflux from veins. In view of the gross degenerative processes that take place during menstruation, endometrial endothelial cells are fully functional and it actually appears that they are perfectly adapted to control the vascular events that take place during menstruation. Endothelial cells during menstruation express upregulated levels of ICAM-1 [79], maintain their high level of proliferation [74] and appear ultrastructurally normal in the presence of surrounding stromal and gland cell degeneration [80].

The same factors that control angiogenesis in other organs also control angiogenesis in the endometrium. It is believed that VEGF by interacting with its receptor VEGFR-1 and VEGFR-2 plays a major role in controlling endometrial angiogenesis [66, 81, 82]. However, there is no clear cyclic pattern of VEGF or VEGF receptor expression [66, 83, 84] as it for example occurs in the ovary [47]. Many of the other angiogenesis-regulating cytokines have also been detected in the endometrium including the positive angiogenesis regulators bFGF, TGF-β, TNF-α, and IL-8 and the negative regulator thrombospondin [85]. Yet, little is known about the functional role that these molecules play in controlling endometrial angiogenesis or the interplay between angiogenesis regulators and the dominating endocrine regulators of the endometrial cycle estrogen and progesterone.

Angiogenesis in the mammary gland

The mammary gland is unique as most of its development occurs after birth. No other anatomical structure is associated with so profound and intense changes in size, shape and function as it occurs in the breast during puberty, pregnancy, lactation and involution.

The arteries that supply the breast are derived from thoracic branches of the axillary artery, the internal thoracic artery and anterior intercostal arteries. Ramifications of these vessels accompany ductal structures, ultimately reaching the capillary network surrounding the alveoli. Growth of the breasts during puberty is associated with intense growth of blood vessels [86]. This has been studied extensively in experimental models such as mice [87–89], rats [90] and rabbits [91]. These analytical experiments have shown that the developed virgin mammary gland has a dense network of periductal capillaries which is well prepared for rapid expansion as it occurs during pregnancy and

lactation. During pregnancy, the pre-existent vascular bed expands by mechanisms of sprouting angiogenesis associated with proliferation of endothelial cells. During lactation, expansion of terminal alveoli distends the capillary network; however, this stage is not associated with increased vascular growth. Likewise, mammary involution is associated with the progressive disappearance of the capillary bed so that a larger fraction of relatively thick-walled venules and arterioles can be found in the involuted mammary gland.

Paralleling the situation in other organs, VEGF and its receptors comprise the best characterized angiogenesis-regulating system in the mammary gland. In contrast to the lack of dynamic cyclic regulation of VEGF/VEGFR expression in the endometrium, expression of VEGF and its receptor is well co-regulated with the expansion of the vascular bed during pregnancy and lactation. In the mouse and rat, VEGF mRNA expression is upregulated in the mammary gland more than 5-fold during pregnancy [89]. Likewise, lactation is associated with a more than 10-fold upregulation of VEGF mRNA levels [89]. The VEGF receptors are similarly upregulated during pregnancy and lactation, albeit not to the same extent as the ligand [89]. These findings strongly suggest that the VEGF/VEGFR system is the primary molecular regulatory system during mammary gland vascular expansion which is similarly supported by the observation that VEGF is rapidly downregulated following the weaning of pups [89]. Little is known about the factors that control VEGF in the mammary gland. There is a striking reciprocal regulation of VEGF and hepatocyte growth factor (HGF) in the breast during pregnancy and lactation. HGF is downregulated when VEGF is upregulated [92, 93] raising speculation about an inverse relationship between HGF and VEGF in the context of mammary gland vascularization [89].

The upregulation of the VEGF/VEGFR system during pregnancy and lactation may have multiple functional consequences that do not necessarily relate exclusively to angiogenesis. The capillary wall in the virgin mammary gland is usually continuous with rare fenestrations of 30–55 nm [87, 94]. There is a marked increase in permeability during lactation associated with an increased number of cytoplasmic vesicles in endothelial cells surrounding alveoli [87, 95]. It is likely that these changes are controlled by VEGF which is a very strong permeability regulating cytokine in addition to its role as an angiogenesis factor [96] and is also known to induce the morphological alterations in endothelial cells that are associated with the appearance of vesiculo-vacuolar organelles [97].

As endocrine regulators, estrogen, progesterone and prolactin have all been implicated in mammary gland angiogenesis. Endothelial cells express estrogen receptors [64] and a number of pro-angiogenic functions of estrogens have been described [62, 98, 99]. Similarly, endothelial cells have been reported to express progesterone receptors [100]. Progesterone induces cell cycle arrest in cultured endothelial cells and it has been suggested that it may act as a negative regulator of angiogenesis in the mammary gland [100]. Lastly, prolactin is critically required for mammary gland development and lactation. It may not

exert direct effects on mammary gland angiogenesis. Yet, a 16 kD fragment of prolactin has been identified that has potent anti-angiogenic functions [101–103]. The role of the 16 kD prolactin fragment on the vascular system of the mammary gland has not been analyzed, but it may well be that it acts as a negative regulator of angiogenesis.

Angiogenesis in the placenta

The placenta serves a multitude of functions. It anchors the fetus to the uterine wall and acts as the fetus' lung, liver and kidney. Placental anatomy is consequently highly specialized [104] and few organs display such anatomical variations within closely related mammals. Placentation occurs through the invasion of fetal trophoblast cells into the maternal endometrium all the way into the spiral arteries where fetal trophoblast cells actual partially line the lumen of the maternal spiral arteries. This process is referred to as pseudo-vasculogenesis (see below) and leads to the transformation of the spiral arteries into a system of low resistance vessels. The mature human placenta is a placenta hemochorialis that forms a discoid organ in which the maternal blood flows directly around the fetal villi. The terminal villi form a surface area of about 12–14 m^2 [105]. In order to facilitate an effective feto-maternal exchange, the villi are highly capillarized. Capillaries in the terminal villi form a coiled network and it is believed that capillary expansion is actually controlling villous expansion [106]. Placental capillaries have a continuous, non-fenestrated monolayer with numerous tight junctions [107, 108].

The VEGF/VEGFR system has been analyzed most extensively for its role in regulating placental angiogenesis [109]. VEGF immunoreactivity and mRNA is detected in the placenta and is primarily produced by fetal and maternal macrophages, villous trophoblasts and some villous fibroblasts [110–112]. The VEGF-related molecules placenta growth factor (PlGF) is also expressed at high levels in the placenta [112, 113]. PlGF binds to VEGFR-1 and not to VEGFR-2. Due to its abundance, PlGF was originally believed to play a primary role during placental angiogenesis. Yet, PlGF-deficient mice do not have a placental or developmental phenotype and PlGF appears to be dispensable for placental angiogenesis [114]. Expression of the angiopoietins has also been described in the placenta [115, 116]. Their functional role during vessel remodelling and maturation has not been analyzed in great detail. Yet, there is evidence that they do not just affect placental endothelial cells, but also trophoblast behavior [117].

Oxygen transport is one of the most important functions of the placenta and recent work has provided evidence that local pO_2 concentrations are critical regulators of placental morphogenesis and angiogenesis. Many of the angiogenesis regulating cytokines including VEGF are regulated by hypoxia [118]. In turn, PlGF is downregulated by hypoxia [119]. There is good evidence to suggest that early placentation occurs in a relative hypoxic atmosphere and

that hypoxia-driven transcriptional regulation plays a major role in controlling the growth of blood vessels in the placenta. It has been shown that aggregates of fetal cytotrophoblast cells virtually occlude the maternal spiral arteries before 12 weeks of gestation leading to sparse flow of blood into the intervillous space [120, 121]. These observations suggest that the trophoblast mediated anchorage to the maternal uterine wall directly controls subsequent vascular morphogenic events in the placenta proper.

Cytotrophoblast-mediated pseudo-vasculogenesis

As discussed above, fetal cytotrophoblasts invade the uterine spiral arteries during normal placentation and transform them to a low resistance system that is responsible for the free flow of maternal blood around the fetal villi. Fetal cytotrophoblasts may even replace the maternal endothelial cells to line the lumen of the maternal spiral arteries. This invasive process is associated with a distinct transdifferentiation of the fetal cytotrophoblast cells towards an endothelial cell phenotype, a process called pseudo-vasculogenesis. Cytotrophoblasts cells change their phenotype during this transdifferentiation process from an epithelial towards and endothelial cell phenotype [122]. This has been elegantly demonstrated by the down regulation of epithelial cell adhesion molecules such as the integrin $\alpha_6\beta_4$ and E-cadherin and the expression of the endothelial cell surface molecules VE-cadherin VCAM-1, PECAM-1, and $\alpha_1\beta 1_1$. This transition from an invasive epithelial cell phenotype towards an adherent vascular phenotype is intricately controlled by oxygen concentrations. Proper early gestational invasion and transdifferentiation of cytotrophoblast cells is consequently a critical requirement for later gestational proper function of the placenta. Disturbed cytotrophoblast invasion during early gestation leads in late gestation to the most frequent maternal complication of pregnancy, called preeclampsia (approx. 3% of all human pregnancies). This life-threatening disease is a direct consequence of a disturbed placentation in early gestation [122–124] that leads to reduced placental perfusion. This can be compensated for most of the pregnancy but results in an uncompensated vitious circle in late gestation leading to the clinical manifestation of preeclampsia [125, 126] which is associated with severe systemic endothelial dysfunction.

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Mechanisms of Angiogenesis
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Arterialization, coronariogenesis and arteriogenesis

Borja Fernández

Dep. Biología Animal, Fac. Ciencias, Univ. Málaga, Campus de Teatinos s/n, 29071 Málaga, Spain

During embryonic life, the early vascular system consists of a network of immature endothelial channels called the primary plexus, which irrigates the growing embryo. Conducting vessels (arteries and veins) develop later, following the metabolic demand of the organ primordia. Embryologists described the development of conducting vessels as a process of investment of the primary plexus with undifferentiated mesenchymal cells, which become committed *in situ* to the musculo-vascular phenotype (reviewed in [1–3]). This process of muscularization or arterialization is currently considered the last stage (maturation) in the whole angiogenic process, when capillaries are invested with pericytes, and arteries and veins with smooth muscle cells. Both cell types can be seen as members of the same fibroblastic lineage with a high phenotypic plasticity [4].

The molecular bases of the process of arterialization began to be understood during the nineties with the discovery of new genes that can regulate the formation of arteries and veins (reviewed in [3]). The Tie/Angiopoietin signaling system appeared to be a key pathway for perivascular cell recruitment and differentiation [5–9]. Angiopoietin-1 deficient mice were found unable to effectively recruit vascular wall precursors, and mutations of the Tie-2 receptor were detected in human patients with venous malformations. We now know that Tie-2/Angiopoietin-1 interactions maintain cell-cell contacts between endothelial cells, stabilizing the vessel wall and promoting vessel maturation. Platelet derived growth factor (PDGF) and its receptor (PDGFR) were also found to be involved in the process of arterialization, as PDGFβ and PDGFR-B mutant mice lack pericytes and smooth muscle cells in particular organs [10–12]. Transforming growth factor (TGF) was also suspected to be involved in arterialization due to its regulatory capabilities on epithelial-mesenchymal interactions, and to the yolk-sac vascular defects detected in TGFβ null mice [13]. All these studies allowed drawing a first theory on the molecular mechanism of differentiation of arteries and veins (reviewed in [3]). Perivascular mesenchymal cells secrete Angiopoietin-1 that binds to Tie-2 receptor in the surface of endothelial cells. A putative chemoattractant signal from endothelial cells then recruits mesenchymal cells to the vessel wall. Upon contact, mesenchymal cells differentiate into pericytes and smooth muscle cells, induced by differentiation factors like PDGF, TGFβ and others. Multiple

molecules and signaling pathways are now known to form part of this differentiation program (reviewed in [14–16]).

For a better understanding of the musculo-vascular differentiation program, it can be useful to inspect the process of differentiation of other muscular tissues. The discovery of master differentiation genes that can regulate the whole process of skeletal muscle differentiation meant a qualitative step forward in this field (reviewed in [17–22]). MyoD, mef2 and related proteins are transcription factors and co-factors that bind to consensus DNA sequences found in the promotor of many skeletal muscle specific genes. The coordinated transcription of this gene pool is able to convert a variety of cell types into skeletal myoblasts. However, to date, no single pathway is known to regulate the entire smooth muscle cell differentiation program.

Possibly, the upstream pathways regulating cell differentiation are more diverse in smooth muscles than in skeletal muscles. The later are strictly specialized in contraction, and this specialization may imply a more deterministic regulation of cell differentiation. On the contrary, smooth muscle cells show wide phenotypic plasticity (contractile and synthetic smooth muscle cells, pericytes, myofibroblasts) that allows diverse cellular activities (contractility, secretion, motility and proliferation). In addition, smooth muscle cells originate in the embryo from a variety of cell lineages, like neural crest cells [23], endothelial cells [24], proepicardial cells [25], cardiomyocytes [26] and locally recruited mesenchymal cells of different embryonic organs [27]. They may thus require a more complex regulation of the differentiation program. The thoracic arteries are a good example. They are composed of smooth muscle cells with two different embryonic origins: the neural crest and the mesoderm. Neural crest-derived and mesoderm-derived arteries show different elastogenic properties [28–30], and the mechanism of investment is different in each embryonic vessel ("centrifugal" *versus* "centripetal" investment) [31]. Despite these differences, differentiation is initiated in both cases by the same stimulus (the blood flow), and leads to an anatomically and histologically continuous arterial tree. Thus, the differentiation program of smooth muscle cells is flexibly coordinated between two independent embryonic cell types, to finally form the same anatomical structure with two different histomorphological characteristics.

The coronary vasculature develops from an extra-cardiac structure, the proepicardial organ, through an evolutionary conserved morphogenetic mechanism already present in primitive vertebrates like sharks [32]. Mesothelial cells of the proepicardial organ delaminate, migrate to the heart and cover its surface forming the embryonic epicardium. Then, they invade and colonize the subepicardial space by a process of epithelio-mesenchymal transformation, giving rise to presumptive angioblasts, fibroblasts, pericytes and smooth muscle cells of the coronary system (for a review see [33]). Recent studies indicate that proepicardial cells have a predetermined fate prior to their migration into the heart. Explanted proepicardial cells can differentiate *in vitro* into coronary smooth muscle cells [34, 35], and the recently discovered gene *bves* is exclu-

sively expressed in the proepicardial organ and in the population of cardiac mesenchymal cells that will form the coronary artery smooth muscle cells [36]. All these experiments demonstrate that presumptive coronary smooth muscle cells are predetermined prior to their differentiation, and even prior to their migration to the embryonic heart.

The embryonic coronary plexus expands and irrigates the trabeculated embryonic heart. When the heart grows into a more compact organ, the invasive capillary plexus makes contact with the developing aortic root in the outflow, and with the coronary sinus in the inflow tracts of the heart, connecting the coronary vasculature to the systemic circulation. This connection initiates coronary arterialization, and the mechanical effect of blood flow is a crucial trigger. Mesothelial-derived mesenchymal cells are recruited to invest the capillary plexus, differentiating into smooth muscle cells. Recruitment and investment start at the aortic root in the coronary ostia, and propagate relatively fast in a proximal-to-distal direction with respect to blood flow, following a constant route through the cardiac *sulci*. Thus, arterialization is initiated with a sudden increase in the blood flow that establishes a proximal-to-distal wave of differentiation in the plexus. Interestingly, coronary veins do not follow the same morphogenetic program as arteries. Veins differentiate later, and are formed by smooth muscle cells with different lineages in the proximal, and probably also in the distal portions of the coronary system [26].

In humans and other mammals, a certain type of coronary artery malformation consists in the lack of one of the two coronary arteries that normally irrigate the heart. These anomalies are probably the result of a defective connection of the embryonic coronary plexus to the aortic root [37–40]. The remaining single coronary artery develops normally, irrigating its presumptive (right or left) cardiac chamber. However, when the arterialization process reaches the interventricular limit, the single coronary artery does not restrict to its presumptive irrigation area, but continues growing distally throughout the irrigation area of the missing artery, until it arrives to an area of the myocardium that is already irrigated. It seems that the musculo-vascular differentiation program is regulated by putative inhibitory signals from the irrigated tissue or from the differentiated vasculature itself.

Researchers on coronariogenesis made another crucial observation that was underestimated until recently. The connection of the coronary plexus to the artic root occurs in tight anatomical association with neural crest derived presumptive nerve ganglia [41, 42]. Moreover, the proximal-to-distal wave of differentiation follows an anatomical route that parallels cardiac innervation [41, 43]. During recent years, a number of nerve-related embryonic genes have been shown to play different roles in the developing vascular system (neuropilin/semaphorin; ephrin/eph; notch/jagged reviewed in [17]). Recently, Mikouyama and collaborators provided the first direct experimental evidence of this morphogenetic relationship [44]. First, neurons and Schwann cells are able to induce vascular differentiation *in vitro*. Second, mutations in genes important for axon guidance alter the nerve branching pattern, and the arterial

tree follows the same altered pattern. Thus, the musculo-vascular differentiation program depends on mechanical stimuli from the blood flow as well as on putative inductive factors from the nervous system. Coronary veins, however, though in close anatomical contact to arteries and nerves, are not directly influenced by nerve induction [26].

FGF-1 and 2 are potent angiogenic factors and can induce capillary tube-formation *in vitro* (reviewed in [45]). They are also mitogenic for a variety of cell types, including smooth muscle cells. During embryogenesis, FGF signals are required for development of mesodermal derivatives including the heart [46, 47]. Localization of FGF-1 ligand and receptors correlates with proliferation and differentiation stages of cardiomycytes and smooth muscle cells [48–50]. However, knocking out FGF-1, FGF-2 or both does not result in cardiovascular defects [51]. It is believed that different FGFs have overlapping functions during embryonic development, and some of the 23 members of the increasing FGF family may compensate for FGF-1 and 2 deficiencies [52, 53]. We generated transgenic mice that overexpress FGF-1 in the heart by coupling the FGF gene to the Myosin light chain promoter [54, 55]. These transgenic mice do not suffer any congenital cardiac malformation. The anatomical course, histology and ultrastructure of the coronary arteries are normal and capillary vessels are similar to those in wild type mice in amount and distribution, but the number of coronary arterioles is increased. Morphometric analyses in the heart of adult transgenic mice showed a moderate increase (~1.5 fold) in the numerical density of arterioles, together with a parallel increase of the amount of arterial branches. Studies in neonatal hearts revealed that the arteriolar overgrowth takes place between the 2nd and the 6th week after birth. Interestingly, in the rat postnatal heart coronary arterioles stop growing after the first month *postpartum* [56], correlating with the downregulation of FGF-1 in smooth muscle cells [48]. These results indicate that FGF-1 overexpression can alter the postnatal terminal differentiation of the arterial system. We have proposed that increased levels of FGF-1 in the heart of postnatal transgenic mice maintain the growth status of the arteriolar network, leading to the moderate overgrowth found in adult animals. This hypothesis is in agreement with previous experiments in rat neonatal hearts using blocking antibodies for FGF-2, which resulted in inhibition of coronary arteriolar growth [57]. The fact that FGF-1 overexpressing hearts develop an overgrown but structurally normal arterial tree supports the idea of a master regulatory role of FGF in the development of the vasculature. In the embryo, FGFs are potent morphogens, regulating the development of whole organs. Liver [58] and nerve [59] development are initiated by FGF signals. Different FGF members can induce the formation of structurally normal extra-limbs in the chick embryo [60], and FGF-10 deficient mice develop neither limbs nor lungs [61].

The altered branching pattern of the coronary arteries in FGF-1 transgenic mice is an interesting finding. FGF-1, 2, 7, and 10 all affect ureteric branching morphogenesis [62], and lung branching requires FGF receptor activation [63].

Concentration gradients of FGF appear to be crucial to maintain the growth of limbs and bones [64, 65]. FGF is normally stored in the extracellular matrix bound to heparan sulfate proteoglycans that serve as local reservoirs of growth factor. During angiogenesis, FGF-2 can be observed accumulated at sites of capillary branching [66, 67]. In a model for coronary arterialization, the mechanical stimulus of the blood flow might liberate FGF protein from its storage in the heparan-sulfated basal lamina. Local accumulations of the growth factor in a permissive environment would then stimulate the progressive proliferation and differentiation of perivascular cells in a proximal-to-distal direction.

Activation of FGF receptor can induce transcription of genes with important morphogenetic potential, like Notch [68]. Upon activation, the Notch receptor undergoes proteolytic cleavage and the inner cellular domain is translocated to the nucleus where it regulates gene transcription. One function of Notch in the vascular system is to determine the arterial *versus* venous identity of vascular precursor cells [69]. In the zebrafish embryo, Notch expressing angioblasts will form the aorta, whereas angioblasts not expressing Notch will form the cardinal vein. It may be of interest to study the possible involvement of the Notch signaling pathway in the predetermination of coronary precursors. The Notch receptor pathway is particularly interesting in the context of the growth stimulatory capacity of FGF. The general function of Notch is to keep cells receptive to differentiation signals. FGF mediated Notch signaling might turn cells susceptible to stimuli from the environment that can eventually transform perivascular mesenchymal cells into mural cells.

The Ephrin/Eph system is another source of instructive signals that may confer arterial and venous identities to the vasculature (reviewed in [70, 71]). Like Notch, Eph receptors and Ephrin ligands are bound to the plasma membrane, limiting signals to cells in contact. The general function of Ephrin/Eph during morphogenesis is to establish spatial boundaries in developing structures [72]. Arterial endothelial cells express the ephrin-B2 ligand, while the corresponding Eph-B4 receptor is expressed only by venous endothelial cells [73]. Ligand-receptor interactions between arterial and venous endothelial cells restrict cell intermingling and stimulate capillary sprouting. This may be a cellular mechanism to restrict angiogenesis to venous capillary segments. Some ephrin and eph members are also expressed in perivascular mesenchymal cells, but their function is still obscure [71]. Interactions with their endothelial cell partners may regulate patterning of the vasculature. Normally, arteries branch into smaller arteries and arterioles, ending in a capillary bed. Ephrin-eph signals may serve to avoid abnormal connections between an artery and a vein.

Ephrin-eph signals might also regulate connections between two developing opposing arteries. Such connections would result in arterial loops or natural bypasses. These types of vessels are called collateral arteries. They are often seen as small arteries or arterioles connecting parallel arterial trees (Fig. 1B). They are not numerous but common in most arterial systems, and have an

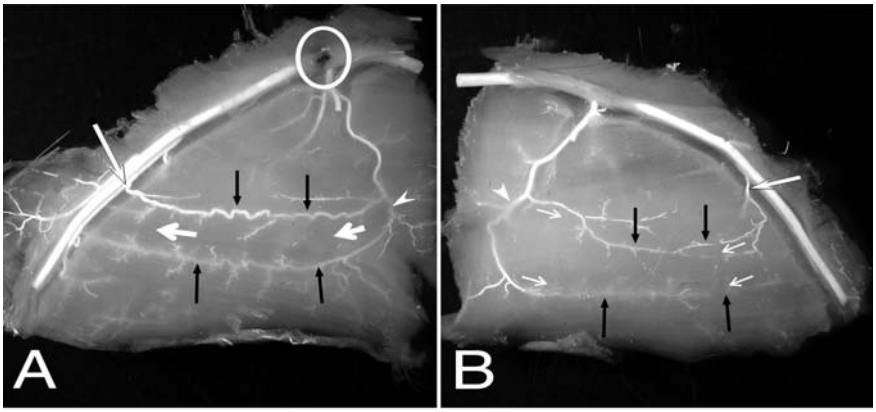

Figure 1. Microphotographs of murine adductor muscles containing collateral arteries filled with bismuth/gelatin contrast agent. In a physiologically normal limb (B), collateral arteries (black arrows) can be identified as small arteries connecting the distal femoral artery (big white arrow) to the *arteria profunda* (big white arrowhead). Small white arrows show the direction of the blood flow. When the femoral artery is occluded (A, white circle), the blood flowing through the collateral arteries changes direction and intensity (white arrows). Two weeks after surgery, the collateral arteries (black arrows) have significantly grown in size and have acquired a corkscrew appearance. (For colored picture see color plate 3)

important physiological function in the adult. When one of the mother arteries suffers a stenosis, the blood is redirected to the collateral artery, which activates a process of remodeling and growth named arteriogenesis. Arteriogenesis results in the formation of a larger artery that can bypass the occlusion area and irrigate the tissue in risk of ischemia (Fig. 1A) (reviewed in [74]). Studies on coronary arteriogenesis using animal models revealed a strong variability in the amount and distribution of pre-existing collateral arteries in different species [75]. The guinea pig heart is an example of the physiological relevance of collateral arteries. The heart of this rodent shows a high density of collateral vessels compared to other species, and occlusion of a main coronary artery in the adult leads to little or no ischemic damage.

Very little information exists on how collateral arteries develop during embryonic or postnatal life. However, some congenital malformations of the vascular system highlight the importance of the collateral circulation. A particular human coronary malformation consists in the anomalous origin of the left coronary artery from the pulmonary artery (ALCAPA or Bland White Garland syndrome). During embryonic life, the left coronary artery develops normally, as it is perfused with oxygenated blood due to the connection of the systemic and pulmonary circulations through the *ductus arteriosus*. After birth, however, the ductus closes and the left coronary artery starts receiving poorly oxygenated blood, coming from the pulmonary artery. Postnatal survival relies on the presence of well-developed collateral arteries that connect branches of the right and left coronary arteries. A right to left shunt is created, and arterial blood can perfuse the left ventricle. A particular strain of

Syrian hamsters shows a spontaneous high incidence of ALCAPA in the population [38, 40]. Collateral connections allow postnatal survival of the animals, which do not suffer any important cardiac decompensation, and grow until adulthood. In affected animals, the right to left shunt stimulates the growth of the collateral system during postnatal life, what allows a proper irrigation of the whole heart.

Price and collaborators studied the postnatal formation of collateral arteries in the gracilis muscle of rats using markers of arterial differentiation [76]. They found that collateral arteries form by arterialization of opposing preexisting terminal arteriole endings, proceeding along capillary pathways. Smooth muscle cells covering the new arteriolar domains may derive from recruited perivascular pericytes and fibroblast, or from mitosis and migration of smooth muscle cells in the terminal arterioles. The same group and others have shown that this type of arteriolar growth occurs in adult tissues subjected to a variety of pathophysiological situations, like chronic hypertension [77], electrical stimulation [78], chronic adrenergic blockage [79] and chronic hypoxia [80].

The discovery during the last decades of molecular pathways regulating angiogenesis has opened the door for new clinical strategies to promote or inhibit capillary growth. Currently, however, many vascular biologists have finally come to the conclusion that capillaries cannot replace an occluded artery, and only arterial collaterals are able to partially compensate the blood flow deficit [81]. Research on the molecular pathways regulating arteriogenesis and collateral formation may lead to a new explosion in the development of anti-ischaemic strategies. The next years may bring new exciting possibilities in this field.

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Mechanisms of Angiogenesis
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Sprouting angiogenesis *versus* co-option in tumor angiogenesis

Robert M.W. de Waal and William P.J. Leenders

Department of Pathology 437, University Medical Centre Nijmegen, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

Introduction

The development of multicellularity of organisms has led to a high degree of cellular specialization. No longer had every cell the obligation to fulfill all of the functions needed for life, like uptake of unprocessed food, defense against external threats and reproduction. Still, for maintenance of cellular viability, an almost permanent supply of oxygen and nutrients to every single individual cell is required. In higher animals this has led to the development of the circulation system, consisting of blood vessels that are perfused by the pump function of the heart, and of specialized oxygen-transporting cells, the erythrocytes. Especially during tissue expansion there is high need of blood supply. A vital mechanism to cope with this condition is the development of new blood vessels as a reaction to hypoxia [1–4]. This mechanism is already operational during early embryogenesis and complements vasculogenesis, the formation of vessels from a primary primitive plexus. Once blood vessels have formed, they still retain the ability to generate new branches via sprouting, which then grow and mature. This hypoxia-driven part of the vessel-forming process is called angiogenesis and occurs both during fetal and adult life [5–7]. In adults, angiogenesis supports tissue expansion as well, e.g., during the female reproductive cycle, but is also involved in tissue repair and remodeling in the later phases of wound healing. Not only tissue expansion is associated with a need of intense blood supply. Organs with a high level of metabolism also require ample supply of oxygen and nutrients, and organs involved in oxygen uptake from the air and in the disposal of metabolic waste products by filtering of blood are obviously densely vascularized as well.

As with many biological processes that need to be tightly controlled, dysregulation of angiogenesis leads to severe complications. One example is retinopathy, a condition that occurs in patients suffering from diabetes and is characterized by extensive neovascularization in the retina, ultimately leading to disruption of the nervous structures of the retina and blindness [8]. Other pathological situations in which angiogenesis plays a prominent role are

rheumatoid arthritis, psoriasis, macula degeneration and cancer [9]. The importance of angiogenesis in cancer can be easily envisioned when one considers that many solid tumors cannot expand beyond a maximum size of 1–2 mm^3 unless a vascular bed develops that supplies tumor cells with the necessary oxygen and nutrients.

How tumors get blood supply

Recent data have shown that the dependence of solid tumors on angiogenesis is not an absolute phenomenon, but that properties of the tumor cells play a paramount role (for a review see [10]). With respect to growth pattern, malignant tumors can be roughly divided in two types: expanding and infiltrating lesions. Likely, the expanding growth pattern is associated with the presence of intercellular adhesion molecules holding the tumor cells together, and a relative absence of proteolytic and migratory activity. Expanding, non-infiltrative tumors are dependent on angiogenesis for optimal nutrition and oxygenation. Other tumors may show an infiltrating phenotype, hematological malignancies being extreme examples. However, tumors are mostly heterogeneous, and mixed phenotypes occur. Tumor lesions with an expanding growth pattern push away the surrounding tissues and vessels and readily develop central hypoxia. In such tumors, hypoxia may induce expression of hypoxia-inducible factor-1 (HIF-1) which is a transcription factor for the potent angiogenic molecule Vascular Endothelial Growth Factor-A (VEGF-A) [2]. Expression of this protein leads to angiogenesis which is accompanied by active ingrowth of vascular sprouts into the tumor lesion and the formation of a tumor vascular bed that will drive further outgrowth [11–14]. Tumors that grow infiltratively may circumvent hypoxia, if they arise in a densely vascularized, well-perfused and large organ, like brain, liver, lung or kidney [15–17]. These tumors then may exploit the existing vasculature, a process called co-option, and grow independent of angiogenesis. Nevertheless, infiltrating tumors still may induce angiogenesis if the factors responsible for vessel neoformation are excreted by the tumor cells constitutively, in a genetically determined fashion as in von Hippel Lindau disease (VHL, [18, 19]) or, when they expand to larger, more homogenous tumor nests, in response to hypoxia.

Although it is well known from clinical oncology that a variety of tumors, many of which are infiltrative, thrive in large, densely vascularized organs, a large number of tumors arise in tissues with limited dimensions like colon, bladder, stomach or skin. Such tumors may initially grow superficially or radially, utilizing oxygen that diffuses from the pre-existent vasculature. However, such tumors will eventually expand by growing in directions perpendicular to the plane of the tissue they arise in and quickly grow beyond the diffusion distance from pre-existent vessels. From this point on they can only expand to form bulky lesions if they acquire the capacity to induce angiogenesis. Therefore, in such tumors a selection pressure towards angiogenesis dependen-

cy is present. Obviously, this selective pressure is absent in tumors that develop in organs with extensive "three dimensional" vascular beds where angiogenesis is not a prerequisite for expansion. In conclusion, patterns of tumor vascularization will range from pure co-option on one side, to complete angiogenesis dependency on the other, and all intermediate phenotypes in between.

Mouse tumor models of cancer

Most mouse tumor models have used subcutaneous implanting for convenience. Tumor take is defined as the formation of a palpable or visible nodule and tumor load can simply be determined by straightforward volume measurement. Obviously, growth of tumors in the avascular subcutaneous space implies a selection for an angiogenic phenotype, just like in the colon or bladder wall as described above. We have shown in our laboratory that the tumor (vascular) phenotype heavily depends on specific tissue characteristics. Large differences exist between the phenotypes of xenografts, derived from the same tumor cell line, in the subcutaneous space and in brain, respectively [10, 16, 20, 21]. In these experiments a human melanoma cell line was used that expressed very little angiogenic activity. After implantation in the subcutaneous space, xenograft lesions grew over two months to small, highly necrotic tumors with only a small rim of viable tumor tissue. When the same cell line was introduced into brain parenchyma by injection in the internal carotid artery, it developed large lesions that completely lacked necrosis within three to four weeks ([16, 20], see also Figs 1A and C). Interestingly, we were able to demonstrate that these tumors indeed grew by co-option of pre-existent vessels, since no sign of vessel activation (vascular permeability, endothelial proliferation or loss of blood brain barrier function) could be observed. Accordingly, these brain lesions were not detectable in magnetic resonance imaging (MRI), even after contrast enhancement with Gd-DTPA, a small paramagnetic diagnostic agent that extravasates from leaky tumor vessels, accumulates in the surrounding tissue and thereby generates an MRI signal [22]. The fact that the dependence on angiogenesis of tumors is largely determined by the vascularity of the host tissue may have biased many preclinical therapeutical studies on angiogenesis-inhibition. Where subcutaneous tumors may be sensitive to such treatment, in clinically relevant organs like brain, the same tumor types may fail to respond to anti-angiogenic therapy, or react by a conversion from an angiogenic to an infiltrative phenotype ([23] and own observation). Since infiltrative tumors may be invisible in MRI scans, this represents a serious complication, since MRI is increasingly regarded as the method of choice to monitor the effectiveness of angiogenesis inhibition therapy. MRI-based evaluation may mistakenly suggest an anti-tumor effect of angiogenesis inhibition therapy. In summary, it is obvious that for application of clinical angiogenesis inhibition therapy, it is crucial to determine in advance the true angiogenesis-dependence of the tumors to be treated, and to be aware of effects of the therapy on diagnostic imaging results.

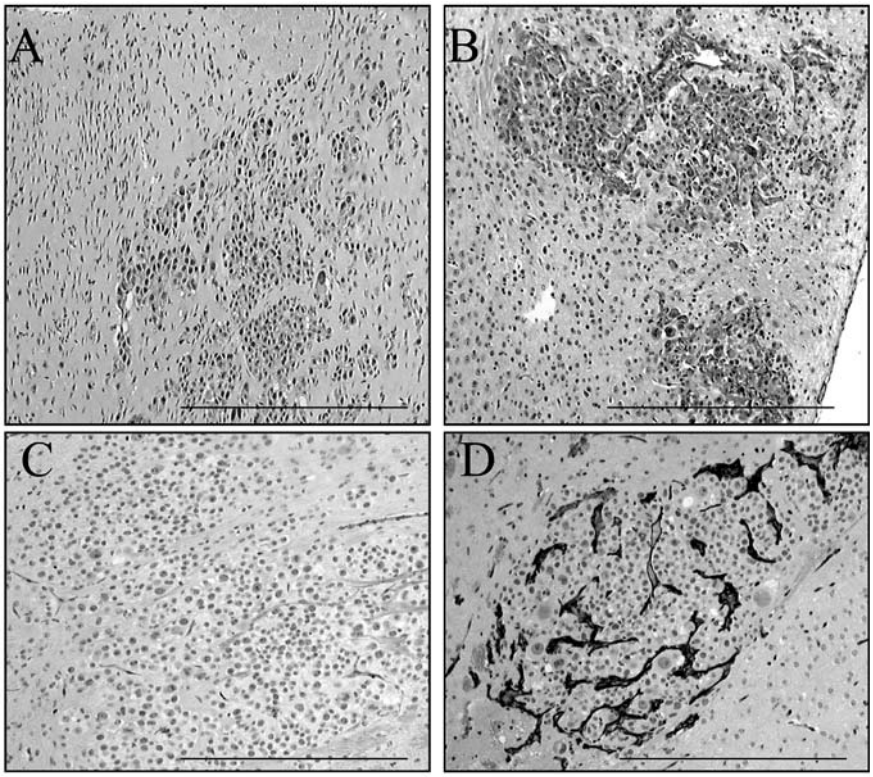

Figure 1. (A, C) Representative example of an infiltratively growing tumor in mouse brain. Mel57 human melanoma cells were injected in the internal carotid artery of mice as described [16]. After 3 weeks animals were sacrificed, the brains removed, fixed in buffered formalin and slices subjected to H&E staining (A) or CD34 endothelial staining (C). Blood vessels in these tumors are all pre-existent, as illustrated by the presence of an intact blood brain barrier and lack of upregulation of CD34 and other endothelial markers (C, and not shown). B/D: Results from a similar experiment, but now with Mel57 cells that were stably transfected with VEGF165. Note the highly dilated vessels in the VEGF-expressing tumors. (B) H&E staining, (D) CD34 endothelial staining. Bars represents 500 μm. Original magnification: 250 ×. (For colored picture see color plate 4)

Tumor dormancy and the angiogenic switch

Many human tumors are accompanied by a dense vascular bed and by hypertrophy of peritumoral vessels. In 1971 the hypothesis was formulated by Folkman and colleagues that this tumor vascular bed was not a modulated and incorporated pre-existent vasculature, but a newly formed one, generated by a process mimicking various aspects of embryonic vessel formation, which was called angiogenesis [24]. As a consequence, the hypothesis emerged that solid tumors are dependent on angiogenesis for growth and also, metastasis. High tumor vascular density was taken as a sign of previous or ongoing angiogenesis. Indeed, micro-vessel density has been shown to correlate with poor prog-

nosis in a number of solid tumor types [25, 26]. This hypothesis received much attention, because it held the promise that inhibition of vascular neoformation could in fact result in inhibition of tumor growth, and possibly even of metastasis as well. The angiogenesis-dependence of solid tumors was verified in numerous studies showing that inhibition of angiogenesis leads to inhibition of tumor growth and even regression of tumors in a number of animal models [27–33]. Key observations confirming that tumors are indeed angiogenesis-dependent were derived from the phenomena of tumor dormancy and the angiogenic switch. Therefore, these will be discussed here in more detail.

In 1976, Gimbrone and Gullino showed that progression from a benign precancerous stage of mammary gland epithelial cells towards malignancy is accompanied by acquirement of angiogenic capacity [34]. Later this phenomenon was named "the angiogenic switch". Many reports state that human tumors can remain "dormant" for years owing to a balance between cell proliferation and apoptosis which is maintained by a lack of angiogenesis or by the presence of angiogenesis inhibitors [35]. When this balance is disrupted, an angiogenic switch may occur, followed by tumor outgrowth. The angiogenic switch has been particularly nicely illustrated in the Rip1-Tag2 mouse, a transgenic mouse in which the large T-antigen was cloned behind the insulin promoter. Rip1-Tag2 mice very reproducibly develop pancreatic islet carcinomas via a defined series of pre-malignant to malignant stages [36]. Sometimes, metastatic tumor lesions that lack angiogenesis may stem from parental lesions in which angiogenesis is markedly present (e.g., Lewis lung carcinoma). This apparently paradoxical phenomenon could be explained by assuming the presence of excessive levels of angiogenesis inhibitors in the circulation. The longer half-life of these endogenous angiogenesis antagonists may cause their systemic concentration to exceed that of stimulators and thus inhibit growth of small metastases at distal sites. This hypothesis formed the basis for the discovery of angiostatin, endostatin, vasculostatin and other endogenous inhibitors of angiogenesis in animal models [37, 38]. However, these inhibitors may be generated by human tumor cells as well [39, 40].

In primary tumors, absence of angiogenesis has been explained by a failure of the angiogenic switch to occur. What triggers this angiogenic switch is largely unknown, but several mechanisms are possible. The first is that, although dormant, the metastatic lesion slowly reaches a size where central hypoxia may develop, which then will induce expression of hypoxia-sensitive genes [41]. One of these genes encodes for vascular endothelial growth factor-A (VEGF-A), the most powerful angiogenic factor. A second mechanism involves DNA damage leading to constitutive expression of angiogenic growth factors like VEGF-A. Finally, dormancy of primary tumor lesions may be maintained by non-tumor related endogenous inhibitors like thrombospondin [42]. Loss of expression of inhibitors will then lead to an angiogenic switch.

Although the concept is broadly accepted, the actual occurrence of tumor dormancy in human patients is surprisingly poorly documented. Barnhill et al. explained the dormant state of micro-metastases of human melanoma by a lack

of significant tumor vascularity and low but comparable rates of proliferation and apoptosis [43]. In addition, there are some reports on the presence of micro-metastases transferred to recipients as dormant lesions in organ transplants [44]. Very recently, we found that removal of a primary colon carcinoma lead to a flare-up of metabolic activity in its liver metastasis and a fall in plasma levels of angiostatin and endostatin (Peeters C. et al. , manuscript in preparation). This would be the first description in a human patient of the phenomenon that led to the discovery of the endogenous inhibitors of angiogenesis. Taken together, there is clear evidence that in a number of tumor types, the state of dormancy is associated with a lack of angiogenesis, implying that in these tumors outgrowth is dependent on the neoformation of a vascular bed.

Angiogenesis

For angiogenesis to occur, profound changes in vessel architecture have to take place. Quiescent vessels are built up by a luminal lining of endothelial cells, a basement membrane consisting of members of the collagen family, laminin and fibronectin, and an abluminal layer of perivascular cells, pericytes in capillaries and smooth muscle cells in larger veins and arteries. During angiogenesis, this structure needs to be temporarily destabilized. This is accomplished by secretion by endothelial cells of metalloproteases (MMPs) which degrade the basal lamina, and secretion of angiopoietin-2 resulting in detachment of pericytes [45, 46]. The ensuing vessel hyper-permeability allows extravasation of fibrinogen and its conversion to fibrin by tissue factor activity which is upregulated in endothelial cells during angiogenesis [47]. Perivascular fibrin serves as a substrate on which newly formed endothelial cells can adhere and migrate to form new sprouts which develop into mature vessels. During maturation, a new basement membrane is formed by the endothelium, and perivascular cells are recruited by secretion of angiopoietin-1 by endothelial cells. Platelet-derived Growth Factors play a role in this process as well [48].

The angiogenic process is tightly controlled by a number of positive and negative regulators.

Many negative regulators consist of proteolytic fragments of larger proteins. Well known examples are angiostatin, a degradation product of plasminogen, and endostatin, a fragment of collagen type XVIII [37, 38]. Both act by inducing apoptosis in endothelial cells via as yet unidentified mechanisms. It has been suggested that endostatin interferes with binding of VEGF to VEGFR2 [49]. Other examples of endogenous inhibitors are tumstatin [50, 51], thrombospondin [52] and soluble Flt-1 (sFlt-1), a secreted splice variant of one of the tyrosine kinase transmembrane receptors for VEGF-A [53]. Because sFlt-1 only consists of the extracellular domain of this receptor, VEGF-A is efficiently captured and neutralized in the circulation.

Among the positive regulators, members of the VEGF family and the angiopoietins play prominent roles. One of the most potent and important

angiogenic factors is VEGF-A. In a number of tumor types, VEGF-A levels correlate with vascular density and poor prognosis [25, 54]. Examples of VEGF-A-induced *in vivo* vascular effects are shown in Figures 1B and D. *In vitro*, VEGF-A induces migration and proliferation of endothelial cells, expression of tissue factor and MMPs by endothelial cells and tube formation. Most of these activities appear to be mediated by VEGF receptor-2 (also referred to as Kinase insert Domain-containing Receptor (KDR, human homologue) or Fetal Liver Kinase (Flk-1, mouse homologue). The other VEGF tyrosine kinase receptor VEGFR1 (also referred to as Fms-like tyrosine kinase (Flt-1)) has been proposed to be involved in migration, not only of endothelial cells, but also of cells of hematopoietic origin like monocytes. VEGFR1 knock-out mice die early in development, because endothelial cells fail to form tubular structures [55]. Whether tyrosine kinase activity of VEGFR1 during embryonic development is necessary is doubtful, since the intracellular part of VEGFR1 is dispensable during embryonic development. Mice lacking only this part of the molecule develop normally [56]. VEGFR1 function in tumor angiogenesis is not unequivocally resolved. We showed that a mutant of VEGF-A that recognizes VEGFR1 but not VEGFR2 was inactive in *in vivo* tumor angiogenesis assays (unpublished results). Accordingly, Placental Growth Factor (PlGF), a VEGFR1-selective ligand, did not induce an angiogenic response as well. The assays in which these activities were determined involved generation of brain tumors of a human melanoma cell line that was transfected to secrete (variants of) angiogenic molecules in the absence of endogenous VEGF-A. However, other groups have reported that in transgenic mice overexpressing PlGF in the dermis, angiogenesis is more prominent than in normal mice [57] and treatment with PlGF induced increased tumor angiogenesis which in turn could be inhibited with an anti-Flt-1 antibody [58]. Whether these phenomena can be explained by competitive displacement of endogenous VEGF-A from VEGFR1 by PlGF, thereby making more VEGF-A available for VEGFR2-binding and activation, remains unsettled.

The recognition that the VEGF-A/VEGFR2 signaling system is crucial for the angiogenic process is based on numerous observations. First, not surprisingly, VEGF-A null mice are not viable, and even heterozygous null mice die at embryonic day 11 [59]. Also, VEGFR2 knock out mice die early during development due to a lack of differentiation of endothelial cells [60]. A viral VEGF family member, VEGF-E, is a selective VEGFR2 ligand and appears to have comparable activities [61, 62]. This again points to a role for VEGFR2, rather than VEGFR1, in tumor angiogenesis. Finally, disruption of VEGFR2 alone was sufficient to prevent angiogenesis in a number of tumor types [63].

The VEGF family

VEGF-A is a member of a larger family of angiogenic growth factors. It also includes PlGF and VEGF-B, both VEGFR1-selective ligands, VEGF-C and D,

ligands for VEGFR2 and VEGFR3 (also referred to as Flt-4, a tyrosine kinase receptor involved in lymphangiogenesis) and the viral variant VEGF-E, a selective VEGFR2 ligand [61]. Apart from VEGFR1 and VEGFR2, additional receptors for VEGF exist, depending on the specific VEGF-A isoform. The VEGF-A pre-RNA is differentially spliced to generate isoforms of 121, 145, 165, 189 and 206 amino acids. $VEGF_{165}$ and $VEGF_{189}$ bind via basically charged domains, encoded by exon 7 in $VEGF_{165}$ and exons 6 and 7 in $VEGF_{189}$ to neuropilins 1 and 2 (via exon 7) and heparan sulfate proteoglycans (via exon 6) (for review, see [64]). The functional importance of the different isoforms has only recently received attention in the literature. The larger isoforms are sequestered by the extracellular matrix and it has been suggested that in this way the extracellular matrix serves as a reservoir of biologically active protein. Bio-active aminoterminal fragments can be quickly released upon cleavage of $VEGF_{165}$ and $VEGF_{189}$ by plasmin and urokinase-plasminogen activator respectively [65, 66]. Recently, an alternative role for matrix-bound VEGF was suggested, based on the observation that endothelial cells were only able to migrate on a matrix to which $VEGF_{189}$ or $VEGF_{165}$ was bound, possibly mediated by a direct interaction between these VEGF isoforms and certain integrin moieties [67].

This finding implies that $VEGF_{121}$ is not able to induce endothelial migration because this isoform has no affinity for matrix components. Interestingly, when $VEGF_{121}$ was expressed in our experimental brain tumor model we found that, in striking contrast to the larger isoforms, only very few blood vessels were present intratumorally. In contrast, $VEGF_{121}$ caused dilation of pre-existent vessels that were pushed aside by the expanding tumor [22]. In some of these dilated vessels, we found numerous proliferating endothelial cells, sometimes filling up the vessel lumen, leading to a glomeruloid microvascular proliferation-like phenotype which is frequently found in human cancer lesions with high angiogenic activity, like glioblastoma multiforme (GBM). Indeed, this observation is suggestive of endothelial cells that do not migrate since directional signals (matrix-bound VEGF) are lacking. An example of a $VEGF_{165}$-expressing Mel57 tumor in mouse brain is given in Figures 1B and 1D.

Targeting angiogenesis and tumor vascularity

Targeting the VEGF/VEGFR2 complex and its downstream signaling events have become main topics of research. Blockade of VEGFR2 signaling has been shown to be an effective way of inhibiting tumor angiogenesis and growth in subcutaneous animal tumor models. To accomplish this, several approaches have been developed, among which using antibodies directed against VEGF-A or VEGFR2 [29, 68]. On the basis of VEGF-A structure, other antagonists were designed as well [69, 70]. Despite an efficient anti-angiogenic capacity of these compounds, the approach of small compound inhibitors is more promising since their synthesis is cheaper and oral administration is possible. Via high-throughput screening, a number of companies

have now developed inhibitors that antagonize tyrosine kinase activity of (a subset of) angiogenic receptors and are very effective in animal tumor models [71, 72]. As shown above, successful treatment of tumors will depend strongly on their dependence on angiogenesis. Although it is probably impossible to exactly determine to what degree a certain individual tumor is angiogenesis-dependent, this may be estimated via a triad of approaches: 1) by contrast-enhanced MRI, which visualizes vascular permeability associated with local VEGF-A production, 2) by analysis of plasma VEGF-A levels, although this will probably reveal preferentially the non-matrix binding isoform $VEGF_{121}$ and not the predominant and most active larger isoforms, 3) by immunohistochemical and *in situ* hybridization analysis of tumor biopsies for expression of VEGF-A isoforms, microvascular density and endothelial cell proliferation. Tumor lesions with a high score in these analyses may be considered for angiogenesis inhibition treatment. It is however possible that they harbor a hidden angiogenesis-independent component, that will emerge only under inhibitory treatment conditions. Therapy should therefore include also an anti-tumor modality. The true challenges are the strictly co-opting tumors. Because changes in vascular parameters in such lesions may be very subtle, it is unclear whether an anti-vascular approach, directed at differentially expressed molecules, is feasible. Such an approach, that almost certainly will have to be combined with anti-tumor therapy, still has to be developed.

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Mechanisms of Angiogenesis
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Hormones and the neovascularization process: role of angiotensin II

Jean-Sébastien Silvestre and Bernard I. Lévy

INSERM U541, Hôpital Lariboisière, 41 bvd de la chapelle, 75475 Paris cedex 10, France

In embryogenesis, the vascular system develops from vasculogenesis in which endothelial cell precursors (angioblasts) differentiate into endothelial cells to form primitive capillary network. The subsequent growth, expansion and remodeling of these primitive vessels into a mature vascular network is referred to as angiogenesis. However, a large body of evidence underscores that both vasculogenesis and angiogenesis also proceed in the adult during pathological conditions such as tumor growth or ischemic diseases. The ability of organisms to spontaneously develop collateral vessels represents an important response to vascular occlusive diseases which determine the severity of residual tissue ischemia. Neovascularization of ischemic cardiac or skeletal muscle may be sufficient to preserve tissue integrity and/or function, and may thus be considered to be therapeutic. In addition, under certain circumstances, including advanced age, diabetes, hypertension and hypercholesterolemia such native angiogenesis is impaired leading to the development of disabling symptoms related to tissue ischemia and supporting the requirement of vascular surgery or therapeutic strategies designed to increase native blood vessel growth. Therefore, understanding the mechanisms of the neovascularization process is of major importance. Numerous factors modulated the angiogenic reaction. Among these factors, Angiotensin II (Ang II) the biological active component of the renin-angiotensin system (RAS) might be involved in both beneficial angiogenesis and pathological vessel growth. This chapter focuses on the role of Ang II in angiogenic process mainly in the setting of ischemia.

Molecular physiology of ischemia-induced neovascularization

Neovascularization in response to tissue ischemia constitutes a natural host defense intended to maintain tissue perfusion required for physiologic organ function. In adult organisms, both hypoxia and inflammation are usually considered to be major stimuli for ischemia-induced neovascularization [1].

Hypoxia-related pathways

The main mechanism of hypoxia-induced angiogenesis involves the rise in hypoxia-inducible factor-1 (HIF-1) protein. HIF-1 binds to specific hypoxia-responsive element in the regulatory regions of several hypoxia-sensitive genes, such as vascular endothelial growth factor (VEGF) [2]. The expression of VEGF receptor type 1, VEGFR1 (Flt-1), mRNA is also hypoxia-inducible [3], indicating that the endothelium must sense hypoxia in order to be maximally receptive to VEGF stimulation. Endothelial cell expression of VEGF receptor type 2, VEGFR2 (KDR), protein is also induced in ischemic tissue via a paracrine mechanisms [4]. Finally, placenta growth factor, which binds to VEGFR1, is induced by hypoxia and positively regulates the neovascularization process in ischemic tissue [5].

In hypoxic condition, VEGF is then secreted and binds to cognate receptor tyrosine kinases (VEGFR1 and VEGFR2) located on the surface of vascular endothelial cells. Receptor ligation triggers a cascade of intracellular signaling pathways that initiate angiogenesis. The serine/threonine kinase Akt, also known as protein kinase B or Rac kinase, has been shown to play a key role in matrix adhesion and integrin-mediated signal transduction and in the suppression of apoptotic cell death induced by growth factor deprivation. VEGF can activate the survival and cell proliferation promoting phosphatidylinositol 3'-kinase/Akt pathway [6, 7]. Enhanced Akt signaling in the endothelium promotes angiogenesis in ischemic limbs of normocholesterolemic rabbits [8]. Akt may contribute to the angiogenic reaction by phosphorylating endothelial nitric oxide synthase (eNOS) leading to a persistent calcium-independent enzyme activation or by inhibiting the apoptotic process [9]. Interestingly, VEGF-induced endothelial cells survival and migration requires the activation of Akt, it is therefore likely that Akt is a downstream effector of VEGF-related pathways [9].

VEGF may also act by rising eNOS expression which plays an essential role in postnatal neovascularization. Endothelial NOS (eNos) knockout mice are characterized by impaired angiogenesis in response to ischemia [10]. Nitric oxide (NO) may also contribute to VEGF related-pathways. In mice ischemic hindlimb, the angiogenic response to VEGF involves the activation of eNOS gene [10]. Similarly, in ischemic heart, the induction of coronary collateralization by VEGF requires the production of NO [11]. Finally, human endothelial NOS gene delivery promotes angiogenesis in the rat ischemic leg, supporting the idea that eNOS works in conjunction with VEGF to promote angiogenesis [12].

Inflammation-related pathway

Neovascularization appears to be also controlled by the inflammatory process that occurs in the ischemic area. Monocytes/macrophages accumulate during vessel growth in ischemic tissues [13]. Arterial femoral ligation during the phase of monocyte depletion resulted in a reduction of blood flow reconstitu-

tion. This inhibition could be reversed by an injection of isolated monocytes, demonstrating then a functional link between the monocyte concentration in the peripheral blood and the enhancement of neovascularization [14]. The presence of these inflammatory cells is associated with local secretion of several angiogenic factors, including cytokines such as IL-2 and TNF-α, growth factors such as VEGF and basic fibroblast growth factor (bFGF), and matrix metalloproteinases (MMP) [15, 16]. Recently, a macrophage-derived peptide PR39 has been shown to inhibit the degradation of HIF-1 α leading to increased VEGF expression and accelerated formation of vascular structures *in vitro* [17]. In addition, cytokines, such as IL-1β, strongly increase HIF-1α activity in cultured human hepatoma cells, emphasizing a possible role of HIF-1 α as a trans-acting factor in inflammatory process as well [18]. During the inflammatory reaction, anti-inflammatory cytokines are also produced and tend to modulate the inflammatory process. Recent studies underlined the involvement of anti-inflammatory cytokines in the angiogenic process. Angiogenesis is increased in ischemic hindlimb of mice deficient for the anti-inflammatory cytokine IL-10 [19]. Such an effect was blocked by MMPs inhibitor, despite a sustained upregulation of VEGF. Activation of MMP pathway is then a primary event in the series of cellular events leading to vessel growth in ischemic leg [20]. MMPs may play a central role in the angiogenic reaction by degrading extracellular membranes and basement membrane structures, allowing endothelial cell migration to occur and resulting in the liberation of growth factors. Leukocytes infiltration in the infarct heart and infarct revascularization is reduced in plasminogen activator/MMP9-deficient mice confirming the crucial link between inflammation, proteinases and ischemia-induced angiogenesis [21].

Bone marrow derived cells-related pathway

Recent studies provide increasing evidence that postnatal neovascularization does not rely exclusively on sprouting of pre-existing vessels, but also involves bone marrow-derived circulating endothelial progenitor cells (BM-EPC). A specific subset of endothelial cell precursor (including human CD34-expressing cells, human and rabbit flk1-expressing cells and mouse sca-1-expressing cells) was shown to home and incorporate into site of neovascularization in ischemic tissue where differentiation into endothelial cells is completed [22, 23]. Moreover, BM-EPC can be grown out of isolated CD34-positive cells *in vitro* and make a significant contribution to blood vessel formation [24, 25]. Finally, the rise in BM-EPC levels may contribute to the pro-angiogenic effect of growth factors, such as VEGF or granulocyte macrophage-colony stimulating factor [22, 26]. The stimulation of mobilization and/or differentiation of bone-marrow derived cells may thus provide a useful novel therapeutic strategy to improve postnatal angiogenesis. In this view, it has been shown that administration of BM-EPC increases blood flow recovery and capillary density in both ischemic hindlimb and myocardium of rodents [27, 28]. In human BM-EPCs

from adults have been found to induce new blood vessel formation in the infarct bed and proliferation of pre-existing vasculature after experimental myocardial infarction [29]. Recent studies also suggest that BM-MNCs without purification of EPC might be sufficient and even more effective cellular source for therapeutic neovascularization. Indeed, BM-MNCs contain various kinds of cell lineage, such as hematopoietic cells, fibroblasts, osteoblasts and myogenic cells, as well as endothelial lineage, such mixed population of BM-MNCs can work both beneficially and harmfully in angiogenesis therapy. BM-MNCs secrete potent angiogenic ligands and cytokines such as basic fibroblast growth factor, VEGF, angiopoietin 1, interleukin 1β and tumor necrosis factor α, incorporate into neocapillaries and enhances collateral perfusion in ischemic myocardium and hindlimb [30, 31]. Similarly, autologous implantation of BM-MNCs in ischemic limbs of patients with peripheral arterial disease improved transcutaneous oxygen pressure, rest pain and pain-free walking time [32].

Angiotensin II and ischemia-induced neovascularization

Growth of new blood vessels is a complex process regulated by numerous factors. Among these factors, Ang II, the main effector peptide of the renin-angiotensin system, may contribute to vessel growth regulation. The physiology of Ang II continues to be a major field of investigation and recent reports suggest that Ang II may be involved in new vessel growth regulations, especially in neovascularization of ischemic tissue.

The renin angiotensin components

Ang II is the active component of the renin-angiotensin system (RAS). This octapeptide hormone plays an important role in the regulation of blood pressure, plasma volume and sympathetic nervous activity. Ang II has also a pathophysiological role in cardiac hypertrophy, myocardial infarction, hypertension and atherosclerosis. It is synthesized via the classical RAS and locally via tissue RAS. In the classical RAS, circulating renal-derived renin cleaves hepatic-derived angiotensinogen to form the decapeptide angiotensin I (Ang I), which is converted by angiotensin-converting enzyme (ACE) to the active Ang II [33]. However, Ang I and II can be generated by other enzymatic pathways. Ang I can be formed by nonrenin enzymes such as tonin or cathepsin, and Ang I can be converted to Ang II by enzymes as trypsin, cathepsin and chymase. Alternatively, Ang I can also be processed into the heptapeptide Ang I(1-7) by tissues endopeptidases. Nevertheless, the quantitative contribution of these alternative pathways to the generation of circulating Ang II remains unclear.

Although the RAS was initially described as a circulating system, many of its components are localized in tissues, indicating the existence of a local tis-

sue RAS, as well [34]. All components of the RAS, except renin, have been detected in the vasculature. It is therefore likely that local angiotensin formation does occur, but depends, at least under normal circumstances, on the uptake of renal renin from the circulation. Tissues may thus regulate their local angiotensin concentrations by varying the number of renin receptors and/or renin-binding proteins, the ACE level, the amount of metabolizing enzymes and the angiotensin receptor density. In addition, the components of tissular RAS may be different from those of circulating RAS. Indeed, non ACE-pathways, such as chymase, may represent an important mechanism for conversion of Ang I to Ang II in heart, kidney and vasculature.

The discovery of specific Ang II receptor antagonists has confirmed the existence of various subtypes of Ang II receptors which are termed AT1 and AT2. Both the AT1 and AT2 receptors have been cloned. In rodents, AT1 have been further subdivided into AT1a and AT1b. Two other angiotensin receptors have been described AT3 and AT4 receptors but the pharmacology, the role and the exact signaling pathways of these receptors are still undefined.

AT1 receptors

AT1 receptors are widely distributed throughout the cardiovascular, renal, endocrine and nervous systems. In vasculature, AT1 are present at high levels in smooth muscle cells and at relative low levels in the adventitia. AT1 receptors are also expressed in some animal models of cultured aortic endothelial cells [33]. AT1 belongs to the superfamilly of G-protein-coupled receptors that contain 7 transmembrane regions. AT1 typically activates phospholipase C (PLC) through the Gq protein, although it may also signal through Gi, G11/13 and Gs. This leads to multiple specific signaling cascades including non-receptor and receptor-associated tyrosine kinase-mediated protein phosphorylation [35]. One of the earliest detectable events resulting from AT1 stimulation is a rapid PLC-dependent hydrolysis of phosphatidylinositol 4,5 bi-phosphate, production of inositol 1, 4, 5-triphosphate and diacylglycerol, calcium mobilization and protein kinase C (PKC) stimulation. Ang II also activates phospholipase AII, and subsequently eicosanoids production which influence mitogene activated protein kinase (MAPK) and redox-sensitive pathways [36, 37]. Indeed, Ang II-stimulated activation of NAD(P)H oxidase occurs through release of arachidonic acide metabolites, triggering PKC activation [38]. However, it is also likely that Ang II augments NAD(P)H oxidase-mediated reactive oxygen species (ROS) production by enhancing the abundance of NAD(P)H subunits mRNA via transcriptional pathways [39]. AT1 receptor also stimulates intracellular members of Janus family kinases Jak2 and Tyk2 [40] and subsequently, vascular growth, remodeling and repairs [33, 35]. Ang II, through its AT1 receptor, promotes cells migration and induces changes in cell shape by induction of focal adhesion kinase (FAK) autophosphorylation leading to FAK binding to Grb2 and an association with the GDP-

GTP exchange protein Sos and Ras which in turns leads to extracellular signal-regulated kinase (ERK) 1/2 activation. [33]. Finally, phosphatidyl inositol 3 kinase (PI3K), which phosphorylate inositol lipids, are also associated with AT1 receptor activation. This may lead to activation of protein serine/threonine kinase Akt/protein kinase B and modulation of the apoptotic process [9, 33].

AT2 receptors

AT2 receptor is mainly expressed in fetal mesenchymal tissues. The expression of this receptor declines after birth. In adult, AT2 receptor expression is detectable in the pancreas, heart, kidney, brain, adrenals and vasculature. In blood vessels, AT2 receptor has been detected in small arteries and capillaries, mainly in endothelial cells [41]. AT2 receptor also contains 7 transmembrane regions. However, the signaling pathways involved in AT2 receptor activation are not fully understood but appear to involve Gαi2 and Gαi3 and protein G-independent pathway. In biochemical studies in cultured neurons, AT2 receptor activates serine/threonine protein phosphatase (PP2A) [42, 43]. PP2A activation results in dephosphorylation and inactivation of growth factor-activated MAPK and, in particular, inactivation of ERK 1/2. Alternatively, AT2 receptor has been shown to promote apoptosis through stimulation of ERK phosphatase leading to dephosphorylation and inhibition of MAPK and Bcl2 [44]. Stimulation of AT2 receptor is also associated with increased generation of bradykinin, NO, and cGMP. In aortic homogenates, AT2 receptor induces inhibition of the amiloride-sensitive Na^+/H^+ exchanger leading to intracellular acidification, stimulation of kininogenase activity, and subsequently bradykinin release and NO production [45]. Finally, in AT2 receptor deficient mice, blood pressure is only marginally elevated compared to wild-type mice. This has been attributed to increase production of vasodilatator prostanoids PGE_2 and PGI_2 suggesting a role for prostacyclins in AT2 signaling [46].

Ang II and hypoxia-related pathways

Hypoxia activates a number of genes that are important in cellular and tissular adaptation to low oxigen conditions. As previously mentioned, cellular events occuring in hypoxic environment are triggered, at least in part, by the specific binding of HIF-1 to the hypoxic response elements of numerous genes including *VEGF*. However, recent evidence suggests that HIF-related pathway may be activated in non hypoxic environment by stimuli such as insulin, insulin-like growth factors 1 and 2 and epidermal growth factor [47]. In addition, Ang II can also increase HIF-1α in vascular smooth muscle cells to levels that are substantially more elevated that the hypoxic treatment. HIF-1α induced by Ang II is located in the nucleus, binds to the hypoxic response element, and is transcriptionaly active [47]. By binding to the AT1 receptor, Ang II activates at

least two separate pathways involved in the induction of the HIF-1 transcription complex. Ang II strongly increases HIF-1α gene expression through activation of the diacylglycerol-sensitive PKC. Ang II also relies on ongoing translation to maintain elevated HIF-1α protein levels by ROS-dependent activation of PI3K pathway [48]. An active HIF-1 complex is thus formed that increases the expression of its target genes, including VEGF. These results shed light on the mechanisms involved in the Ang II modulation of angiogenesis, and suggest that the effect of Ang II in HIF-1 signaling may be involved in Ang II pro-angiogenic effect.

Ang II and growth factor related pathways

Growth factors are involved in the regulation of the angiogenic process and may represent a putative target for Ang II-induced angiogenesis. In the setting of ischemia, Ang II administration raises angiographic score in ischemic tissue in association with a marked increase in VEGF protein content [49]. In addition, Ang II angiogenic effect is prevented by both AT1 receptor blockade and VEGF neutralizing antibody. Thus, pro-angiogenic action of Ang II is mediated by AT1 receptor through the sustained activation of VEGF production within the ischemic tissue [49]. RAS has also been shown to modulate the angiogenic response to electrical stimulation in the rat skeletal muscle through the activation of the VEGF-dependent pathway [50]. In addition, renin gene transfer in Dahl S rats with low plasma renin activity restores angiogenesis and VEGF expression associated with electrical stimulation [51]. Finally, treatments with anti-VEGF neutralizing antibody or antisense oligomers against VEGF mRNA reduce Ang II/AT1 receptor-induced angiogenesis in hamster sponge granulomas [52]. Ang II angiogenic effect is associated with a rise in eNOS protein content. This increase is hampered by treatment with neutralizing antibody against VEGF suggesting that eNOS actes downstream VEGF. In this view, angiogenesis in eNOS-deficient mice is not improved by Ang II treatment despite an increase in VEGF protein contents demonstrating that eNOS is required for Ang II-induced vessel growth and lies downstream Ang II-VEGF signaling in the revascularization process after ischemia [49] (Fig. 1).

In addition, Ang II may stimulate the production of other growth factors such as basic fibroblast growth factor [53]. Interestingly, basic fibroblast growth factor has been shown to modulate eNOS level and nitric oxide production leading to endothelial cell differentiation into vascular tubes [54]. Ang II also induces angiopoietin-2 and VEGF expression in an *in vivo* corneal assay [55]. Angiopoietin-2 is the ligand for Tie2 receptor, disrupts blood vessel formation and initiates the angiogenic process.

Interestingly, chymostatin, an inhibitor of chymase, markedly inhibits both upregulation of VEGF mRNA and angiogenesis in granulation tissues treated by basic fibroblast growth factor [52]. Hence, one can speculate that local chy-

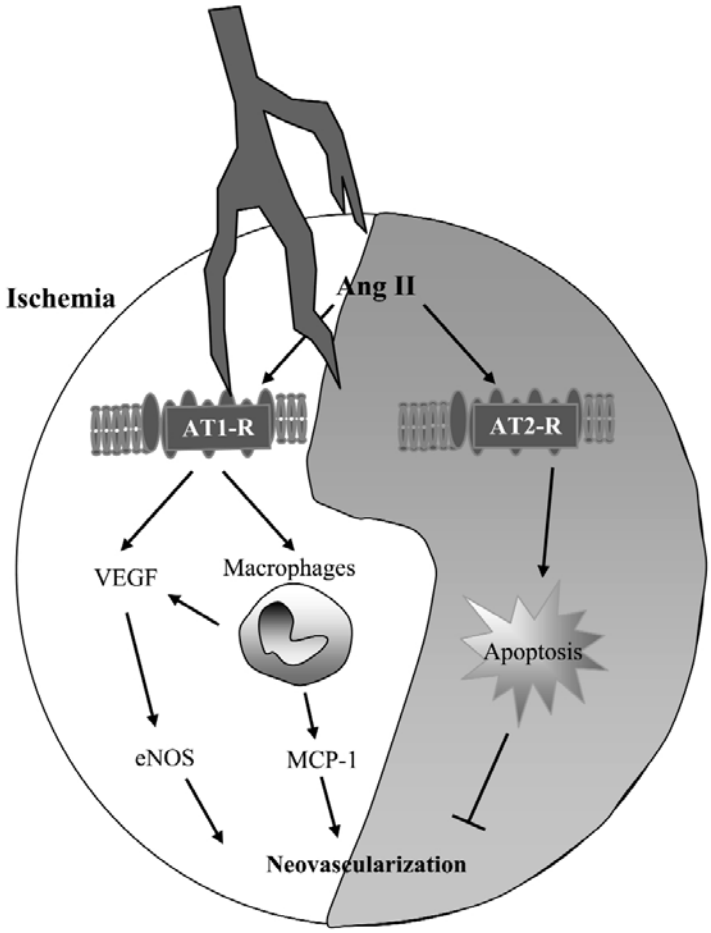

Figure 1. Ang II and post-ischemic neovascularization. AT1 receptor positively regulates the angiogenic process in the setting of ischemia. Conversely, AT2 receptor activation hampers new vessel growth, suggesting that Ang II may tightly regulate the revascularization reaction. (For colored picture see color plate 5)

mase-Ang II-VEGF pathway may operate in some tissue as the primary mediator of angiogenesis. Recently reported mechanisms of AT1 receptor activation, such as receptor transactivation of tyrosine kinase receptors suggest that Ang II may have growth factor and cytokine-like properties by alternative pathways. Receptor transactivation may be defined as the process whereby ligand stimulation of one receptor leads to activation of another distinct receptor. It has been suggested that mitogenic response to AT1 receptor may be mediated by activation of epidermal growth factor receptor. Ang II can transactivate epidermal growth factor receptor by tyrosine kinase phosphorylation, ROS activation or cleavage of the epidermal growth factor receptor [33]. Similarly,

in vascular smooth muscle cells, Ang II may transactivate the platelet-derived growth factor B-receptor, leading to tyrosine phosphorylation of Shc proteins, resulting in subsequent complex formation between Shc proteins and the platelet-derived growth factor receptor [56]. Other studies have demonstrated that Ang II also induces rapid transactivation of the mitogenic insulin-like growth factor I receptor [57].

Ang II and inflammation-related pathways

As previously mentioned, the inflammatory reaction plays a key role in the neovascularization process. Recent work has shown that Ang II has significant pro-inflammatory actions in the vasculature walls, and as a result may affect new vessel growth. Ang II influences the expression of pro-inflammatory molecules in the vessel wall that influence multiple steps in monocyte recruitement into the injured vessel. In endothelial cells, Ang II upregulates vascular cell adhesion molecule-1, intercellular adhesion molecule and E-selectin expression through a ROS-dependent pathway [58]. These are important adhesion molecules that bind very late antigen-4 on the surface of circulating leukocytes, initiating their recruitment into the vessel wall. In vascular smooth muscle cells, Ang II stimulates the production of vascular cell adhesion molecule-1, chemokine monocyte chemotactic protein-1 (MCP-1) and the cytokine IL-6 [58]. MCP-1 is a small chemokine that specifically attracts monocytes and T lymphocytes expressing the CCR-2 receptor. Interestingly, MCP-1 administration has been shown to activate the neovascularization process in mice ischemic leg [16]. IL-6 is a glycoprotein abundantly secreted by activated macrophages and vascular smooth muscle cells. IL-6 has paracrine actions to promote smooth muscle cell proliferation involving the local production of PDGF [59]. The mechanisms by which Ang II induces expression of inflammatory genes overlap those typical of the pro-inflammatory cytokines, such as TNF. Specifically, Ang II activates the potent cytoplasmic transcription factor, nuclear factor NFκb, a protein that controls networks of chemokine-modulating, growth factor-modulating, translational control and cellular survival genes [58]. Ang II activates the translocation of the sequestered cytoplasmic NFκb complex through targeted proteolysis of the Iκb inhibitors but also induces the processing of the DNA binding form, NFκb1. NFκb activation appears to be downstream from the NAD(P)H oxidases and is involved in Ang II-induced activation of IL-6, MCP-1 and vascular cell adhesion molecule-1 [60–62].

Our own work has underlined the crucial link between Ang II, inflammation and the angiogenic process [63]. Ang II raises macrophages infiltration in the Matrigel model in mice, in association with an increase in cyclooxygenase type II protein content. Furthermore, blockade of cyclooxygenase type II activity completely hampers the Ang II-induced cell ingrowth [63]. Similarly, infiltration of inflammatory mononuclear cells, including macrophages and T lymphocytes, and expression of VEGF and MCP-1 are suppressed in the ischemic tissues of AT1a knock-out mice. The impaired angiogenesis in AT1a-deficient

mice is rescued by intramuscular transplantation of mononuclear cells [64]. Taken together these results indicate that the Ang II/AT1 receptor pathway promotes neovascularization by supporting inflammatory cells infiltration and angiogenic cytokine expression.

The AT2 enigma

It is generally accepted that most of the well-known Ang II functions in the cardiovascular system are attributable to AT1. Among these, Ang II-induced activation of the neovascularization process is mediated by AT1 receptor activation. Little information is available regarding the physiological roles of AT2 and its signal-transduction pathway. Several lines of evidence suggest that the AT2 receptor might mediate opposite effects to those related to AT1 receptor activation. AT2 receptor activation has been shown to suppress the Ang II-induced stimulation of endothelial cell proliferation [65] whereas AT2 receptor blockade enhances the Ang II angiogenic effect in the rat subcutaneous sponge granuloma [66]. One important emerging function of the AT2 receptor concerns its pro-apoptotic role. AT2 promotes apoptosis in a wide variety of cell types and in vascular smooth muscle cells of spontaneously hypertensive rats *in vivo* [67, 68]. Mechanistically, AT2 has been shown to upregulate the pro-apoptotic protein Bax in cultured vascular smooth muscle cells [69]. These findings suggest a putative anti-angiogenic effect of AT2. However, number of recent reports indicate that AT2 in cardiovascular tissues may be growth promoting and share at least in part a common signaling pathways with AT1. Hence, AT2 mediates cardiac hypertrophy resulting from pressure overload and aortic hypertrophy induced by Ang II treatment [70, 71]. One of the important reasons for the disparity may be due to different experimental conditions. In particularly, AT2 expression is known to be unstable or even absent in cultured cells.

Recently, our group uncovered that in AT2-deleted mice, the vessel growth is specifically increased in the ischemic hindlimb with no effect on the non-ischemic contralateral hindlimb [72]. Consistent with these *in vivo* observations, cultured vascular smooth muscle cells transfected with an AT2-expression vector also exhibit decreased rates of DNA synthesis [73]. Another study also reports an antiproliferative influence of the AT2 receptor on different experimental models [65, 66]. Taken together, these results suggest that the AT2 receptor may inhibit neo-vessel growth *in vivo*. The increase in revascularization observed in the ischemic hindlimb of AT2-deleted mice is associated with a marked increase in the cell survivor factor Bcl-2 and a decrease in the number of apoptotic cells [72]. Bcl-2 can prevent and delay apoptosis induced by a wide variety of stimuli suggesting that Bcl-2 controls a distal step in the final common pathway for cell death [74]. The AT2 receptor has been shown to induce apoptosis in PC12W cells and confluent R3T3 cells [75]. Activation of the AT2 receptor also inhibits MAPK, resulting in the inactiva-

tion of Bcl-2 and the induction of apoptosis [76]. Hence, one can speculate that the AT2 receptor may control vessel growth associated with tissue ischemia through the regulation of the apoptotic reaction (Fig. 1).

The ACE inhibitor paradox

Ang II has pleiotropic actions at multiple points in the neovascularization process. However, ACE inhibition, has also been shown to rise cardiac capillary length density in stroke-prone spontaneously hypertensive rats [77] and to promote ischemia-induced angiogenesis in ischemic rabbit and rat hindlimbs [78, 79]. In addition, hypertension-induced impairment of neovascularization is reversed by ACE inhibition in ischemic leg of spontaneously hypertensive rats [80]. ACE catalyzes the conversion of Ang I to Ang II and the breakdown of bradykinin into inactive peptides. Hence, the pharmacological effect of ACE inhibitors may be in part mediated via inhibition of Ang II formation but also via bradykinin accumulation. Bradykinin is generated from the action of kallikreins on their substrate kininogen and acts by at least two bradykinin receptor subtypes, B_1 and B_2. The B_2 receptor is constitutively expressed in various tissues and is responsible for the majority of bradykinin effects. In contrast, B_1 has higher affinity for kinin metabolites and its expression is induced in pathological conditions. Activation of the B_2 receptor, leads to the release of nitric oxide and prostacyclin which modulate numerous biological function [81]. In addition, several lines of evidence underline the putative role of bradykinin in the modulation of angiogenesis. Bradykinin activates angiogenesis of coronary venules [82] and, in synergism with interleukin-1, enhances the angiogenic process in the rat subcutaneous sponge granuloma [83]. Local delivery of tissue kallikrein gene has been shown to stimulate angiogenesis in both ischemic and normoperfused skeletal muscle, through the activation of nitric oxide synthase and cyclooxygenase-2 [84, 85, 86]. Activation of the bradykinin-related pathway may thus account for increased angiogenesis in response to ACE inhibition. Recent studies confirms this hypothesis since ACE inhibition-induced vessel growth in ischemic leg is impaired in mice-deficient in bradykinin B2 receptor [87] (Fig. 2). An unresolved issue is whether the potentiation of bradykinin effects by ACE inhibitors is caused only by blocking bradykinin enzymatic hydrolysis. B_2 receptor is a G-protein-coupled receptor that is rapidly desensitized and internalized in response to high agonist concentrations. ACE inhibitors have been shown to inhibit and/or partially reverse the bradykinin-induced internalization of the B_2 receptor and to reactivate signaling events initiated by the B_2 receptor [88]. In fact, ACE inhibitors may augment bradykinin effects on B_2 receptor indirectly only when enzyme and receptor molecules are sterically closed, possibly forming a heterodimer [89]. Taken together, these data suggest that ACE inhibition may benefit patients with myocardial ischemia and with essential hypertension presenting lower limb vascular insufficiency.

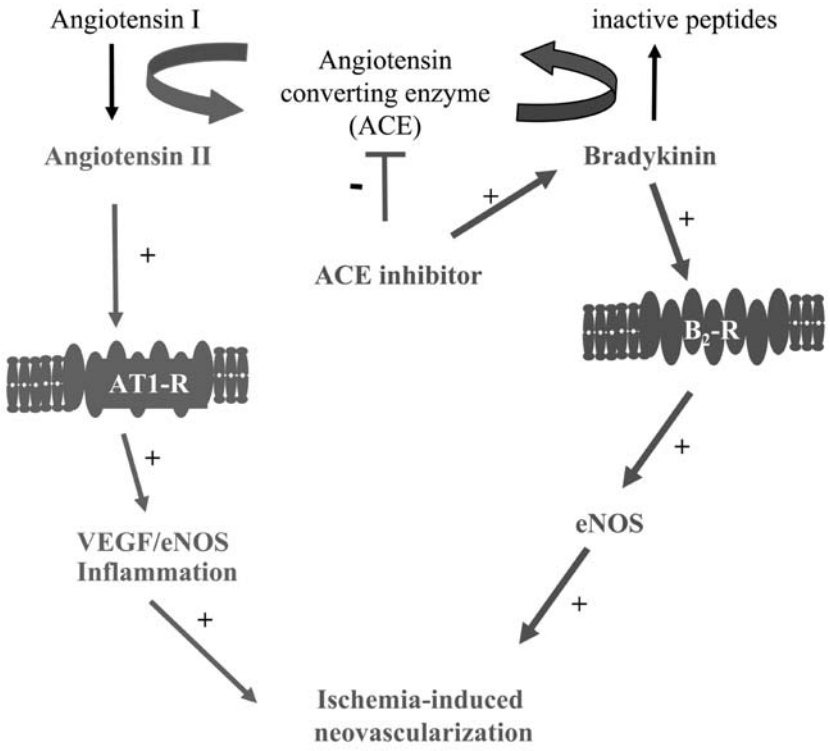

Figure 2. The ACE inhibitor paradox. Ang II has been shown to stimulate the angiogenic process through AT1 stimulation and activation of VEGF and inflammation-related pathways. ACE inhibitor inhibits Ang II formation but also blocks the breakdown of bradykinin. Activation of the bradykinin signaling may likely account for increased neovascularization in response to ACE inhibition. AT1, Angiotensin II receptor type I; B_2R, Bradykinin receptor type 2. (For colored picture see color plate 5)

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Mechanisms of Angiogenesis
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Can tumor angiogenesis be inhibited without resistance?

Kathy D. Miller, Christopher J. Sweeney and George W. Sledge, Jr

Division of Hematology and Oncology, Department of Medicine, Indiana University, 535 Barnhill Drive RT-473, Indianapolis, IN 46202, USA

Angiogenesis, the process of new blood vessel formation, plays a central role in both local tumor growth and distant metastasis [1] Because normal endothelial cells are genetically stable, anti-angiogenic therapy was initially touted to be "a treatment resistant to resistance" [2]. Initial xenograft studies supported these theoretical predictions – widespread activity, limited toxicity and no resistance [3]. For a time it was argued that disease control if not outright cure was close at hand.

Unfortunately, millions of years of evolution has yet to be thwarted by three decades of research. Emerging laboratory and clinical data suggest resistance to anti-angiogenic therapy is a very real problem. This chapter reviews potential mechanisms of acquired and *de novo* resistance to anti-angiogenic therapy, both theoretical and practical, then suggests strategies to combat such resistance.

Resistance to anti-angiogenic therapy

Substantial preclinical *in vitro* and *in vivo* data, as well as emerging clinical data, suggest distinct anti-angiogenic activity for several existing, commonly used agents [4]. However, these agents fail to cure most malignancies. Mechanisms of resistance to the anti-angiogenic effects of cytotoxic agents likely also apply to novel anti-angiogenic agents. Theoretical and (often) substantiated mechanisms of acquired and *de novo* resistance to anti-angiogenic therapies are described below.

Endothelial cell heterogeneity

Initial expectations of widespread activity and limited resistance assumed that "normal" endothelia, incapable of mutating to a resistant phenotype, had been merely usurped by the "malignant" tumor. Moreover, all endothelial cells were presumed similar, if not identical; therefore anti-angiogenic agents were pre-

dicted to be equally effective regardless of the tumor type or anatomic location. If endothelial cells are heterogenous, the potential for selective sensitivity, a frequent euphemism for resistance, exists.

Normal embryonic development requires endothelial heterogeneity. Developing endothelium is dynamic and capable of differential gene expression based on the physiologic requirements and microenvironment of the associated tissue. For example endothelia in the brain and testes express high levels of the Mdr protein thereby limiting exposure to potentially harmful xenobiotics [5–7]. Though the evolutionary advantage of organ-specific endothelial gene expression is clear, the mechanisms that control such expression have not been fully elucidated.

Differences in endothelial function become apparent when comparing the results of *in vitro* studies using different sources of "normal" endothelial cells. Vascular cell adhesion molecule-1 (VCAM-1) expression is induced on human umbilical vein endothelial cells (HUVEC) by both tumor necrosis factor alpha (TNF- α) and interleukin-1 alpha (IL-1α), whereas only TNF-α induced VCAM-1 expression in human dermal microvascular endothelial cells (HDMEC) [8]. The differential response was explained by distinct expression patterns of the CXC chemokine and interleukin-8 (IL-8) receptors.

St. Croix and colleagues recently compared the gene expression patterns of vascular endothelial cells derived from normal and malignant colorectal tissues. Of 170 transcripts analyzed, almost half (79) were differentially expressed in tumor-associated endothelial cells (TEC) compared to normal endothelium. Similar but not identical expression patterns were found in TECs from metastatic lesions and primary tumor sites. [9] The human herpes virus 8, thought to be the etiologic agent for Kaposi's sarcoma, multicentric Castleman's disease and AIDS-associated primary effusion lymphoma, alters gene expression in human dermal microvascular endothelial cells [10]. Schlaifer et al. found expression of the energy-dependent efflux pump, P-glycoprotein (P-gp) in TECs but not in human umbilical vein endothelial cells [11, 12]. Vincristine with the P-gp antagonist verapamil, but not vincristine alone, inhibits angiogenesis induced by mouse sarcoma 180 cells suggesting P-gp expression in TECs has functional significance [13].

Individual differences in endothelial sensitivity to anti-angiogenic agents should also be expected. Rohan and colleagues found up to a 10-fold difference in the response to growth factor-stimulated angiogenesis in the corneal micropocket assay among 12 inbred mouse strains. Even more importantly, differential sensitivity to angiogenesis inhibitors was seen between mouse strains, with one demonstrating complete resistance to both TNP-470 and thalidomide [14]. Similarly, Pandey and colleagues found inherited differences in angiogenic *versus* angiostatic activity in estrogen-induced rat pituitary tumours [15]. The genetic factors underlying these differences were not explored, though genetic polymorphisms affecting target molecules or critical metabolic pathways seem plausible. Variation in sensitivity from patient to patient seems likely as well. Polymorphisms of the 3' untranslated region of

VEGF and of the VEGF promoter have been reported though the clinical relevance of these polymorphisms is uncertain [16, 17].

Tumor cell heterogeneity

Tumor cell heterogeneity, whether in expression of angiogenic factors or sensitivity to hypoxia, also results in resistance to anti-angiogenic therapy [18]. Invasive cancers commonly express multiple angiogenic factors. At least six different pro-angiogenic factors were identified in each of 64 primary breast tumors studied by Relf and colleagues with the 121-amino acid isoform of VEGF predominating [19]. Genetic instability of the tumor may result in modulation of both the amount and type of pro-angiogenic factors expressed [20]. Given such redundancy in angiogenic pathways, it seems naive to assume that inhibition of a single factor would produce a sustained clinical effect. In addition, some pro-angiogenic factors display tissue specificity. A VEGF isoform, endocrine gland-derived vascular endothelial growth factor (EG-VEGF), induces proliferation, migration and fenestration in capillary endothelial cells derived from endocrine glands (ovary, testis, adrenal and placenta) but has little effect on other endothelial cell types [21].

Production of the same pro-angiogenic factor does not guarantee the same response to anti-angiogenic therapy. Though Wilms tumor and neuroblastoma both predominantly produce VEGF, Wilms tumor is growth inhibited whereas neuroblastoma is resistant to a VEGF-directed monoclonal antibody [22]. The mechanism underlying this resistance has not yet been elucidated. More recently, Kerbel et al. have shown that disruption of p53 in tumor cells reduces sensitivity to anti-angiogenic metronomic therapy [23]. Chronic hypoxia selects p53 mutant tumor cells resistant to hypoxia-induced apoptosis. Indeed, in most long-term xenograft studies of anti-angiogenic therapy, tumors eventually progressed (though more slowly than controls) despite continued treatment [24–26].

Hypoxia is a key signal for the induction of angiogenesis, often via the hypoxia-inducible factors (HIF-1 and HIF-2) [27–29] HIF-1$\alpha^{-/-}$ tumors have decreased hypoxia-induced VEGF expression and are less vascular but (perhaps paradoxically) have accelerated growth *in vivo* compared to HIF-1$\alpha^{+/+}$ tumors due to decreased hypoxia-induced apoptosis [30]. Yu and colleagues isolated tumor cells based on their relative proximity to perfused vessels and compared HIF-1α expression and *in vivo* growth characteristics [31]. In heterogeneous tumors HIF-1$\alpha^{+/+}$ cells were located in the perivascular areas and were much more highly dependent on proximity to blood vessels for their growth and survival *in vivo* than the HIF- 1$\alpha^{-/-}$ cells [32].

Erythropoietin production is tightly modulated by hypoxia and HIF-1α. Although the principal function of erythropoietin is to stimulate the maturation of erythroid precursors, erythropoietin modulates a host of cellular signal transduction pathways in endothelia and pluripotent stem cells. Erythropoietin stimulates endothelial cell proliferation and migration as well as erythropoiesis

and vascular resistance. Erythropoietin inhibits apoptosis through two distinct mechanisms that involve maintenance of genomic DNA integrity and preservation of cellular membrane asymmetry [33].

Anti-angiogenic therapy assumes a uniform response to hypoxia, hypoglycemia and waste product accumulation – the physiologic stresses produced by effective inhibition of angiogenesis. Hypoxia may be chronic due to consumption/diffusion limitations or periodic resulting from transient reductions in tumor blood flow (so-called cyclic hypoxia) [34]. In contrast to normal vasculature, tumor microvessels frequently lack complete endothelial linings and basement membranes with arterio-venous shunts and blind ends being common [35]. As such, blood flow through tumors tends to be sluggish [36]; cyclic hypoxia is quite common (occurring in as many as half of tumor vessels) suggesting that the cancer cell's natural environment is one of recurring hypoxic insults. Tumor cells by definition must evolve mechanisms to resist such cyclic hypoxia merely to survive. Indeed some cancer cells may remain viable for prolonged periods of hypoxia [37–39].

A VEGF/VEGFR-2 autocrine loop supports the growth and migration of leukemic cells [40]. In response to leukemia-derived pro-angiogenic and pro-inflammatory cytokines, endothelial cells release increasing amounts of another vascular endothelial growth factor family member, VEGF-C. In turn, interaction of VEGF-C with its receptor VEGFR-3 (FLT-4) promotes leukemia survival and proliferation. VEGF-C protected leukemic cells from the apoptotic effects of three chemotherapeutic agents. [41, 42] VEGF tyrosine kinase receptors have been found on some solid tumor cells as well, suggesting that such autocrine/paracrine loops may have widespread importance. (S. Rafii, personal communication)

Impact of the tumor microenvironment

The complex interaction between tumor cells and host microenvironment has been recognized for over a century [43, 44]. More recently, Fidler and colleagues implanted human renal cell carcinoma (HRCC) cells obtained from a surgical specimen into different organs of nude mice; tumors were then recovered and established in culture. The cell lines each had a unique karyotype indicating that the local environment selected for different subpopulations of HRCC. Only those HRCC cells implanted orthotopically (that is under the renal capsule) metastasized [45]. Colorectal lung metastases only form tumors in athymic mice if implanted in the lung; implants in either the colon or skin did not form tumors [46]. Similarly, human colon cancer cells did not metastasize unless implanted orthotopically in the cecum [47]; injected human prostate cancer cells grew preferentially to transplanted human bone rather than transplanted human lung or native mouse bone [48].

To study angiogenesis and tumor growth at a secondary site, Gohongi and colleagues implanted a gel impregnated with basic fibroblast growth factor

(bFGF) or Mz-ChA-2 tumor in the cranial windows of mice without tumors, mice with subcutaneous tumors or mice with orthotopic cholangiocarcinomas. The concentration of transforming growth factor-beta1 (TGF-beta1) in the plasma of mice with orthotopic cholangiocarcinoma was 300% higher than that in the plasma of mice without tumors or with subcutaneous tumors. Similarly, angiogenesis in the cranial window was substantially inhibited in mice with orthotopic tumors but only minimally effected by subcutaneous tumors [49]. In a xenograft pancreatic cancer model, orthotopic pancreatic tumors grew faster, expressed VEGF, maintained vascular density and hyperpermeability compared to subcutaneous tumors. As in other models, orthotopic but not subcutaneous tumors metastasized similar to advanced human pancreatic cancer [50].

The tumor microenvironment protects the endothelial compartment. Medium conditioned by colon cancer cells increases extracellular signal regulated kinase-1/2 (Erk-1/2) phosphorylation and decreases apoptosis of HUVECs compared to medium conditioned by non-malignant cells [51]. The resistant phenotype can be reproduced *in vitro* by the addition of VEGF and/or bFGF to HUVEC culture systems. HUVEC anti-apoptotic pathways stimulated by VEGF and/or bFGF include (but are by no means limited to): p44 mitogen-activated protein kinase (MAPK), c-jun-NH2-kinase (JNK), phosphoinositide 3-OH kinase (PI-3-kinase), Bcl-2, inhibitors of apoptosis (IAP) and survivin [52–56]. Pericytes invest mature vasculature and provide critical survival signals to vascular endothelial cells. Differences in pericyte coverage among tumor types have obvious implications for vessel maturation, survival and sensitivity to anti-angiogenic therapies [57].

As many pro- and anti-angiogenic factors are contained in or released from the extracellular matrix, differential sensitivity based on site of disease may be anticipated. For example, treatment with the matrix metalloproteinase inhibitor batimastat had different effects on tumor progression and growth depending on the site of tumor implantation [58]. Predicting the effect of an individual intervention in such a complicated and interrelated system as the tumor microenviroment is fraught with hazards. The potential for unintended consequences must be kept in mind. For instance, the proteolytic action of the MMPs releases angiostatin from the extracellular matrix. Thus, MMP inhibition may actually increase angiogenesis by decreasing angiostatin release [59].

The tumor microenvironment affects drug delivery. Pluen and colleagues studied the diffusion of macromolecules and liposomes in tumors growing in cranial windows (CWs) and dorsal chambers (DCs). For the same tumor types, diffusion of large molecules was significantly faster in CW than in DC tumors. The slower diffusion in DC tumors was associated with a higher density of host stromal cells which synthesize and organize collagen type I [60]. These preclinical findings may seem far removed from the clinical setting at first glance. However mixed responses (i.e. regressions in lung metastases but growth in liver metastases within the same patient) have been observed in early phase clinical trials of anti-angiogenics [61–65]. Though the mechanisms

underlying these mixed responses have not been explored in the clinic, their frequency argues for the critical role of the tumor microenvironment.

Compensatory responses to treatment

As chemotherapy induces tumor cell death, the production of pro-angiogenic peptides decreases, leading to regression of the tumor-associated vasculature with increasing tumor hypoxia, stimulating an increase in VEGF production [66, 67]. The increased VEGF production in areas of tumor hypoxia may stimulate brisk angiogenesis, essentially rescuing areas of tumor that are sublethally injured. In an *in vivo* model with rat 13 762 mammary carcinomas, treatment with cyclophosphamide resulted in tumor hypoxia with increased VEGF production and increased tumor CD31 staining detectable within 24 hours [68]. It seems reasonable to expect VEGF production to increase in response to treatment with the "pure" anti-angiogenics as well. Indeed, VEGF levels increased after therapy with doxorubicin and a VEGF receptor tyrosine kinase inhibitor [69].

Tumor growth may be independent of angiogenesis

Vessel co-option, growth by intusseception, vascular mimicry and vasculogenesis may decease a tumors dependence on classical angiogenesis (sprouting and elongation of existing vessels). The sensitivity of these alternative means of establishing circulation to anti-angiogenic therapies has not been studied.

Holash and colleagues documented that a subset of tumors initially grows by co-opting existing host vessels. This co-opted host vasculature does not immediately undergo angiogenesis but instead regresses, leading to a secondarily avascular tumor and massive tumor cell loss. Ultimately, the remaining tumor is rescued by robust angiogenesis at the tumor margin [70]. Kunkel and colleagues studied systemic treatment with DC101, a monoclonal antibody against vascular endothelial growth factor receptor (VEGFR) in an orthotopic intracerebral glioma model. Tumor volumes and microvessel density in animals treated with DC101 were reduced compared with IgG and PBS controls. Though systemic inhibition of VEGFR-2 blocked angiogenesis and inhibited glioblastoma growth, there was increased co-option of pre-existing cerebral vessels with a distinct growth pattern in the residual tumors. In mice treated with DC101, there was a significant increase in small satellite tumors clustered around, but distinct from, the primary tumor. The satellites contained central vessel cores, i.e. co-opted vessels. Tumor cells often migrated long distances along the co-opted host vasculature to reach the surface and spread over the meninges [71].

Passalidou and colleagues described a group of non-small cell lung carcinomas without morphological evidence of neo-angiogenesis; neoplastic cells

filled the alveoli with the only vessels belonging to the trapped alveolar septa. The vascular phenotype of all the vessels in the non-angiogenic tumors was the same as that of alveolar vessels in normal lung: LH39 positive and alphaVbeta3 variable or negative. This pattern was distinct from the vessels in angiogenic tumors [72].

Intussusceptive microvascular growth refers to vascular network formation by insertion of interstitial tissue columns, called tissue pillars or posts, into the vascular lumen and subsequent growth of these columns, resulting in partitioning of the vessel lumen. Patan and colleagues used intravital microscopy to observe the growth of the human colon adenocarcinoma (LS174T) *in vivo* [73]. Both intussusception and endothelial sprouting occurred at the tumor periphery. In the central regions intussusception led to network remodeling and occlusion of vascular segments, interfering with vessel patency and causing heterogenous perfusion and hypoxia thus perpetuating angiogenesis [74]. Interestingly, in mammary tumors of *neuT* transgenic mice, both sprouting and intussusceptive angiogenesis was observed simultaneously in the same nodules [75].

Vascular mimicry refers to the unique ability of some aggressive tumor cells to form tubular structures and patterned networks in three-dimensional culture, mimicking embryonic vasculogenic networks [76]. Several adhesion factors were exclusively expressed by highly aggressive (vasculogenic) melanoma cells. Down-regulation of VE-Cadherin expression or restoration of EphA2 ligand binding in the aggressive melanoma cells abrogated their ability to form vasculogenic networks [77, 78]. Multiple vascular cell-associated markers were identified by RNase protection assay in invasive ovarian cancer cells that lined the vascular structure. Tumor cells lined 7 to 10% of channels containing red blood cells in patient tumor sections from advanced high-grade ovarian cancers. By comparison, all vascular areas in benign tumors and low-stage cancers were endothelial lined [79, 80].

Postnatal vasculogenesis refers to incorporation of bone marrow derived endothelial progenitor cells (EPCs) into growing adult vasculature. Using transgenic mice constitutively expressing beta-galactosidase under the transcriptional regulation of an endothelial cell-specific promoter (Flk-1/LZ or Tie- 2/LZ), Asahara and others have identified EPCs in the neovasculature of developing tumors [81–83]. The role of EPCs was further documented by Lyden and colleagues using the angiogenic defective, tumor-resistant Id-mutant mice. Transplantation of wild-type BM or VEGF-mobilized stem cells restored tumor angiogenesis and growth, while donor-derived EPCs were detected throughout the neovessels of tumors and Matrigel-plugs in an $Id1^{+/-}$ $Id3^{-/-}$ host. Incorporated EPCs were associated with VEGF-receptor-1-positive myeloid cells. Targeting either VEGFR1 or VEGFR2 alone partially blocked the growth of tumors in this model; inhibition of both VEGFR1 and VEGFR2 was necessary to completely ablate tumor growth [84]. Recent data suggest that inflammatory breast cancer, a rare but highly aggressive form of the disease, relies almost entirely on vasculogenesis as opposed to angiogenesis, apparently due to the inability of the cancer cells to bind endothelial cells [85].

Pharmacokinectic resistance – the dose and schedule required for anti-angiogenic activity is not clinically attainable

Maximal anti-angiogenic therapy typically requires prolonged exposure to low drug concentrations, exactly counter to the maximum tolerated doses administered when optimal tumor cell kill is the goal [86]. Three recent reports confirm the importance of dose and schedule. In all three the combination of low, frequent dose chemotherapy plus an agent that specifically targets the endothelial cell compartment controlled tumor growth much more effectively than the cytotoxic agent alone [24, 87, 88].

Dose and schedule are also critical for the novel anti-angiogenics. Constant exposure to low non-cytostatic doses of interferon was more effective in downregulating bFGF expression in the laryngeal cancer cell line HlaC79 than high doses [89]. 10 000 units of IFN-α administered daily was more efficacious in inhibiting the growth of bladder cancer in a murine orthotopic model than the 70 000 units given in two or three divided doses over one week or as one injection per week [90]. Daily subcutaneous administration of 5000 or 10 000 units per day produced maximal reduction in tumor vessel density, bFGF and MMP-9 expression (at both the mRNA and protein levels) and serum levels of bFGF.

What might explain the paradoxical (at least to classically trained medical oncologists) observation that lower dose interferon is more effective in inhibiting angiogenesis? In summary, interferons bind to receptors on the cytoplasmic membrane and activate the Janus kinase (JAK) family of protein tyrosine kinases. The JAK pathway then activates the activators of the transcription family of proteins (STATS), which in turn activate multiple genes that control the immune system, growth and hematopoiesis. A family of proteins termed cytokine inducible SH2 proteins (CIS) negatively regulates cytokine signals. Interferon-γ induces the activation of one of the CIS family, suppressors of cytokine signaling-1 (SOCS-1). It is therefore hypothesized that the low constant doses of interferon activate the JAK-STAT pathway and downregulate bFGF and MMP-9 without activating the SOCS-1 inhibitor. In contrast, higher doses also activate SOCS-1, shifting the balance to an angiogenic or neutral phenotype [91].

The natural inhibitors of angiogenesis angiostatin and endostatin are cleared rapidly from the circulation when administered as an intravenous bolus [92, 93]. It is likely that the overall balance of pro- and anti-angiogenic factors remains tilted toward angiogenesis for substantial periods with such bolus administration. As expected, the most profound effects in preclinical models maintained constant exposure with continuous infusions [94–96].

Natural history of tumor growth and patient selection

Angiogenesis inhibitors significantly curtail primary tumor growth and establishment of metastases in several pre-clinical minimal disease models. Overt

shrinkage of large, well-established tumors is less common. Hahnfeldt and colleagues have explored a model of tumor growth under angiogenic signaling [97]. This model considers growth of the tumor vasculature to be explicitly time dependent (rather than dependent on tumor volume) and to be under the control of distinct positive and negative signals arising from the tumor. Overall the model parallels Gompertzian kinetics with tumor growth slowing as tumor size increases. Tumor growth eventually reaches a plateau as the action of stimulators is offset by the increasing production of vascular inhibitors by the primary tumor. Anti-angiogenic therapies act to lower this plateau tumor size, hopefully to a level compatible with asymptomatic host survival. Importantly, the final tumor size is dependent only on the balance of positive and negative angiogenic factors and is *independent* of tumor size at the start of treatment. The model also predicts initial tumor *growth* with some inhibitors of angiogenesis before stabilization at the plateau size. This early growth could easily be interpreted (perhaps misinterpreted) as resistance.

Potential means of thwarting resistance to anti-angiogenic therapy

The reality of human tumors and initial clinical experience with novel anti-angiogenic agents confirms that resistance remains an obstacle. While occasional trials have demonstrated modest clinical efficacy [98–101], results have largely not lived up to the initial expectations. Understanding the potential mechanisms of anti-angiogenic resistance suggests several possible means to ameliorate or bypass such resistance.

Use standard therapies with anti-angiogenic intent

Chemotherapeutic agents have long been developed based on the concept of maximum tolerated dose, and with the assumption that the cancer cells are the sole - or at least primary - target. Numerous chemotherapeutic agents have anti-angiogenic activity at dose levels far lower than those required to kill cancer cells [4]. Chronic low-dose chemotherapy (so-called “metronomic therapy”) may be potently anti-angiogenic, though this effect seems most pronounced when the chemotherapeutic agent is combined with a specific endothelial agent [24, 102].

Combine anti-angiogenic agents with standard chemotherapy regimens

Extensive preclinical data support this combined approach, with multiple anti-angiogenic and chemotherapeutic agents having additive or synergistic combinatorial activity [24, 102–105]. The mechanistic rationale for many of these combinations is poorly understood, and not intuitive as both radiotherapy and

chemotherapy depend on an effective blood supply for therapeutic efficacy. A potential explanation may lie in the inherent inefficiency of the tumor vasculature. Anti-angiogenic therapy "normalizes" flow initially resulting in improved tissue oxygenation and increased delivery of cytotoxic agents [36].

Potential interactions between VEGF and chemotherapeutic agents have been extensively examined. VEGF is anti-apoptotic for endothelial cells via several pathways, including induction of the anti-apoptotic proteins Bcl-2 and A1, activation of the PI 3-kinase/Akt signaling pathway, stimulation of NO and PGI2, and increased FAK tyrosine phosphorylation [106]. This survival function may play a role in the protection of tumor endothelial cells against the anti-angiogenic effects of commonly used chemotherapeutic agents. For instance, Sweeney and colleagues demonstrated that VEGF protects endothelial cells against docetaxel, an effect reversed by an anti-VEGF monoclonal antibody [105].

The anti-apoptotic effects of VEGF may not be limited to endothelial cells. Neuropilin-1, a receptor important in neuronal guidance, is a newly identified co-receptor for VEGF [107] and is highly expressed by some tumor cells [108–110]. In these tumors, VEGF acts as an anti-apoptotic factor, potentially protecting tumor cells against chemotherapeutic agents. It is reasonable to expect that the combination of a chemotherapeutic agent with an agent targeting VEGF will increase the therapeutic efficacy of both the cytoxic and the anti-angiogenic.

Combine multiple anti-angiogenic agents

As tumor progression is associated with expression of increasing numbers of pro-angiogenic factors, the use of multiple anti-angiogenic agents to simultaneously attack this multiply redundant process may thwart resistance to individual agents. This approach is of course not unique to anti-angiogenic therapy, having previously been used to limit resistance to cytotoxic, antimicrobial and antiviral therapies. The combination of anti-angiogenic agents has been tested in preclinical models with success, e.g., interferon and TNP-470 [111] and angiostatin with endostatin [112].

Combine anti-angiogenic agents with other biologically targeted agents

The epidermal growth factor receptor and HER-2 both regulate VEGF in human tumors; their blockade reduces VEGF production and angiogenesis [113–119]. Given the plethora of indirect influences on angiogenesis, might we be able to utilize the combination of biologic agents as a means of inhibiting angiogenesis? Might we be able to combine anti-angiogenic agents with anti-growth factor receptor agents as a means of overcoming resistance? This strategy was effective in preclinical tumor models [120] and is currently under

clinical investigation with combinations of anti-angiogenic agents and trastuzumab in patients with HER-2 positive breast cancer.

Conversely, anti-angiogenic agents might offer a means of overcoming resistance to growth factor-targeting agents. Recent data from Viloria-Petit et al. suggests that increased production of VEGF represents one mechanism by which tumor cells escape anti-EGFR monoclonal antibody therapy [121]. The combination of a VEGF-targeting agent with an anti-EGFR agent might thereby limit resistance to growth factor receptor therapy.

Use anti-angiogenic therapy as adjuvant therapy

It is a rare treatment that is more effective for large tumors than for small. Tumor progression results in resistance to all anti-cancer therapies. One means of thwarting the development of drug resistance associated is to treat cancers when they are small. The adjuvant setting (or similar minimal residual disease setting) is the logical place to accomplish this goal.

The use of anti-angiogenics as adjuvant therapy has its own potential barriers. Physicians are frequently loath to use agents in the adjuvant setting until there is evidence of activity in advanced disease. The toxicity of chronic anti-angiogenic therapy remains largely unexplored, as is the toxicity of combinations of chemotherapy with anti-angiogenic therapy. Though intuitively the impact of angiogenesis inhibition is expected to be greatest in patients with micrometastatic disease, proof of this concept will require commitment of substantial human and financial resources to a randomized adjuvant trial. Recent studies illustrate the importance of population-specific feasibility trials, especially for agents administered chronically [122, 123]. Experience gained in patients with advanced disease is meaningful, but even large trials of patients with metastatic disease provide long-term safety data in only a limited number of patients. Population-specific feasibility studies can identify toxicities that might not be acceptable in an otherwise healthy patient population, thereby limiting exposure and avoiding premature closure of a large adjuvant trial [124].

Use anti-angiogenic therapy as targeted therapy

Anti-angiogenic therapy has been used as a general therapy given on a population basis, rather than as a targeted therapy given to patients with a specific molecular phenotype. It is reasonable to ask whether we can call failure to respond to a therapy "resistance" if the target at which the therapy is aimed is not present in the tumor. If a patient's tumor does not express VEGF and therefore fails to respond to an anti-VEGF therapy, is the tumor resistant or is the therapy merely misguided? As insensitivity due to lack of therapeutic target results in resistance at the *patient* level, proper targeting is a means of overcoming such resistance. Ideal targets are biologically relevant, reproducibly

measurable and definably correlated with clinical benefit. Examples of molecular targets fulfilling such criteria include estrogen receptor or HER-2 for breast cancer, c-kit for gastrointestinal stromal tumors or bcr-abl for chronic myelogenous leukemia.

At present we are unable to point to any truly targeted anti-angiogenic therapy. It is reasonable, if not critical, to require mandatory tissue collection for testing as part of the development of anti-angiogenic agents. Though we lack validated assays for most of the anti-angiogenic therapeutic targets, the availability of tissues for testing will speed development and validation of appropriate assays.

Conclusion

Early enthusiasm for anti-angiogenic therapy, justified by the impressive preclinical data, has given way to clinical reality: resistance continues. Acknowledging this fact is not to suggest that anti-angiogenics will find no place in the therapeutic armamentarium. Rather it is to remind us that the way forward lies in advancing our knowledge of fundamental cancer biology.

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Cellular and physical mechanisms of blood vessel growth

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Role of pericytes in vascular morphogenesis

Christer Betsholtz[1], Per Lindblom[2] and Holger Gerhardt[3]

[1] *Laboratory of Vascular Biology, Division of Matrix Biology, House A3, Plan 4, Department of Medical Biochemistry and Biophysics, Scheels vag 2, Karolinska Institutet, 17177 Stockholm, Sweden*
[2] *Vascular Development Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC 2A 3PX, UK*
[3] *Vascular Biology Laboratory, Cancer Research UK, 44 Lincoln's Inn Fields, London WC 2A 3PX, UK*

Introduction

The cardiovascular system constitutes the first functional organ system of the developing mammalian embryo [1]. In the mouse, a functional cardiovascular system is required for development beyond embryonic day 8.5 (E8.5). If its proper formation fails, embryonic development arrests and a number of secondary effects occur, likely due to lack of proper oxygenation and nutrition of the growing embryo. The simple role of the cells of the vasculature as structural components of a tubular network solely devoted to metabolic exchange has been challenged by recent observations suggesting that the endothelial cells also deliver signals to neighboring cells, thereby playing a role in inductive signaling in development [2]. Besides the reciprocal signaling between the endothelium and the mural cells within the vascular wall, endothelium-derived paracrine signals also seem to play pivotal roles in organogenic processes, such as the formation of the pancreas and the liver [2].

In studies of the embryonic vasculature, the focus of attention has mainly been the endothelial cells. However, mural cells are present already in the first primitive vascular networks that coalesce in the embryo and extraembryonic tissues, and continue to invest the vessels that form by angiogenesis. Mural cells are called vascular smooth muscle cells (vSMC) when they encircle larger vessels, and pericytes when they reside in the wall of small vessels such as capillaries and postcapillary venules. Although the nature of the pericytes has been controversial, most evidence now suggests that they constitute a phenotypic variant of vSMC and that a continuum of phenotypes exist between the typical vSMC of an elastic artery and the typical solitary pericytes of the capillary bed. However, it is important to appreciate that pericytes have distinct features in different organs and that this may reflect on their ontogeny, morphology, marker expression and function.

A number of functions have been proposed for pericytes. These include the sensing of hemodynamic forces and, through contraction, the regulation of

capillary blood flow. Moreover, pericytes have been proposed to play a role in blood vessel morphogenesis by signaling to the endothelial cells and regulating their proliferation and differentiation, and by depositing extracellular matrix. However, to a large extent, these suggestions lack firm experimental evidence *in vivo*, and therefore the pericyte function(s) remain controversial. For historical notes and for detailed information on pericyte distribution, anatomy and *in vitro* functions in co-culture with endothelial cells, the reader is referred a series of comprehensive review articles by other authors [3–8]. The present review focuses on conclusions drawn from the analyses of gene-targeted mice with primary pericyte defects.

Ontogeny of mural cells

Mural cells arise from the mesoderm in most parts of the body and from the ectoderm (neural crest) in the head region [9, 10]. Independent of their origin, they appear to be recruited to the vessels by at least two different modes. First, they can appear by induction, i.e., by differentiation of immature mesenchymal cells surrounding the endothelial tube (mesenchyme that may either be of mesoderm or neural crest origin). This is typically seen surrounding the developing axial arteries, such as the dorsal aorta, where concentric layers of mesenchymal cells condense around the vessel and start to express SMC markers [9]. It is likely that this mesenchymal condensation and differentiation process is dependent on inducers released by the endothelial cells. Signaling through the transforming growth factor β (TGFβ) pathway has been implicated in this process. TGFβ induces the expression of SMC markers in undifferentiated mesenchymal 10 T1/2 cells *in vitro* [11]. Additionally, mouse mutants of several components of the TGFβ signaling pathway display defective vSMC development [12–15], and mutations in some of these components give rise to hereditary hemorrhagic telangiaectasia in humans, a vascular abnormality including mural cell deficiencies [16, 17]. However, it is still not clear from the observed effects *in vivo* whether TGFβ is a direct inducer of vSMC differentiation in the undifferentiated perivascular mesenchyme, or whether effects of TGFβ on the endothelial cells, mediated by the ALK-1 and ALK-5 receptors [18], affects endothelial release of other vSMC inducers that are distinct from TGFβ.

The second mode of mural cell recruitment is through migration and proliferation of pre-existing mural cells, or mural cell progenitors, in association with angiogenic sprouting, or in conjunction with vessel growth in size and remodeling into arteries, the latter coinciding with the formation of a thicker mural cell coat. This process is selective rather than inductive, since a pre-existing pool of mural cells or committed progenitors becomes expanded by cell proliferation and/or moved by cell migration. This mode of mural cell recruitment depends, at least in part, on platelet derived growth factor B (PDGF-B) signaling via platelet derived growth factor receptor β (PDGFRβ). PDGF-B is expressed by the sprouting endothelium and recognized by the

mural cells, which express PDGFRβ. Hence, a paracrine loop between the endothelium and the mural cells ensures that the latter are co-recruited when new vessels are formed from pre-existing vessels or when vessels enlarge in size and acquire a thicker mural cell coat. The PDGF-B/PDGFRβ dependent co-recruitment appears to be associated with angiogenic modes of vessel formation [19]. The mural cell deficiency observed in PDGF-B and PDGFRβ null mice is most severe in organs known to be vascularized by angiogenesis. Conversely, induction of mural cells from undifferentiated mesenchyme may be the dominating mode of mural cell recruitment in vasculogenesis, and this mode of mural cell formation is independent of PDGF-B/PDGFRβ signaling [19]. Pericytes of the sinusoidal vessels in the liver (also called Ito cells) are atypical in the sense that they do not express PDGFRβ and, as expected, they develop independently of PDGF-B [19]. The origin and mechanism of recruitment of these pericytes are unclear.

One of the problems encountered when studying pericytes is the shortage of available markers for these cells. In addition, none of the existing markers is a pan-pericyte marker and none is absolutely specific for pericytes, or even vascular mural cells. The identification of pericytes therefore has to rely on morphological criteria in combination with marker expression. Using multiple markers, and by simultaneous labeling of the endothelium, pericytes can be faithfully identified in most tissues, but as a principle, the expression of existing markers cannot be used for cell fate-mapping studies of the mural cell lineage, because the markers available are all dynamic in their expression. The choice of marker(s) for any given study therefore has to depend on the tissue of interest, the age of the animal and whether quiescent or actively remodeling vasculature is studied. Pathological situations present specific challenges, since the vessel and tissue architecture is often abnormal, which impacts on pericyte morphology as well as marker expression. Much of the discussion below is centered on angiogenesis in the central nervous system (CNS), and on the timing and importance of pericyte recruitment in conjunction with angiogenic sprouting in the CNS. Both in pre- and postnatal CNS, pericytes can be identified using reagents that detect PDGFRβ or its mRNA [20], desmin [21], the NG-2 chondroitin sulphate proteoglycan [22] and regulator of G-protein signaling (RGS)-5 mRNA [23, 24]. In addition, the gene-trap reporter transgene *XlacZ4* can be used as a pericyte nucleus marker in the developing CNS [25].

Role of PDGF-B in pericyte recruitment

As mentioned above, PDGF-B released from the endothelial cells mediates recruitment of pericytes. This has been particularly well documented in the developing brain, which is invaded by the first angiogenic sprouts from the surrounding perineural vascular plexus on late embryonic day 9 (E9). Pericytes invest these sprouts from the onset of sprouting. PDGF-B is particularly strongly expressed by the endothelial cell situated at the sprout tip, the

tip-cell [26]. Presumably, this ensures pericyte spreading along the vessel in the direction of sprouting, as the PDGF-B concentration would be assumed to be higher at the tip than in the sprout stalk and further back in the developing vascular plexus (Fig. 1A, B). Besides the tip cells, PDGF-B expression is also high in developing arteries, which coincides with the need for mural cell pro-

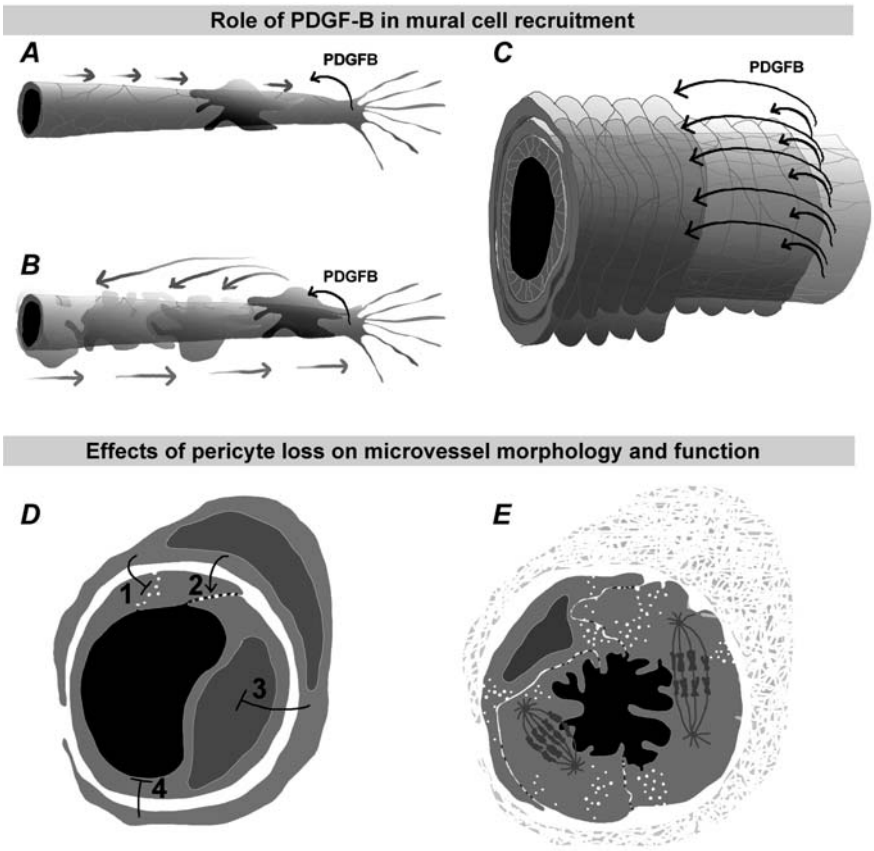

Figure 1. Role of PDGFB in mural cell recruitment and effects of pericyte loss on vessel morphology and function. (A) Schematic model of PDGF-B expression and function in pericyte recruitment in conjunction with angiogenic sprouting. PDGF-B expressed by the endothelial tip cell promotes pericyte co-migration. (B) Alternative angiogenic scenario: the leading pericyte (pink*) "hitch-hikes" with the migratory tip cell and responds to tip-cell derived PDGF-B by proliferation, leaving daughter cells behind to invest the elongating sprout. C) Role of PDGF-B in arterialization. PDGF-B produced by the endothelium stimulates mural cell proliferation thereby increasing their density and triggering organization in concentric layers typical of arterial vascular smooth muscle cells. D) Hypothetical role of pericytes in CNS microvessels deduced from mouse mutants of the PDGF-B/PDGFRβ signaling pathway. Pericytes inhibit vesicle transport and transcytosis (1), stimulate maturation of endothelial junctions (2), inhibit endothelial proliferation (3) and inhibit endothelial luminal membrane formation (4). E) Absence of pericytes leads to increase vesicle transport and transcytosis, the formation of abnormal inter-endothelial junctions, increased endothelial cell proliferation and increased luminal membrane surface with numerous membrane folds. (*For colored figure see color plate 6)

liferation in conjunction with arterialization [19] (Fig. 1C). PDGF-B stimulates pericyte proliferation *in vivo* [19]. It is unclear if pericytes also migrate in response to PDGF-B *in vivo* (Fig. 1A). This is difficult to study, since the substrate for the pericyte, the abluminal endothelial surface, is not fixed in its position, but rather actively moving relative to surrounding cells. Our current understanding is that endothelial migration takes place mainly at the tip of the sprout and that endothelial proliferation occurs mainly in the sprout stalk [26]. Pericytes extend up to but not ahead of the endothelial tip cell [27]. Hence, it is possible that pericytes "hitch-hike" with tip-cells and proliferate in response to PDGF-B release from these cells, leaving daughter cells behind to invest the elongating sprout (Fig. 1B). This model needs further testing, but it is compatible with the distribution of the small number of residual pericytes in the brain of PDGF-B or PDGFRβ null embryos; rather than being located close to the point where the vessels entered the CNS (i.e., close to the meningeal surface) they are associated with vessels deep into the brain tissue, suggesting that their relocation from the original perineural location to sites along intra-cerebral vessels is not dependent on PDGF-B/Rβ signaling.

The importance of the endothelium as a source of PDGF-B relative to other sources, such as hematopoietic cells and neurons, has been confirmed by tissue-specific genetic PDGF-B ablation [28, 29]. Once released from the endothelial cells, PDGF-B needs to remain localized in the vicinity of the source. Extracellular retention of PDGF-B is mediated by a retention motif, which constitutes a conserved stretch of basic amino acid residues located at the C-terminus of PDGF-B as well as in certain splice variants of PDGF-A and VEGF family members [30, 31]. PDGF-B is not alternatively spliced, but can be proteolytically processed to generate a molecule that lacks the retention motif. The PDGF/VEGF family retention motifs have affinity for heparin and heparan sulphate proteoglycans (HSPG) [32–34]. It is hypothesized that PDGF-B and other members of the PDGF/VEGF family become concentrated at the cell surface or in basement membranes and other extracellular matrix (ECM) structures by binding to HSPGs. If HSPGs contribute the major targets for the retention motifs *in vivo*, and if so, to what extent there is specificity in the growth factor-HSPG interaction, is unclear. The biological importance of the PDGF-B retention motif has been demonstrated by targeted deletion of the PDGF-B retention motif *in vivo* [35]. In different types of experiments it has been confirmed that PDGF-B lacking the retention motif is hyper-secreted from cells, but has intact PDGFRβ binding and activation capacity [35–37]. PDGF-B retention motif deficient mice show reduced numbers of pericytes, but in addition, their pericytes do not invest the vessel walls properly, but become partially detached from the abluminal endothelial surface [35]. It is tempting to speculate that PDGF-B retention helps generating a gradient or localized depot of PDGF-B protein that promotes pericyte association with the endothelial cells surface and basement membrane. This might involve PDGF-B dependent expression of cell adhesion molecules or integrins. It is also possible that periendothelial PDGF-B gradients/depots help guiding peri-

cyte migration along the abluminal endothelial surface. For deeper mechanistic insight, additional information is required, including the identity of the relevant *in vivo* target for the PDGF-B retention motif.

Role of pericytes in prenatal angiogenesis in the CNS

Much of our knowledge about the role of pericytes in embryonic angiogenesis stems from the analyses of the vascular abnormalities in mice lacking PDGF-B or PDGFRβ. These studies demonstrate that angiogenic sprouting *per se* into the CNS (and likely also in other organs) does not require pericytes. This is important to keep in mind, as there are situations and locations, such as the growing corpus luteum, where pericytes have been suggested to migrate ahead of the endothelial cells in the sprouting process [38, 39]. It is unclear if pericyte pioneers are indeed required for the angiogenic sprouting in these cases, but at least we can conclude that pioneering pericytes are not generally required for angiogenic sprouting in the developing mouse embryo.

The vessels devoid of pericytes develop a series of abnormal features, including endothelial hyperplasia, hypervariable diameter and tortuosity, abnormal endothelial junctions, signs of increased vesicular transport, and increased leakage of plasma and erythrocytes [20, 40] (Fig. 1 D, E). Moreover, rather than displaying smooth luminal surface, the pericyte-deficient capillaries show a large increase in luminal membrane folds, suggesting an excess of luminal membrane production [40] (Fig. 1E). Although these vessels likely have impaired perfusion, they are apparently sufficiently functional to promote an overall normal embryonic development, with only slight retardation in growth (~10% lower weight) until E17–19. However, at this age there is rapid onset of microaneurysm formation, microhemorrhage and edema formation, leading to embryonic death just before or at birth. Live E18.5 PDGF-B and PDGFRβ negative fetuses delivered by Caesarean section failed to start breathing properly, possibly because of pericyte deficiency and vascular dysfunction in the lungs [41, 42]. While all these effects occur as a consequence of pericyte deficiency, several of them might be secondary. VEGF-A is upregulated in PDGF-B null embryos [40], and VEGF-A is known to induce increased vascular permeability, endothelial junctional changes and endothelial proliferation, i.e., some of the effects observed in the absence of PDGF-B. The cause of the VEGF-A upregulation may be local vascular dysfunction, but more likely, it is associated with placental dysfunction since it was noted also in the liver, in which pericytes develop independently of PDGF-B/Rβ signaling [19]. Moreover, VEGF-A upregulation coincides in time with the occurrence of placental abnormalities due to placenta pericyte deficiency [40, 43]. Thus, VEGF-A upregulation may occur secondary to systemic ischemia as a result of placenta dysfunction, and may contribute to the vascular abnormalities of PDGF-B and PDGFRβ null mutants. However, the endothelial hyperplasia and the irregular capillary diameter

observed in these mice were apparent before the development of placental abnormalities and VEGF-A upregulation, suggesting that changes in endothelial proliferation and differentiation are direct consequences of the pericyte deficiency. How pericytes control endothelial cell proliferation and capillary diameter is unclear. The latter may relate to luminal membrane formation and turnover. Interestingly there are *Drosophila* mutants showing defects in the trachea system that are similar to those observed in the capillaries of PDGF-B/PDGFRβ knockouts. In these mutants, which show an increased diameter and tortuosity of the tracheal tubes, the surface of the apical membrane (corresponding to the luminal membrane of blood vessels) is increased and abnormally folded [44, 45]. In summary, currently available evidence suggests that pericytes play a role in angiogenesis in the prenatal CNS by regulating endothelial cell proliferation and differentiation. However, we lack insight into the signals that mediate these effects and their mechanisms of action.

Role of pericytes in retinal angiogenesis

The retina constitutes a peripheral part of the CNS, which is not vascularized before birth. Vascular sprouts start to spread over the retinal surface from a vascular plexus surrounding the optic nerve immediately after birth, and reach the peripheral retinal margin by approximately postnatal day 8 (P 8). Subsequently, the deeper capillary plexuses form by sprouting from the superficial plexus, and remodeling of the vessels of the superficial plexus shape a hierarchical pattern of arteries, veins and intervening capillaries.

Similar to the situation in the developing brain, pericytes appear around the retinal vessels from the onset of sprouting and invest the entire expanding plexus up to the tip-cell, which expresses PDGF-B strongly. Pericyte processes make focal contact with the tip-cells, which may facilitate their response to tip-cell-derived PDGF-B.

The importance of pericytes in the retina has not been possible to address in the PDGF-B or PDGFRβ knockouts, because of their lethality before/at birth. However, extensive evidence has accumulated from the study of viable mutants involving the PDGF-B and PDGFRβ genes [28, 35, 46], and by systemic injections of neutralizing PDGFRβ antibodies [47]. These studies provide compelling evidence for the critical role of PDGF-B and PDGFRβ in pericyte recruitment to retinal vessels. However, unlike the situation in the rest of the CNS, pericyte deficiency severely abrogates angiogenic sprouting and the formation of a primary retinal vascular plexus. In PDGF-B retention motif deficient mice (in which retinal vessels show a dramatic reduction in pericyte density from the onset of sprouting), the number of sprouts was lower, and the plexus that formed was highly irregular with a notable shortage of branch points [35]. A similar effect was observed by Uemura and colleagues following retinal injections of PDGFRβ antibodies [47]. Severely abnormal vessel

formation was also observed in mice in which PDGFRβ signaling was abrogated by a mutation switching the intracellular domain of PDGFRβ for that of PDGFRα [46]. In all these models, the retinal vascular abnormalities progressed and led to severe deterioration of the retinal histology, often combined with bleedings and secondary ocular opacities.

Pericyte recruitment is broadly deficient in the genetic models of PDGF-B/PDGFRβ signaling defects, but the retina stands out as the most sensitive site for pericyte loss. This was also apparent in studies of endothelium-restricted PDGF-B conditional knockouts [28]. These animals displayed a wide inter-individual variation in the extent of Cre-lox recombination at the PDGF-B locus, and consequently a spectrum of pericyte-deficient states were generated, ranging from 20% to more than 90% reduction in pericyte densities. Intriguingly, when overall pericyte counts in the CNS were lower than 50% of normal, the animals invariably developed a condition similar to diabetic proliferative retinopathy. The proliferative changes always took place within the superficial vascular plexus, from which vessels extended into the vitreous, and they always occurred at sites where the deeper plexuses had regressed completely. It is therefore likely that pericyte deficiency in the retina initially triggers vessel regression, which primarily affects the deeper plexuses. Subsequently, and perhaps because of resulting retinal hypoxia, vessels at the retinal surface start to proliferate, resulting in the formation of high-density vascular tufts at the retinal surface, which penetrate into the vitreous. The pathogenesis of these defects show close resemblance to diabetic proliferative retinopathy in humans.

Conclusions

Studies of angiogenesis in the developing CNS show that pericytes invest the vessel sprouts from the onset of sprouting and extend up to the endothelial tip-cell. The longitudinal spreading of pericytes along the elongating sprouts is controlled by the tip-cell, which secretes the pericyte mitogen PDGF-B. Thus, pericyte recruitment is an integrated part of angiogenic sprouting. The sprouting process can proceed relatively unaffected in the absence of pericytes, although in the retina highly abnormal vessel plexuses are formed when pericyte recruitment fails. Thus, the pericytes do not appear to control the polarization of the sprout into distinctive tip- and stalk regions and they do not appear to regulate tip-cell migration. However, without pericytes, the newly formed vessels acquire super-numerous endothelial cells with abnormal morphological features and junctional arrangement. Therefore, pericytes appear to control the proliferation and differentiation of endothelial cells in the newly formed microvessels. Lack of such control leads to the formation of rupturing microaneurysms, and to a pathological situation reminiscent of diabetic microangiopathy.

Summary

Pericytes are solitary, smooth muscle-like mural cells that invest the wall of microvessels. For a long time, the functional significance of the presence and distribution of pericytes in the microvasculature was unclear. However, in recent years, the application of experimental genetics to the PDGF-B/PDGFRβ signaling pathway in mice has provided a range of mutants with primary defects in pericytes, allowing for studies of the physiological consequences of pericyte deficiency in developmental angiogenesis and adult physiology. Interestingly, some of the phenotypic consequences of these mutations resemble human diseases, such as diabetic retinopathy. The studies have also led to the discovery of critical mechanisms involved in pericyte recruitment and differentiation. The present review focuses on genetic data suggesting that pericytes take active part in developmental angiogenic processes.

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Mechanisms of Angiogenesis
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Role of monocytes and macrophages in angiogenesis

Leni Moldovan and Nicanor I. Moldovan

Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University, 473 W 12th Ave., Room 305A, Columbus, OH 43210, USA

Introduction

Formation of new blood vessels in the embryo takes place through vasculogenesis, which consists of the development of vascular structures originating from cellular precursors, the angioblasts [1]. Later on, the expansion or regeneration of microvascular beds relies on angiogenesis, i.e., the formation of new capillaries thought to derive from pre-existent endothelial cells (EC) by the mechanism of sprouting [2]. In both cases, the formation of new blood vessels proceeds based on the properties of a single cell type [3]. However, the simplicity of this model is challenged by the ubiquitous presence of other cell types at the scene of birth of new microvessels. For example, hematopoietic stem cells positive for CD45 (a leukocyte marker in adult organisms) are consistently found at the advancing front of capillaries in the mouse embryos [4]. Similarly, monocytes/macrophages (MC/Mph) are associated with the developing capillaries in various angiogenic settings in adult animals, as described below. The presence of MC/Mph is considered thus far mostly from the point of view of the secretion of regulatory factors. Nevertheless, new data as well as the re-examination of older data suggests that MC/Mph may also contribute both mechanistically and structurally to the formation of capillaries.

Presence of MC/Mph within angiogenic fields

Chronic inflammation

During tissue repair, pathological remodeling or tumoral growth, the inflammatory infiltrate, composed mainly of MC/Mph, often precedes or accompanies angiogenesis (for an extensive review, see [5]). The contribution of angiogenesis to rheumatic arthritis, and to other auto-immune conditions such as systemic lupus erythematosus, systemic sclerosis, Sjogren's syndrome, mixed connective tissue disease, polymyositis/dermatomyositis and systemic vasculites, is well known [6]. The matrix metalloproteases (MMP) and their tissue inhibitors were consistently involved in the pathogenesis of these condi-

tions, and in the associated angiogenic process, besides the increased levels of vascular endothelial growth factor (VEGF) [7].

An intimate association of new blood vessels with a subpopulation of Mph was also found in the chronic inflammation of the lung [8]. Mph were the earliest cells detected surrounding the vessels in an area of cerebral injury as well [9]. Furthermore, immunohistochemical analysis of the cellular infiltrate, in a murine corneal model of angiogenesis, revealed that neither T lymphocytes, mast cells nor Mph in a more mature stage of development were part of the infiltrate that preceded the ingrowth of new blood vessels. Instead, the early infiltrating cells were mostly inflammatory MC [10]. Conversely, the abolishment of MC is known to reduce both angiogenesis and wound healing [11]. More details about the role of MC/Mph in wound-related angiogenesis are presented below.

The relationship of MC/Mph with angiogenesis may be addressed in the reverse way as well. If it is true that these cells, when specifically primed, have a facilitator role in angiogenesis, then when they accumulate in tissues, a concurrent angiogenic process would be expected to occur. This is the indeed the case with atherosclerotic plaques, where the accumulation of MC/Mph play an essential role [12] and where a concurrent angiogenesis takes place [13–15]. Remarkably, the angiogenic inhibitors have a potent blocking effect on plaque formation in experimental animal models [16–18].

Communication between MC/Mph and EC is bi-directional. EC may stimulate MC to produce the broad-spectrum monocytic MMP-9 protease [19]. This suggests that not only capillary advancement may be facilitated by MC presence, as described before, but also the mobility of MC can be stimulated by EC. Similarly, both *in vitro* [20] and *in vivo* [21] the capillaries were shown to chemoattract tumor cells and not only *vice versa*, as commonly considered.

Mph infiltration in angiogenesis-dependent tumors

The development of several types of solid tumors depends upon their vascularization (for reviews, see [22–24]). In gliomas [25], carcinomas [26, 27], non-small cell lung cancer [28], cardiac myxoma [29] or malignant melanomas [30], there is a positive correlation between Mph infiltration, vascularity and tumor growth or malignancy. However, the relationship between MC/Mph presence and tumor development is nuanced. A recent study [31] showed that tumor formation depends on monocyte chemoattractant protein 1 (MCP-1) secretion and MC infiltration. Low levels of MCP-1 followed by modest MC infiltration resulted in tumor growth due to increased angiogenesis, whereas high levels of MCP-1 secretion were associated with massive MC/Mph infiltration into the tumor mass, leading to its destruction. Similarly, tumor-associated lymphocytes and Mph may have dual roles in lung cancer, inducing either tumor promotion or destruction, depending on their number [32]. These studies demonstrate that, depending on the level of infiltration,

tumor-associated MC/Mph have a biphasic effect. At lower numbers they support tumor angiogenesis (with or without increase in tumor growth), but limit tumor development when present in a higher proportion.

Arteriogenesis

Another major contribution of MC to vasculogenesis in the adult mammals is associated with arteriogenesis (formation of collateral circulation in the ischemic tissues [33, 34]). Basically, the mechanism of arteriogenesis consists in the enlargement of pre-existent arterioles. MC/Mph are instrumental in remodeling of arterioles by destroying their elastic lamina, and allowing smooth muscle cells to proliferate and relocate [33]. Consequently, increased local levels or purposeful administration of MCP-1 and other MC chemoattractants modulate the arteriogenesis as well [35, 36]. The process is also controlled by the vascular endothelial growth factor receptor-1 (VEGFR-1) [37]. The importance of this mechanism is underscored by the fact that its impairment in diabetic patients leads to decreased arteriogenesis [38]. For an in depth review of arteriogenesis, see chapter by Heil and Schaper in this book.

MC/Mph and the pro-angiogenic factors

VEGF

In physiologic and pathologic angiogenic settings, specific local conditions such as hypoxia or lactate concentration induce the resident cells to secrete factors that attract and activate not only EC, but also MC. One of the most important pro-angiogenic factors is VEGF, (also known as the vascular permeability factor), which promotes EC migration and proliferation and increased vascular permeability, also contributing to angiogenesis [39]. VEGF action was considered to be restricted to EC. However, it was discovered that MC express the functional VEGFR-1 (VEGFR-1/Flt-1) and respond in a number of ways to VEGF exposure [40] (more details about this topic are presented elsewhere in this book).

An indication for a role of MC during the angiogenic processes may come just from the sharing of functional receptors for VEGF with EC. A major population of MC in human peripheral blood express Flt-1 on the cell surface, which is involved in their VEGF-induced chemotactic response [41, 42]. Additionally, $CD34^+$/Flt-1^- mononuclear hematopoietic cells from human cord blood can be induced to differentiate into Flt-1^+ cells by culturing them in the presence of hematopoietic cytokines [42].

The significance of these findings is emphasized by the impact of VEGF production not only on angiogenesis, but on Mph infiltration as well. Within venous thrombi, for example, increased VEGF levels were related to the occur-

rence of MC, EC and "spindle" cells [43]. VEGF increased the recanalization and organization of thrombi in a manner dependent upon MC recruitment in a rat model of inferior vena cava thrombosis [44]. In human ovarian cancer xenografts, the VEGF expression positively correlated with the number of MC/Mph present within the tumor stroma and with a higher vascularization, but not with the rate of tumor growth [45].

Thus, various cells within wounds, tumors, thrombi, and in other physiological or pathological pro-angiogenic settings produce VEGF, which induces the recruitment of MC besides that of EC. A positive feedback seems to operate here, since during this process MC/Mph are activated and in turn secrete more VEGF. Numerous factors were reported to induce VEGF secretion by MC or Mph. In wounds these are hypoxia, lactate [46] or relaxin [47]. Others are lipopolysaccharide [48], prostaglandin E(2) [49], as well as microenvironmental factors present in rheumatoid arthritis [50] and in tumors [51].

MCP-1

Similarly to VEGF, MCP-1 was largely considered an agent specific for one cell type, in this case for MC. Recently, MCP-1 was found, however, to be chemotactic for EC as well, which displays the CCR2 receptor for MCP-1. In these studies MCP-1 induced EC chemotaxis *in vitro* and angiogenesis *in vivo* [52, 53].

There is evidence that MCP-1 is angiogenic, both directly or indirectly. For example, when implanted into rabbit cornea, MCP-1 exerted a potency similar to the more specific VEGF-A_{121} [54]. In addition, a paracrine angiogenic loop described for a particular type of cancer (head-and-neck squamouos-cell carcinoma [55]) is probably more general. These tumor cells secrete MCP-1 and TGF-β1 that chemoattract and activate MC/Mph, respectively. Upon activation, Mph secrete TNF-α and IL-1, which in turn stimulate tumor cells to produce the pro-angiogenic chemokines IL-8 and VEGF. MCP-1 was found in other tumors, e.g., carcinomas [27, 56], cardiac myxoma [29] or breast cancer [57]. Significantly, in these studies MCP-1 was also present within infiltrating Mph, thus closing the positive feedback loop.

Wound healing and clot recanalization were two other situations where MCP-1 production was associated with MC/Mph recruitment and increased vascularization. When exposed to VEGF [58] or to brief ischemia [59], EC synthesize MCP-1. This and the macrophage inflammatory protein-1alpha (MIP-1α) are abundant in acute wounds, and for this reason the wound repair was expected to be altered in MIP-1$\alpha^{-/-}$ and MCP-$1^{-/-}$ mice [60]. Wound angiogenesis, epithelialization and collagen synthesis were indeed delayed in MCP-$1^{-/-}$mice, but not in MIP-1$\alpha^{-/-}$ mice. Interestingly, no change in the total number of wound Mph was observed in MCP-$1^{-/-}$ mice, which suggests that the role played by MCP-1 in healing wounds consists most likely in influencing the effector state of Mph (and possibly of other cell types) rather than sim-

ply modulating their number. In addition, endogenous MCP-1 increased with maturation of experimental venous clots in rats [61]. In the same study, MCP-1 injected into clots induced an accelerated resorbtion, by a mechanism that involved increased cellular infiltration, prior to the formation of capillaries.

Other angiogenic cytokines

Activated MC/Mph can secrete a wide array of factors that, on one hand, may induce and support processes involved in tissue vascularization, and on the other hand may help regulating these processes, by the inhibition of angiogenesis. These chemokines may either directly modulate EC migration and/or proliferation, or indirectly induce other cells to secrete angiogenic factors [5].

Activated human Mph secrete IL-8 [62], a potent angiogenic chemokine. In a rat thrombosis model, when animals were treated with IL-8, significantly more leukocytes and spindle cells (fibroblasts and EC) were noted and neovascularization was increased [63]. Similar evidence was provided by others, using an implanted sponge model of angiogenesis in the rat [64], but paradoxically EC *in vitro* were found to be non-responsive angiogenically to IL-8 [65]. This observation opens the possibility that MC themselves could be the targets for the pro-angiogenic effect of IL-8, as suggested elsewhere [66].

Other factors produced by activated MC/Mph that contribute to angiogenesis, act through their chemotactic and/or proliferative effects upon EC (for reviews see [5, 67]). Granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) induce EC migration and proliferation, in addition to their hematopoietic effects [68]. Transforming growth factor alpha (TGF-α) was shown to be angiogenic *in vivo* [69]. TGF-β increased tumor vessel density and Mph infiltration [70] and its inhibition reduced tumor vascularization [71]. Activated Mph also produce a platelet derived growth factor (PDGF)-like molecule [72], and the PDGF-D isoform of this factor stimulates tumor angiogenesis [73], although other isoforms might be inhibitory [74]. Prostaglandins, especially those of the E series, are released by activated MC/Mph and support tumor angiogenesis as well [5, 75].

Mph and anti-angiogenesis

Secretion of anti-angiogenic factors

Angiogenesis is a tightly regulated process, and it is expected that a fine balance of facilitating and inhibitory conditions will actually decide where, when and how many microvessels will occur in a specific micro-volume of tissue. In this respect, when appropriately stimulated, MC/Mph produce, besides angiogenic factors, thrombospondin-1, a very potent inhibitor of angiogenesis [76].

Furthermore, the metalloelastase known as the matrix metalloprotease 12 (MMP12, the equivalent of mouse metalloelastase, MME), which is one of the enzymes assisting Mph migration in the tissue, releases angiostatin from plasminogen [77]. For this reason and for the anti-tumoral effects following its purposeful expression in tumors [78], MME may be considered as being anti- or pro-angiogenic, depending on the circumstances (see below).

Induction of EC apoptosis by Mph

Apoptosis is a cellular function important for capillary development. While the angiogenic molecules VEGF and basic fibroblast growth factor (bFGF) have anti-apoptotic effects on EC [79], an accompanying apoptosis seems to be necessary for the normal progress of angiogenesis. Endogenous expression and exogenous application of TGF-β1 induced both capillary formation and apoptosis in glomerular EC in culture, and both apoptosis and capillary formation were uniformly and entirely absent in EC transfected with a decoy TGF-β receptor [80]. Moreover, the apoptotic Fas/Fas ligand (CD95) system was believed to control angiogenesis beneath the retina [81] in some models but not in others [82], possible due to a non-apoptotic mechanism of action [83]. In fact, the engagement of Fas receptor with the agonist anti-Fas antibody Jo2 was shown to stimulate angiogenesis in an *in vivo* Matrigel assay [84].

The cellular turnover, with its apoptotic component, was aptly identified as a factor during lumen formation in a culture model of capillary tube formation [85]. Supposedly, the apoptosis of some cells facilitates the relocation, insertion and migration of others. Proteolytic enzymes released by the dying cells may help with the dissolution of the extracellular matrix (ECM) in these phenotypically homogenous cultures. As an illustration of the complexity of the situation, Mph can be anti-angiogenic through a pro-apoptotic mechanism. Besides scavenging apoptotic bodies from cells undergoing programmed cell death, they can also induce apoptosis of normal cells [86]. Using injected toxic liposomes to eliminate Mph in the anterior chamber of the rat eye, it was showed that Mph induce apoptosis *in vivo* in normal vascular EC during programmed capillary regression. Mph elimination resulted in the survival of EC that would normally die during ocular maturation, and the persistence of functional capillaries.

Implications for angiogenesis of ECM penetration by MC/Mph

Role of matrix-degrading enzymes

In order to form new capillaries, EC need to migrate. Since the tissues oppose a mechanical resistance, the cells must create a “path” by degradation of the ECM [87]. ECM, which consists of basement membrane and interstitial stro-

ma, has a complex molecular structure and accordingly there are matrix-degrading enzymes specific for each component. The most important are the matrix metalloproteinases (MMPs) which are expressed by many cell types, including activated inflammatory cells, tumor cells, stromal cells and EC [87–89]. Consequently, their role and biological effects differ according to the cells of origin, the specific ECM component(s) degraded, and the response of surrounding cells.

The dissolution of ECM was considered so far mostly from the point of view of its molecular mechanisms [90, 91]. Morphological observations were presented much less frequently in the literature [92]. Nevertheless, it was recently suggested that the subcellular distribution of the proteolytic enzymes (e.g., the preferential accumulation at the leading edge of the advancing cells) is as important as is their biochemical nature [93].

The analysis of the temporal pattern of MMP expression during the healing of skin wounds in the mouse showed that after an initial outburst of more common collagenases and of their inhibitors, at later stages of healing the Mph-specific MME became dominant, and it was clustered around vascular structures [94]. This suggested that Mph are present and active during the penetration of ECM by capillaries. ECM degradation has other important angiogenic consequences. Several angiogenesis inhibitors are products of the proteolysis of different matrix components. The fact that angiostatin, endostatin and several other anti-angiogenic peptides are cleavage products of matrix proteins was mentioned previously. Interestingly, a very efficient enzyme producing them is the Mph-specific MMP12/MME [77], again supporting the idea that these cells act on both sides of the angiogenic balance.

Intercellular cooperation during formation of capillaries

Even in homogenous EC cultures *in vitro*, the development of a "capillary" involves several different functions, accomplished by different cellular sub-populations, split *ad hoc* in at least three functionally distinct phenotypes [85]. One cell sub-population, displaying phagocytic morphology, migrates through the gel and creates "channels" (or "tunnels", as defined below) which set the pattern of the capillary network. These tunnels are later lined by a second sub-population of cells characterized by the presence of intracellular vacuoles. The vacuoles fuse with the plasma membrane in a tubular fashion, thus creating the capillary lumen. A third sub-population of cells migrate and self-interpose between the EC involved in lumen formation, a process that results in the enlargement of the capillary tube. This study emphasized the importance of a pre-formed "path" [85], in the process of new capillary tubes formation [93].

In an early *ex vivo* model of capillary outgrowth from adult vessels in fibrin matrices, it was also shown that the ability of mobile EC to insert among near-by cells is based on the pre-existence of a capillary lumen, even an immature one. The process was named "guided migration" of EC [96], defined as loco-

motion of cells along pre-existing, non-perfused capillary-like structures [95]. In its simplest form, this could be the tunnel produced by a forerunner cell that penetrates a three-dimensional ECM.

During the infiltration of tissues, MC actively penetrate ECM. Therefore, a permanent degradation of the matrix is expected to occur around and behind the migrating MC/Mph, the level of which depends on the ECM density. We previously described the association of MC/Mph penetration with mouse metalloelastase (MME) immunostaining [97] in a model of mouse cardiomyopathy based on transgenic expression of MCP-1 in adult mouse cardiomyocytes (MCP-1 hearts) [98]. This progressively induced an inflammatory infiltrate, of which the MC/Mph population represented a main component [99]. We observed a system of tissular "tunnels" with a cross-sectional size comparable to the diameter of the infiltrating cells [97], similar to those produced by the penetration of phorbol ester-activated Mph in a fibrin matrix *in vitro* [100] (phorbol ester also induces the expression of MME [101]).

The nature and fate of these tunnels was not considered so far, despite the previous empirical evidence for their existence [100]. They are tubular spaces of lower density, containing extracellular fluid and degradation products of the ECM. In histological sections, the tunnels may look as "empty" when their content is extracted. *In vivo*, the tunnels may slowly collapse under the pressure of the nearby tissue, or may be occupied by other cells and/or by their secretory products [102]. It provides a mechanistic reason for why the cells align when they penetrate the ECM. It would be more economical for the cells in the column to follow in the tunnel the cell at the leading front, than to migrate independently. This process, similar to the advancement of a group of people in the snow, is reminiscent of the "cohort migration" of tumor cells [103]. Based on the above observations, we suggested that in certain conditions the first step of capillary formation, the drilling of a path, may be accomplished by MC/Mph besides (or instead of) EC [102], because MC/Mph are so well equipped for tissue penetration and matrix engulfing [104].

To reach intra-tissular positions, MC/Mph must pass first through endothelial basal lamina and sometimes through elastic laminae, a process that depends on the expression of MME [105] or similar proteases. When we assessed by immunochemistry the presence of MME in the MCP-1 hearts, in addition to positive MC/Mph (Fig. 1, insert) we found MME positive tunnels spreading throughout the myocardium, thus giving us an indication for their relationship with the penetrating cells [97]. The explanation of the immunopositivity of tunnels may reside in the binding of the shed MME to residual elastin fragments, as shown by immunostaining in the media of abdominal aortic aneurisms [106], or in the direct shedding of the enzyme similar to another molecule with affinity for elastin, the 67-kDa cell surface elastin binding protein [107].

Various recent studies proposed that EC in the lining of larger or smaller vessels of adult organisms can be replaced with bone marrow derived, circulating endothelial progenitor cells (EPC), by a process named "maintenance

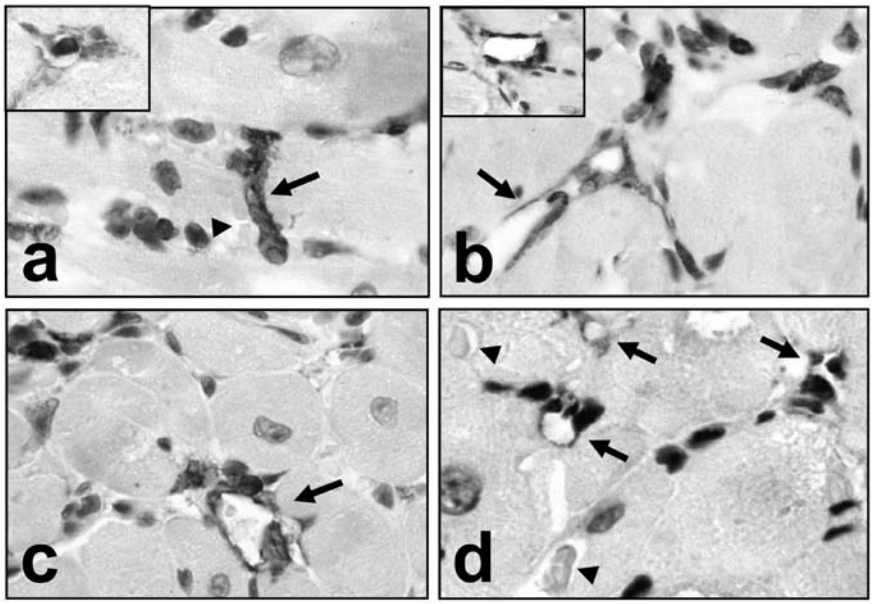

Figure 1. Potential contribution of mononuclear leukocytes to formation of microvascular conduits in the hearts of transgenic mice expressing MCP-1 under a cardiac-specific promoter. (a) Structure positive for CD18 (pan leukocyte antigen, arrow) running in parallel with a capillary, identified by erythrocytes (arrowhead). Note the counterstained (blue*) nucleus of a cell apparently penetrating the extracellular matrix. *Insert:* MC/MPh positive for MMP12 and presenting a surrounding proteolysis rim. (b) Space in the myocardium, lined by CD18 positive cells (arrow). *Insert:* a lumen edged by Mac-3 positive macrophages. (c) Erythrocytes present within a CD18 positive microvascular structure (arrow). (d) Structures populated by CD34-positive cells (arrows). Note that *bona fide* capillaries (identified by erythrocyte content, arrowheads) are CD34 negative. All samples were analyzed by DAB immunocytochemistry (brown*) with hematoxylin counterstaining (blue*). Negative controls (omission of the primary antibody) were all negative (not shown). Original magnifications: ×120. Reproduced with permission from [102]. (*For colored picture see color plate 7)

angiogenesis" [108]. Fresh angiogenic tufts can apparently be initiated in extravascular positions, also through a mechanism resembling angioblast-based vasculogenesis [109]. Additionally, the re-vascularization and re-canalization of experimentally isolated intravascular thrombi seems to proceed based on the angiogenic potential of the blood-borne cells trapped inside [110–112]. The contribution of EPC to neo-vascularization in the adult animals is comprehensivelly addressed elsewhere in this book by Nishimura and Asahara, and in other previously published reviews on this topic [113, 114].

Based on our observations as discussed previously, we suggested that the cooperation between penetrating and phagocytic activity of MC/Mph on one side and tissular insemination and proliferation of EPC on the other side is potentially important for the inflammation-driven angiogenesis [115]. In this hypothesis, EC derived from the blood-borne mononuclear EPC, or from nearby microvessels by sprouting, may be assisted during their ECM penetration by

actively migrating MC/Mph. The "leading" MC may modify the ECM by drilling a tunnel, which would be thereafter colonized either by capillary sprouts, or by EPC [115b]. At the same time, MC may nurture and/or attract the cells following them, by secretion of angiogenic and differentiation factors. Alternatively, a tunnel made in the ECM and occupied by multiple MC may contain a small fraction of EPC, which would further differentiate (or trans-differentiate, if derived directly from MC, as discussed below) into EC covering the tunnel wall. This may proceed through a mechanism similar to the covering with EC of bare plastic grafts interposed in the circulation [116], but acting at a much smaller scale. Experimentally, this possibility is supported by the recolonization with fresh EC of a pre-existent capillary lumen after it had lost its native endothelium, in an experimental subretinal neovascularization model in the monkey [117]. In this study, re-endothelialization occurred after laser photocoagulation by migrating activated EC within the confines of the old basement membrane (therefore in the previous microvascular tube), which formed a scaffold and pathway for the orderly reconstitution of the same microvessel. This process was based on EC migration, with minimal or no EC division.

This alternative model would account for angiogenesis in places where sprouting from nearby microvessels is difficult or impossible, due to local conditions, such as those found in large non-vascularized thrombi (and consequently placed distantly from "mother vessels") and in dense or necrotic tissues. Deep penetration of MC, based on cell surface-limited proteolysis in clots and other fibrin and collagen-rich matrices, or in certain necrotic tissues, would leave behind lasting tunnels. Some of these tunnels would be filled, due to the blood pressure, by adjacent erythrocytes, and/or by other blood cells, including EPC [118].

Recent experimental data also support a role for blood borne mononuclear cells (including bone marrow derived progenitors) in organization, vascularization and recanalization of thrombi [44, 61, 119, 120]. Older studies have described in detail the structural modifications accompanying these processes *in situ* in fibrin-rich thrombi, including the clearance by cell-mediated proteolysis and phagocytosis of the interfibrillar spaces, with formation of "channel"-like tubes, further "colonized" by endothelial progenitors [110, 112, 121–123]. The common themes are: (1) involvement of pluripotent mononuclear cells and (2) phagocytic and/or proteolytic activity of MC/Mph in modifying the thrombus to a pro-angiogenic state. These cells, besides a nurturing function, may therefore assist the engraftment of thrombus-trapped or incoming EPC.

Trans-differentiation of MC into EC?

Suggestions from in vivo *observations*

The mononuclear phagocytes present in the peripheral blood consist of a heterogeneous mixture of different cell sub-populations. The question how to

define a cell type is more than an issue of terminology [124]. For example, the very nature and relationship between MC and Mph is still a matter of debate [125]. How are they related? How soon after tissular recruitment a MC becomes Mph? Or maybe a subpopulation of MC already have Mph traits while still being in the circulation? Some researchers consider this as being the case [126]. The definition of cellular phenotype traditionally relied on the expression of different surface markers. However, between various cell types there are significant overlaps, that fall in two categories: synchronic (when two cell populations at a specific moment are considered and their properties compared), and diachronic (when a given cell population is followed in time as its properties change, acquiring the features of another cell type, such as during trans-differentiation [127, 128]). For a thorough analysis of the phenotypic overlaps between MC/Mph and EC from both points of view, see [129].

Figure 1 presents CD18 positive leukocytes lining apparently bloodless, as well as blood-containing, conduits in the MCP-hearts [98, 99]. These situations involving a pre-existent vascular "tube" and non-endothelial cells which cover it and adopt endothelial functions, suggest an alternative mechanism of vasculogenesis. Recently it was discussed in the literature another instance where MC/Mph participating in the foreign body reaction apparently acquire endothelial-like properties upon exposure to the blood [130, 131].

Another example of "vascular mimicry" is the way the cytotrophoblasts help anchor the fetus to the mother and establish the blood flow to the placenta. These cells invade uterine interstitium and penetrate the vasculature from downstream, replacing the maternal endothelium. To acquire this new function, they modify the repertoire of adhesion receptors and surface molecules, such as to resemble the replaced EC. They start expressing VE-cadherin, platelet-endothelial cell adhesion molecule-1 (PECAM-1) and alpha-4 integrins, while decreasing the synthesis of E-cadherin [132]. Similarly, in tumors a variable proportion of cells lining the microvasculature were found to be of tumoral origin [133]. In particular, highly invasive melanoma cells seem to be able to replace the endothelium [134–136].

These data suggest the ability of cells of various origins to adapt to the functions they are required to accomplish, by an extensive modification of their phenotype. Regarding the establishment of blood conduits, alternative mechanisms to sprouting are therefore likely to exist. Besides capillary splitting (also called "intussusceptive angiogenesis" [137]) and capillary fusion [138], another one seems to be the colonization of pre-existent microvessel-like tube, either by committed or by opportunistic cells which would then acquire endothelial functions. This hypothesis is also supported by the occurrence of interstitial fluid "channels", before EC organization, in a model of lymphatic vessels formation during mouse skin regeneration [139].

In vitro *cultivation of EPC and induction of MC-to-EC conversion*

The *in vitro* differentiation of bone marrow-derived, peripheral blood mononuclear cells is still the subject of an active research. Blood is the source of cells that, given the right conditions (angiogenic growth factors and appropriate adhesion substrate), may differentiate both *in vivo* and *in vitro* into cells displaying at least some endothelial traits [140, 113, 141]. The potential role of these cells in angiogenesis is supported by their ability to accelerate restoration of blood flow in ischemic limbs [142, 143] and in thrombotic veins [119, 120]. Moreover, bone marrow-derived cells could restore vascular structures and regenerate infarcted myocardium [114, 144, 145]. However, it has been thus far very difficult to clearly define the phenotype of these cells.

Several recent studies aimed at characterizing the *in vitro* differentiation of cultured peripheral blood MC [146–153]. In all situations, the authors started from adherent (non-lymphocytic) mononuclear blood cells or, in one case [153], from GM-CSF cytokine-mobilized leukapheresis products that were further selected for the presence of CD34 on their surface. In this latter study, after about 6 weeks in culture, most of the cells started to express both the monocytic marker CD14 and also the endothelial markers VE-cadherin, eNOS and von Willebrand factor, even if the cells did not eventually form a network of tubular structure in Matrigel. A report by Zhao et al. [152] showed that, depending upon the culture conditions, peripheral blood MC could acquire a variety of phenotypes: endothelial, epithelial, T lymphocytes, neuronal or hepatocyte. The stimulus for endothelial differentiation was once again VEGF, which induced expression of VEGF-R2, VEGF-R3 and von Willebrand factor. Interestingly, the cells that differentiated from blood MC became *Ulex Europaeus* lectin positive, were uptaking acetylated LDL (both considered characteristics of EC phenotype) and did not proliferate, but they secreted pro-angiogenic growth factors [150]. Although the cell culture conditions were different in each of these studies, they presented some common features: upon cultivation, these cells (1) maintained some of the MC and/or hematopoietic markers, (2) displayed MC activation and Mph differentiation markers and at the same time (3) started expressing EC markers (such as VE-cadherin, eNOS, von Willebrand factor). Together, these data further support the notion that peripheral blood MC have the capability to trans-differentiate into cells expressing an EC phenotype, given the appropriate microenvironmental conditions, which are present in many pro-angiogenic situations *in vivo*.

Conclusions and perspectives

Angiogenesis is a complex, seemingly multicellular process. For this reason, the understanding as well as the ability to manipulate angiogenesis may depend on the attention given to these cellular “collaborators” that either promote or mitigate neovascularization. Among them, MC/Mph play a key role.

They provide soluble pro- and anti-angiogenic factors and process the ECM making it more prone for capillary penetration or for the engraftment of the endothelial precursors. Additionally, they may provide cellular components of the neovessels in the form of the EPC sub-population, or as cells potentially able to progress to an EC status.

Therefore the MC/Mph, as well as the associated bone-marrow derived EPC could be considered targets of therapeutic angiogenesis or of anti-angiogenic treatments. This is indeed an active research field, with the purpose of improving the perfusion of infarcted myocardium [145, 154] or ischemic limbs [143], and for the re-vascularization of intravascular thrombi [44, 61, 119].

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Mechanisms of Angiogenesis
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Bone marrow-derived endothelial progenitor cells for neovascular formation

Hiromi Nishimura and Takayuki Asahara

Department of Regenerative Medicine, Institute of Biomedical Research and Innovation, 2-2 Minatojima-minamimachi Chuo-ku, Kobe, 650-0047, Japan

Introduction

As the fertilized ovum undergoes repeated cell divisions to increase the cell population for tissue or organ formation exchange of material by simple diffusion becomes unable to nourish all cells. Before facing such a situation, the developing embryo begins to harbor the blood island, which consists of hematopoietic stem cells and angioblasts. Hematopoietic stem cells and angioblasts differentiate into blood cells and vascular endothelial cells in the blood islands, respectively. Then, the blood islands begin fusing with each other and form vasculature with blood cells in its lumen [1, 2]. With the cardiovascular development, the exchange of material by the circulatory system becomes effective and capable of nourishing the growing tissue or organ. In the adult, hematopoietic stem cells in the bone marrow continue asymmetric cell divisions and supply blood cells throughout the individual's life span. However, it was unknown whether hemangioblasts or other stem cells that can differentiate into vascular endothelial cells exist or not. Folkman [3] documented that the extent of tumor growth was dependent upon neovascularization and suggested that this relationship might be based on the angiogenic growth factors that were released by neoplasms. Since his report, neovascular formation derived from pre-existing neighbor vessels has been called angiogenesis. Subsequent investigations revealed the potential of using recombinant formulations of such angiogenic growth factors to expedite and/or augment collateral blood flow in the ischemic tissue. This novel strategy to treat ischemic disease was termed therapeutic angiogenesis [4]. In the developing embryo, vessel formation is initiated by the fusion of blood islands. Then, vascular endothelial cells are supplied by cell division and differentiation of angioblasts in the foci of neo-vascular formation, which is called vasculogenesis [1]. After vessel formation by vasculogenesis, vascular elongation occurs. The vascular endothelial cells in this stage are supplied from endothelial cells of neighboring vessels, which is called angiogenesis [2]. As stated above, vascular formation in the embryo consists of vasculogenesis and angiogenesis, but only angiogenesis has been discussed in relation to adult neovascular formation [1].

What is the endothelial progenitor cell?

In 1997, Asahara et al. discovered that CD34-positive mononuclear cells in the human peripheral blood incorporated into the foci of vascular injury and differentiated into vascular endothelial cells [5]. When cultured on fibronectin-coated dishes with growth factors, such as vascular endothelial growth factor (VEGF) and/or basic fibroblast growth factor, the CD34-positive cells express endothelial nitric oxide synthase (eNOS), kinase insert domain receptor (KDR) and CD31. They then become spindle-shaped cells and take up acetylated low density lipoprotein (LDL), which are features of the endothelial lineage. Human CD34-positive mononuclear cells were isolated from peripheral blood and marked with red fluorescent dye. Then, they were administered to nude mice in the ischemic hindlimb model. After six weeks, some of the vascular endothelial cells in the hindlimb muscle demonstrated red fluorescence, which indicated that administered human CD34-positive mononuclear cells were incorporated into the ischemic muscle tissue and differentiated into endothelial cells [5]. A bone-marrow transplantation model was used in order to exclude the possibility that the cells stained with red fluorescent were not the administered cells but the mouse endothelial cells that had taken up the dye. Bone marrow mononuclear cells from *Flk-1/lac Z* or *Tie-2/lac Z* transgenic mice were transplanted to the background of immuno-deficient mice that were lethally irradiated before the bone marrow mononuclear cell transplantation. The donor *Flk-1/lac Z* or *Tie-2/lac Z* transgenic mice were genetically modified to express bacterial β-galactosidase under the control of an endothelial cell-specific promoter. In other words, only endothelial cells express bacterial β-galactosidase in these mice. It is possible to identify the cells expressing bacterial β-galactosidase by X-gal staining or immunohistochemistry. After bone marrow reconstruction by the transplanted cells, both physiological and pathological neovascularization models were examined in the recipient mice. We can observe the physiological neovascularization in the adult when the corkscrew-like arteries are formed in the endometrium of the proliferative phase [6]. The uteri of recipient female mice whose bone marrow was reconstituted with cells from *Tie-2/lac Z* transgenic mice were examined. In the uteri of mice in the late proliferative phase, frequent incorporation of cells into the vascular structures was observed by X-gal staining. This result indicates that a certain population of bone marrow-derived cells were incorporated in the spiral arteries and differentiated into endothelial cells. We created ischemic models by removing femoral arteries of recipient mice [6]. After two weeks, the hindlimb muscles were examined. Sections stained with X-gal demonstrated that the neovasculature of the ischemic lesions frequently comprised *Tie-2*-expressing cells in the vascular structures. In the next experiments, mouse syngeneic colon cancer cells were implanted subcutaneously into the recipient mice and tumor samples were excised after two weeks. X-gal staining revealed that bone marrow-derived endothelial cells were incorporated even in the developing tumor

tissue [6]. These results indicate that a certain population of bone marrow cells is recruited in the foci of neovascular formation and differentiates into vascular endothelial cells in the setting of both physiological and pathological neovascular formation. These cells are now identified as endothelial progenitor cells (EPCs). The discovery of EPCs indicates that postnatal neovascularization does not rely exclusively on sprouting from pre-existing blood vessels (angiogenesis). Instead, EPCs are released from bone marrow to be incorporated into and thus contribute to postnatal physiological and pathological neovascularization, which is consistent with postnatal vasculogenesis. Since these findings, neovascularization is classified by the origin of endothelial cells. Angiogenesis is derived from pre-existing vessels; vasculogenesis is from bone marrow-derived EPCs. Currently, both are considered to be involved in neovascular formation, even in the adult [7].

Pre-clinical animal research of EPCs

EPCs are released from bone marrow into circulating peripheral blood as CD34-positive mononuclear cells and incorporated into the foci of neovascularization. EPC supply and incorporation are modulated by various kinds of intrinsic and extrinsic factors [8]. Physiologically, the menstrual cycle affects the supply of EPCs. The number of EPCs in peripheral blood increases in the proliferative phase to form spiral arteries and decreases in the luteal phase. It has also been demonstrated that endogenous stimuli like tissue ischemia or exogenous cytokine therapy by VEGF, granulocyte macrophage-colony stimulating factor (GM-CSF) or granulocyte-colony-stimulating factor (G-CSF) mobilize EPCs from bone marrow into the peripheral blood and thereby contribute to neovascularization of ischemic tissues. The development of regional ischemia in both mice and rabbits increases the frequency of circulating EPCs. In mice, the effect of ischemia-induced EPC mobilization was demonstrated by enhanced ocular neovascularization after corneal micropocket surgery in mice with hindlimb ischemia compared with that in non-ischemic control mice. In rabbits with hindlimb ischemia, circulating EPCs were further augmented after pretreatment with GM-CSF, with a corresponding improvement in hindlimb neovascularization. These findings indicate that circulating EPCs are mobilized endogenously in response to tissue ischemia probably via cytokine secretion from ischemic tissue or the exogenously administered cytokines, and thereby augment neovascularization of ischemic tissues [8]. Besides EPC mobilization from bone marrow into peripheral blood, recruitment of EPCs from peripheral blood into the ischemic tissue is another important factor for the EPC contribution to the neovascular formation. A recent study revealed that adhesion molecule-like integrin subunits, such as alpha5, beta1, alpha(v) and beta5, play an important role in the EPC accumulation of EPCs in damaged endothelium [9]. In addition, a local injection of stromal cell-derived factor-1 (SDF-1) into athymic nude mice with ischemic hindlimb

muscle succeeded in accumulating human EPCs in the injected sites and augmented revascularization. Human EPCs expressed SDF-1 receptor, CXCR-4, and migrated toward SDF-1 in the migration assay [10].

To estimate the potential of EPCs in therapeutic strategies to promote postnatal neovascularization, administration of *ex vivo*-expanded human EPCs into athymic nude mice with hindlimb ischemia or nude rats with myocardial ischemia was performed. The results demonstrated that blood flow recovery was markedly improved and the rate of limb loss was significantly reduced in the athymic nude mice with hindlimb ischemia [11]. In the ischemic myocardial rat models, ventricular dimensions and fractional shortening was significantly improved in the EPC-administered group as compared to the control group. Furthermore, the extent of left-ventricular scarring was significantly less in rats receiving EPCs. And in both the mouse hindlimb ischemia model and the rat myocardial ischemia experiments, capillary density was significantly greater in the EPC-administered group and labeled human EPCs were detected in the foci of neovascular formation [12]. To test these favorable effects of EPCs in improving ischemic pathologies of mice or rats in the clinical settings, a large animal study was necessary. Recently, a preclinical study of catheter-based, intramyocardial transplantation of autologous EPCs in a swine model of chronic myocardial ischemia demonstrated the therapeutic potential of cell-based therapy, with attenuation of myocardial ischemia and improvement in left ventricular function [13]. These favorable results suggest a therapeutic capability of EPC transplantation in clinical settings. However, the limitation of autologous EPC isolation from peripheral blood also needs to be considered. Modifying EPC function is one of the candidates for overcoming the cell number limitation. Transplantation of heterologous EPCs transduced with adenovirus encoding VEGF enhanced neovascularization and blood flow recovery more potently than mock-transduced EPCs [14]. The quantity of EPCs used in the study was 30 times less than that required in previous experiments [14]. Other gene transfer, such as that of human telomerase reverse transcriptase (hTERT) could also provide a novel therapeutic strategy. Overexpression of hTERT enhanced EPC migration toward VEGF, inhibited starvation-induced apoptosis and promoted total differentiation of EPC colony appearance in an *in vitro* study. Even *in vivo*, hTERT-induced EPCs dramatically improved postnatal neovascularization in terms of limb salvage, perfusion and capillary density in comparison with that of mock-transduced EPCs [15].

Clinical research on EPCs

As the kinetics of EPCs are closely related to ischemia, it is necessary to investigate the effects of coronary risk factors on EPC kinetics and function. Dimmeler et al. [16] reported that patients with coronary artery disease (CAD) have fewer EPCs in peripheral blood. They evaluated risk factor score by age, sex, hypertension, diabetes, smoking, positive family history of CAD and LDL

cholesterol levels. The value of the risk factor was significantly correlated with a reduction of EPC levels. The migratory response was also impaired in the patient with CAD. This effect was exacerbated by smoking and hypertension [16]. It was also demonstrated that EPCs from patients with type II diabetes exhibit impaired proliferation, adhesion and incorporation into vascular structures [17]. Statins, which lower the cholesterol levels of peripheral blood, contribute to the primary and secondary prevention of CAD. However, the evidence suggests that statins possess favorable effects independent of cholesterol reduction. As a partial explanation, statins were demonstrated to mobilize EPCs from bone marrow and induce adhesiveness by integrin upregulation [10, 18]. These kinds of epidemiologic results strongly suggest the clinical potential of EPCs to treat ischemic disease. One of the most promising strategies is injecting EPCs isolated from one's own peripheral blood into the ischemic region. In order to mobilize CD34-positive mononuclear cells from bone marrow into peripheral blood, G-CSF was administered to patients in our institute. After administration on 4–7 consecutive days, an apheresis method was used to obtain maximum numbers of mononuclear cells from peripheral blood. The surface antigen, CD34, was used to purify the obtained mononuclear cells. The CD34-positive mononuclear cells, EPCs, were injected directly into the muscle of the ischemic lower limb or ischemic myocardium by a catheter system. Compared with animal experiments, expansion of EPCs *ex vivo* is not suitable for clinical settings. Until the safety of the animal products that are necessary for the EPC expansion will be established, freshly isolated EPCs were used for therapeutic angiogenesis. Further basic research, with improved understanding of the mechanisms governing homing and incorporation of EPCs, will still be necessary to optimize the conditions of therapeutic angiogenesis by EPCs.

Umbilical cord blood-derived EPCs and embryonic stem cell-derived vascular progenitor cells

There are several other sources of cells that can differentiate into vascular endothelial cells and contribute to neovascular formation. Murohara et al. [19] reported that umbilical cord blood contained CD34-positive cells and they were capable of differentiating into vascular endothelial cells and forming neovasculature in animal ischemic models. He classified the cells as EPCs and also reported that the proliferative ability of cord blood-derived EPCs is more potent than that of bone marrow-derived EPCs. Besides the individual *in vitro* capacity, we must consider the limitation of the EPC numbers isolated from cord blood [19]. Under current available methods, it is difficult to obtain sufficient numbers of EPCs from cord blood to treat adult ischemic disease. Yamashita et al. [20] succeeded in making mouse embryonic stem (ES) cells differentiate into vascular smooth muscle cells and endothelial cells *in vitro*. They cultured E-cadherin-positive and Flk-1-positive ES cells on feeder cells. The ES cells differentiated into both vascular smooth muscle cells and

endothelial cells. They named the obtained cells vascular progenitor cells [20]. Compared with bone marrow-derived EPCs, ES-derived vascular progenitor cells have advantages as to the cell-supply issue, but, on the other hand, ES-derived vascular progenitor cells must overcome many ethical and practical problems before being used in a clinical setting.

Future direction

The basis of our investigation is gene therapy for ischemic diseases. This was intended to facilitate vascular formation in ischemic tissue and was called therapeutic angiogenesis. Originally, physiological and pathological vascular development in the adult had been considered synonymous with angiogenesis. But the finding that EPCs home in on sites of neovascularization and differentiate there into endothelial cells is consistent with "vasculogenesis", through which the primordial vascular network is established in the embryo. The therapeutic recovery of the blood flow will attenuate the functional impairment of the ischemic organ or tissue. Recently, reconstituting organ function by cell transplantation has received great attention. Except for the hematopoietic system, therapeutic angiogenesis is the leading area of this type of regenerative medicine. Probably, the clinical effectiveness of EPC transplantation for ischemic disease will be established in the very near future. To compensate for the functional cell loss of an organ or tissue, stem or progenitor cells of the organ will be transplanted. This kind of functional impairment is frequently associated with reduced blood flow in the organ. And patients with ischemic disease often have a limited capability of neovascular formation. Without sufficient capacity for neovascular formation, transplanted stem or progenitor cells will not be nourished and cannot survive. Therefore, therapeutic angiogenesis will become basic and essential for the development of regenerative medicine. Therefore, the combination of therapeutic angiogenesis and stem/progenitor transplantation is quite relevant to facilitating organ regeneration. Administering stem or progenitor cells of the organ and EPCs will be one of the strategies for regenerative medicine. Until the recent reports by Matsumoto et al. [21] and Lammert et al. [22], the role of endothelial cells in organogenesis was thought to be restricted to providing a blood supply for the regenerating tissue or organ. However, they demonstrated that endothelial cells are necessary for embryonic organogenesis even before they form vessels because endothelial cells supply some essential signals for differentiation [21, 22]. Applying these results to adult tissue regeneration from stem cells, we can predict a close relationship between endothelial cells and stem cell differentiation. Concomitant EPC administration may be beneficial, in this respect, as well as controlling the differentiation of the transplanted stem/progenitor cells.

In conclusion, the era of regenerative medicine by cell transplantation is now beginning. The strategy for neovascular formation, including EPCs, will become an essential factor in regenerative medicine for all organs.

Summary

A certain population of mononuclear cells in the peripheral blood is capable of contributing to new vessel formation by differentiating into endothelial cells. These cells were discovered by Asahara in 1997 and named endothelial progenitor cells (EPCs). In the previous hypothesis, the endothelial cells of newly formed vasculature were considered to be derived only from nearby pre-existing vessels in the adult. However, it is demonstrated that the bone marrow-derived EPCs are incorporated in the foci of both physiological and pathological neovascular formation. Furthermore, clinical usefulness of EPCs from human peripheral blood is also suggested from animal experiments. If EPCs are administered to immunodeficient animals in ischemic disease models, neovascular formation is augmented and ischemia-induced tissue damage or functional disorder is attenuated. These results indicate that administering EPCs could be a new clinical strategy to treat ischemic disease, diabetic retinopathy or neoplasm in which the promotion or inhibition of neovascular formation is critical. In this chapter, we have showed the significance and potential of EPCs in the basic and clinical settings of neovascular formation.

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Mechanisms of Angiogenesis
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Can angiogenesis be exercised?

Jalees Rehman

Krannert Institute of Cardiology and Indiana Center for Vascular Biology and Medicine, Indiana University School of Medicine, 975 W. Walnut Street, IB 441, Indianapolis, IN 46202, USA

Introduction

"Exercise is good for the heart." This adage is frequently used and makes intuitive sense, thus prompting numerous research endeavors attempting to confirm its validity with scientific methodology. The cardioprotective and vasculoprotective effects of regular exercise that have so far been identified range from reduction of cardiovascular risk factors like obesity and hyperlipidemia to mitigation of ischemia reperfusion injury and improvement of endothelial function [1]. Recent research has also focused on an additional cardio- and vasculoprotective aspect of exercise: angiogenesis (the term angiogenesis in this chapter will be used to describe the growth of capillaries, collateral blood vessels or both).

Exercise has been frequently used as an outcome measure of therapeutic angiogenesis, since increased exercise tolerance suggests improved cardiac or skeletal muscle perfusion. Recent studies, however, show that exercise is not only an outcome measure of therapeutic angiogenesis, but may itself regulate angiogenesis. Many animal and some human studies so far have shown that angiogenesis is enhanced by regular exercise [2–4]. Many of the human studies, which have demonstrated correlations between the participation in exercise programs and the presence of collateral blood vessels, have had to grapple with the selection bias, that patients with collaterals are more likely to participate in an exercise program than those who do not have sufficient collateral flow. Animal studies, on the other hand, have been repeatedly able to demonstrate that regular exercise can indeed enhance the formation of new vasculature.

In the past years the focus of research has been the identification of mediators of this effect. Most studies have hypothesized that acute or single bouts of exercise have angiogenic effects by changes in shear stress, release of growth factors and cytokines or the mobilization of angiogenic cells. Regular or chronic exercise is then seen as a series of repeated pro-angiogenic episodes which may ultimately result in the growth of new blood vessels.

Current models of exercise-induced angiogenesis hypothesize that there are three major ways by which exercise can induce or enhance angiogenesis:

1. Changes in shear stress which then either mechanically or by increased expression of growth factors induce the growth of blood vessels
2. Localized hypoxia in the skeletal or cardiac muscle, which in turn induces growth factors promoting the proliferation of blood vessels
3. Systemic mobilization of angiogenic cells which may home to areas of muscle ischemia and locally induce angiogenesis

This review will focus on the mediators of these effects which in all cases appear to be either pro-angiogenic growth factors or pro-angiogenic cells. The mechanical effects of shear stress changes during exercise and how they may contribute to angiogenesis have been previously reviewed [2, 5]. Due to the cutting edge nature of the topic and the required brevity, this chapter cannot be a comprehensive overview of the topic and is likely to be out-of-date by the time it is published. For more extensive reviews of the topic, please refer to [2, 6, 7].

Exercise effects on growth factors

Exercise appears to acutely increase the expression of vascular endothelial growth factor (VEGF) mRNA, but not of basic fibroblast growth factor (bFGF) (also known as FGF-2) mRNA in human subjects. A recent study measured mRNA levels of the pro-angiogenic cytokines VEGF and bFGF as well as the mRNA levels of the alpha and beta subunits of Hypoxia Inducible Factor-1 (HIF-1) in skeletal muscle biopsies of volunteer subjects 30 minutes after an episode of one-legged cycle ergometry exercise [8]. While VEGF mRNA levels increased markedly, bFGF levels were not significantly affected by the exercise. The mRNA level of the beta-unit of transcription factor HIF-1 was also increased, but the alpha unit mRNA levels were not. This study did not examine the HIF-1 alpha protein levels. Considering that HIF-1 alpha, which is a major transducer of hypoxia signaling, appears to be regulated on a post-transcriptional level [9], future studies may need to measure HIF-1 protein levels after acute exercise. Acute bouts of exercise do not raise plasma VEGF levels, instead there appears to be a trend towards lower circulating VEGF levels after acute or chronic exercise [8, 10].

A second study was able to confirm some of these findings, by demonstrating that a single bout of exercise on a knee-extensor ergometer in sedentary volunteer subjects led to a substantial increase of skeletal muscle VEGF mRNA but not of bFGF mRNA [11]. Exercise was acutely associated with an anticipated drop in intracellular pO_2, and exercising the subjects in conditions of external hypoxia led to a more prominent decrease of intracellular pO_2. Interestingly, this additional decrease in intracellular pO_2 did not result in increased VEGF mRNA production. Therefore, no clear correlation between VEGF mRNA and intracellular pO_2 could be shown. A follow-up study by the same group then demonstrated that regular exercise training in humans using

single leg knee extension [12] showed increased blood flow and capillary density in the trained leg after 8 weeks, thus supporting the idea that regular exercise can enhance angiogenesis, at least at a capillary level. Interestingly, the induction of VEGF mRNA in the trained muscle after an acute bout of exercise was attenuated when compared to the untrained muscle. The baseline VEGF mRNA levels were not significantly different in the pre-trained and post-trained legs. The study also demonstrated that bFGF mRNA did not change significantly with acute or chronic exercise confirming that VEGF may be more relevant to exercise induced angiogenesis than bFGF.

Another study suggests that the increases in skeletal muscle capillary density of rats following exercise training may be due to Angiotensin II mediated increases in VEGF levels [13], as blockade of either the Angiotensin receptor or Angiotensin Converting Enzyme (ACE) was able to mitigate exercise-induced angiogenesis as well as the exercise induction of VEGF. This study also supported the role of VEGF in exercise-induced angiogenesis by showing that virtually all the effects of exercise on capillary density could be blocked by pre-treating animals with a VEGF antibody. The duration of exercise training that is required to induce capillary angiogenesis was shown to be about two weeks, and was preceded by a time period of marked increase in skeletal muscle VEGF expression, when compared to sedentary controls, in a recent study using a rat model [14]. While this study further supported the notion of VEGF as a key player in exercise-induced angiogenesis, it was also able to demonstrate that the expression of Angiopoietin-1 was also significantly elevated during the first two weeks of the study.

One study hypothesized that the angiogenic effects of exercise were due to chronic hypoxia. Therefore, chronic hypoxia was induced by allowing rats to inspire reduced oxygen levels. Interestingly, chronic hypoxia did not result in capillary angiogenesis, but instead downregulated the expression of VEGF mRNA and attentuated the exercise induction of VEGF mRNA [15]. In summary, while multiple cytokines and growth factors appear to be involved in the exercise enhancement and induction of angiogenesis, it appears that VEGF is a key mediator of the angiogenic response in animal as well as human studies

Exercise effects on angiogenic cells

Since exercise can acutely mobilize mononuclear cells like natural killer cells, monocytes and lymphocytes [16], a recent study examined whether a bout of exercise could acutely increase cells, which are involved in the angiogenic and arteriogenic response [17]. The study was able to demonstrate that exercise acutely mobilizes endothelial progenitor cells that co-express the endothelial marker VE-Cadherin as well as the stem and progenitor cell marker AC133, increasing their circulating levels by nearly 300%. In addition to mobilizing rare circulating endothelial progenitor cells (EPCs), which

participate in neovascularization and vascular repair [18], exercise was also able to increase the number of monocyte/macrophage derived endothelial-like cells that can be obtained by culturing circulating mononuclear cells for four days. This monocyte/macrophage derived cell population can also enhance neovascularization [19], possibly by secreting angiogenic growth factors like VEGF [20].

Since VEGF can act as a chemoattractant for endothelial progenitor cells [21] as well as monocyte/macrophage derived endothelial-like cells [22], exercise induction of growth factors in the skeletal muscle and concomitant mobilization of cells into peripheral blood may result in a homing of the released cells to the skeletal muscle. After migrating into the tissue such cell populations could contribute to neovascularization by either incorporating into the nascent vasculature or by secreting angiogenic growth factors that could in turn attract additional mature or progenitor vascular cells. Future studies will be needed to discern the mechanism responsible for the increase in EPCs or monocyte/macrophage derived endothelial-like cells as well as the time pattern of the increase. The increase in circulating EPCs within just 10 minutes of exercise, suggests that this is due to mobilization of EPCs from bone marrow or the marginal pool via beta-2-adrenergic mechanisms, similar to that of lymphocytes [16]. Exercise-induced changes in shear stress [5] may also lead to mechanical dislodging of cells and thus contribute to the increases of circulating cell numbers.

The mobilization of angiogenic cells may also mediate non-local exercise angiogenesis, where angiogenesis occurs in areas which are not being directly exercised or experiencing hypoxia. An example of such non-local effects of exercise is the induction of angiogenesis in the brain (motor cortex) in rats after exercise training [23, 24].

Enhanced external counterpulsation

Enhanced External Counterpulsation (EECP) is a therapeutic approach that is characterized by sequential inflation and deflation of cuffs wrapped around the calves of a patient (see [25, 26]) for a review). EECP appears to be efficacious in treating chronic refractory angina, by mechanisms that are not yet fully understood. One proposed mechanism is the increase of angiogenesis and arteriogenesis as a result of changes in shear stress, similar to that of exercise. The mediators of this proposed effect still need to be determined, but may involve growth factors and circulating cells. While EECP is not really exercise itself, it may mimic some of the beneficial effects of exercise. It may be especially useful in patients who are not able to exercise due to musculoskeletal problems or in patients who have limited exercise tolerance due to their atherosclerotic disease. In the latter group of patients, EECP may well serve as a bridge to improve the patients' exercise tolerance to the point at which they may be able to exercise sufficiently to support angiogenesis and vascular remodeling.

Conclusions

A number of human and animal studies have demonstrated that exercise training can not only induce specific angiogenic mediators like VEGF, but also enhance angiogenesis and collateral blood flow in animal models. Most studies have looked at local angiognesis responses i.e., skeletal muscle angiogenesis in response to skeletal muscle exercise, hypothesizing that local tissue hypoxia may be required. Systemic angiogenesis is less well established and needs to be studied for therapeutic purposes. Coronary angiogenesis may, for example, be induced by skeletal muscle exercise that does not necessarily induce significant cardiac ischemia. Likely mediators for this non-local effect are systemically mobilized cells as well as systemic changes in shear stress (Fig. 1). Identifying the specific mediators of exercise-induced angiogenesis would allow for the development of novel therapeutic strategies which could help patients who are victims of the vicious cycle (Fig. 2) involving cardiovascular disease and reduced exercise tolerance.

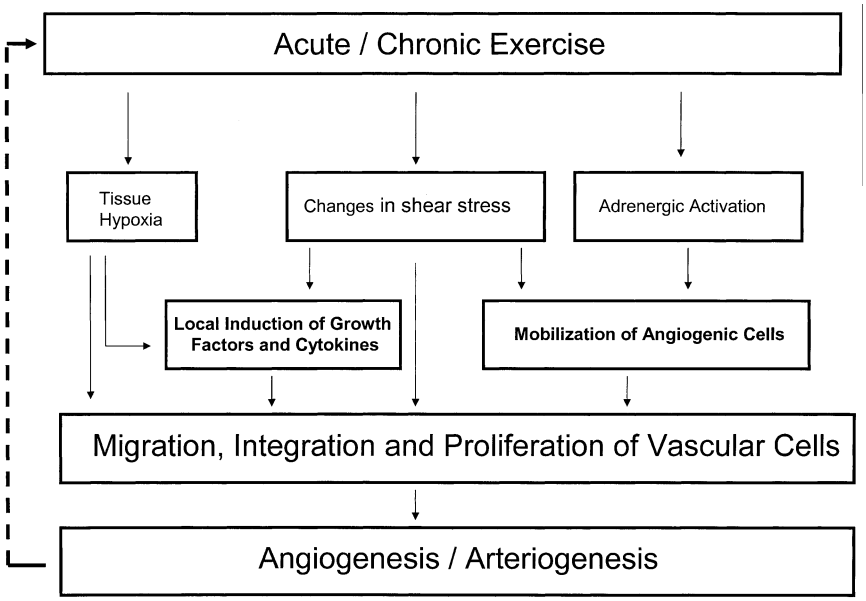

Figure 1. Overview of the postulated mechanisms in exercise-induced angiogenesis

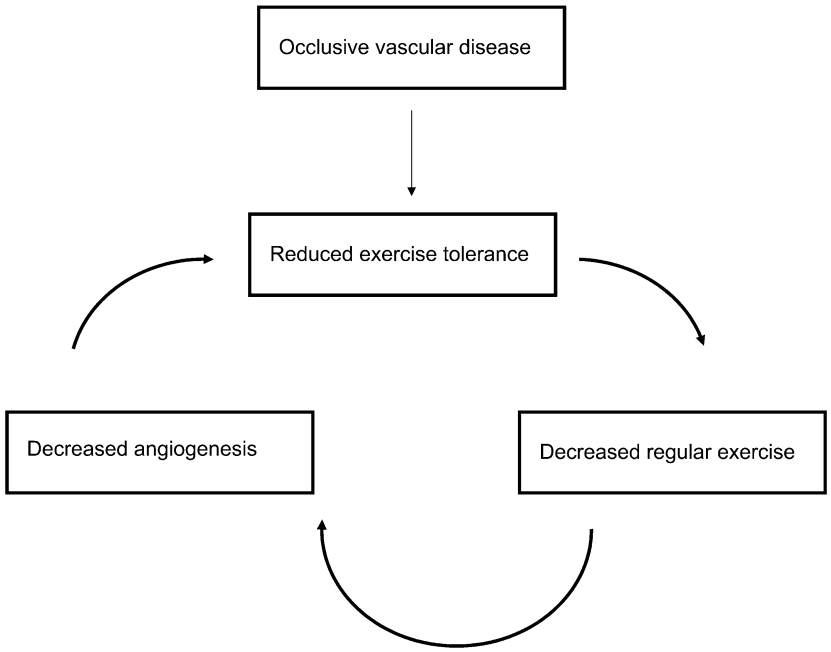

Figure 2. The vicious cycle of reduced exercise tolerance and further reduction and compensatory angiogenesis

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Mechanisms of Angiogenesis
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Angiogenesis – a self-adapting principle in hypoxia

Hugo H. Marti

Institute of Physiology and Pathophysiology, University of Heidelberg, Im Neuenheimer Feld 326, D-69120 Heidelberg, Germany

Introduction

Normal tissue function in mammals depends on adequate supply of oxygen. Alterations in oxygen homeostasis are caused by augmented oxygen consumption or a compromised oxygen delivery resulting in tissue hypoxia. Examples for changes in consumption include increases in muscle workload or increased neuronal activity, an example for the latter maybe the narrowing and occlusion of blood vessels as a result of atherosclerosis [1]. Thus, tissue oxygenation and finally cell survival is not only influenced by the metabolic activity of the tissue but equally well by the vascular system that feeds oxygen and nutrients to the respiring tissue. Therefore, the central role of the vascular system for proper organ function becomes immediately apparent, in that the vascular system must be able to adapt to physiologically altered metabolic demands within a given tissue area. For example, rats housed in complex environments as compared to animals kept in standard cages generate more new synaptic connections. This neuronal plasticity promotes capillary formation by generating long-lasting increases in metabolic demand [2]. These results indicate the existence of inherent self-adapting mechanisms that link metabolic demand to vascular oxygen supply and thus angiogenesis. To understand these adaptive responses, an in depth characterization of the mechanisms that control vascular morphogenesis is needed. These also have far-reaching clinical implications, as many pathological conditions such as tumor growth, ischemic diseases or blindness are associated with an unbalanced blood vessel formation.

During recent years it has emerged that oxygen availability affects several aspects of vascular morphogenesis. The identification of hypoxia-inducible angiogenic factors such as vascular endothelial growth factor (VEGF) and the subsequent discovery of the hypoxia-inducible transcription factors HIF-1 and HIF-2 which control oxygen-dependent gene expression has broadened our understanding of the molecular events that govern oxygen-dependent vessel growth.

In this chapter, I will summarize the mechanisms of oxygen-dependent vessel growth. I commence with a presentation of the current concept of the mole-

cular oxygen sensors followed by a short description of the hypoxia-inducible angiogenic growth factors, and will then present the concept of self-adapting angiogenesis.

Oxygen sensing mechanisms

For most organisms, oxygen homeostasis is an absolute requirement for survival. Changes in oxygen concentrations therefore have to be detected to enable an adequate acute or chronic reaction. Hypoxia induces a variety of specific adaptation mechanisms at the cellular, local and systemic level. These mechanisms aim at maintaining cellular ATP levels and to ensure cell survival. At a systemic level, increased erythropoiesis augments the oxygen-carrying transport capacity of the blood resulting in increased delivery of oxygen to the tissues that are in need of oxygen. Locally, as a short-term reaction, tissue perfusion is increased through vasodilatation mediated by nitric oxide (NO). NO is generated by a family of three NO synthases all of which are activated during hypoxic exposure [3–5]. The growth of new blood vessels represents more of a long-term local adaptation. Finally, at the cellular level, activation of anaerobic glycolysis provides energy for the cell even in the absence of oxygen.

Acute responses often entail changes in the activity of pre-existing proteins, whereas chronic responses normally involve changes in gene expression [6]. Many of the processes that are driven by hypoxia are based on transcriptional regulation and depend on the activation of hypoxia-inducible factor-1 (HIF-1) and HIF-2.

Hypoxia-inducible transcription factors control oxygen-dependent gene expression

The HIFs are heterodimeric transcription factors that function as global regulators of oxygen homeostasis facilitating both oxygen delivery and adaptation to oxygen deprivation. To date, three members of the HIF family have been identified. Each is composed of a α subunit (HIF-1α, HIF-2α or HIF-3α) and a β subunit, which is also called arylhydrocarbon receptor nuclear translocator (ARNT) [7]. Among the three HIF-α isoforms, HIF-1α and HIF-2α appear closely related [8, 9]. However, HIF-2α has a more restricted tissue expression pattern [10, 11]. By contrast, HIF-3α appears to negatively regulate the hypoxic response [12]. HIF targets include genes involved in angiogenesis, vasomotor control, energy metabolism and apoptosis, as well as erythropoiesis [7]. As a consequence of theses various functions, HIFs are also implicated in the pathophysiology of many human diseases [13]. Whereas HIF-1β/ARNT is constitutively expressed, the production of HIF-1α protein is induced in hypoxic cells and is activated at physiologically relevant oxygen levels, thereby ensuring fast and adequate response to hypoxic stress [14]. HIF-1 binds to

the promoter/enhancer elements of hitherto more than 25 known hypoxia-inducible genes (among them *erythropoietin* and *VEGF*) and stimulates their transcription [7]. HIF-1α mRNA is constitutively expressed in virtually all organs and cells [15] implicating a regulation of HIF-1 activity at a posttranscriptional level. Indeed, HIF-1α is an oxygen-labile protein that is very rapidly stabilized under hypoxic conditions. Exposure of cells to hypoxia for less than 2 min already revealed nuclear HIF-1α protein induction [16]. In normoxic cells HIF-1α protein levels are downregulated by ubiquitination and rapid proteasomal degradation, a process that is mediated by the von Hippel-Lindau tumor suppressor protein (pVHL) (see below) [17]. As a result of this normoxic degradation, HIF-1α protein is undetectable in most normoxic tissues [18]. However, expression of HIF-1α increases dramatically in hypoxic tissues, e.g., during chronic hypoxia or ischemia [19, 20].

HIF hydroxylases "measure" tissue pO_2

Oxygen availability influences various steps in HIF activation and recent studies have suggested that at least two steps in this process involve enzymatic hydroxylation of specific amino acid residues in the α-subunit by a specific group of oxygenases. In normoxia, oxygen availability enables a specific prolyl hydroxylation within the oxygen-dependent degradation domain of HIF-1α. This prolyl hydroxylation allows binding of pVHL leading to ubiquitination and proteasomal degradation of HIF-α subunits [21, 22]. Three isoforms of the HIF prolyl hydroxylases were identified in mammalian cells and termed prolyl hydroxylase domain enzymes (PHD1–3) [23, 24]. All three enzymes are widely expressed but show a distinct maximal expression pattern. While PHD1 mRNA is most abundant in testis, PHD2 mRNA is much increased in adipose tissue, and PHD3 mRNA is most abundant in the heart and placenta [25]. Interestingly, the expression of PHD2 and PHD3, but not PHD1 are induced by hypoxia [23], suggesting a possible role for these inducible enzymes in a negative feedback pathway responsible for enhanced degradation of HIF-α after reoxygenation [26]. Further studies will be required, though, to define the relative importance of the three PHD enzymes in the physiological regulation of HIF. Oxygen availability also enables asparaginyl hydroxylation of the C-terminal transactivation domain of HIF-α, blocking interaction with transcriptional coactivators [27]. This event is governed by a specific asparaginyl hydroxylase, termed factor-inhibiting HIF-1 (FIH-1) [28, 29].

All these enzymes use dioxygen in the hydroxylation reaction and, as a consequence, are inactive during hypoxia. The lack of hydroxylation results in stable HIF-α able to form a DNA-binding heterodimer with HIF-β/ARNT. The formed heterodimer then recruits the transcriptional coactivators at the transactivation domain enabling transcriptional activity. Therefore, HIF hydroxylases, providing a direct link between the availability of molecular oxygen and regulation of HIF, act as direct oxygen sensors [25, 26, 30].

Angiogenic growth factors are stimulated by hypoxia

The cellular and molecular mechanisms underlying vascular morphogenesis are in part governed by the activation of HIF-1 and HIF-2 which in turn modulate expression of VEGF and its receptors. Furthermore, several angiogenic factors, such as platelet-derived growth factor (PDGF), various angiopoietins and their receptors are upregulated during hypoxia, although no evidence for their direct regulation by HIFs have been provided so far. Undoubtedly, a proper development of the vascular system requires the concerted action of multiple signaling systems. In the following, I will highlight the most important angiogenic systems that are regulated in an oxygen-dependent manner.

Vascular endothelial growth factor constitutes the major angiogenic factor

VEGF (also termed VEGF-A) is the most important specific regulator of endothelial cell growth and differentiation and also acts as a survival factor for endothelial cells [31]. It exists as several isoforms, derived from a single gene by alternative splicing [32]. In humans, at least five isoforms ($VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$, and $VEGF_{206}$) have been identified, which differ in their capacity to bind heparin-rich matrix components and receptors [33]. VEGF is expressed in virtually all cells of the body, however, expression in endothelial cells has mainly been found *in vitro* in cell culture, while it is hardly detectable *in vivo* [34]. These findings indicate that VEGF acts as a paracrine factor on neighboring endothelial cells carrying the VEGF receptors rather than in an autocrine fashion. Various receptors for VEGF have been identified, VEGF receptor (VEGFR)-1, VEGFR-2 and the neuropilins [35]. While all VEGF isoforms bind to VEGFR-1 and VEGFR-2, the neuropilins function as coreceptors only for specific isoforms [36].

The most important inducer of VEGF gene expression is hypoxia, which was demonstrated for the first time in the perinecrotic areas of glioblastomas [37, 38], although a variety of mechanisms as well as many cytokines and growth factors have been shown to modulate the expression of VEGF [33]. Under hypoxic conditions VEGF expression is mediated through the activation of both HIF-1 and HIF-2 [11, 39]. In addition, VEGF upregulation during hypoxia is also achieved by an increase in the stability of its mRNA [40] and by the efficient hypoxic translation of the VEGF mRNA which is mediated by an internal ribosomal entry site [41]. Also, gene expression of both *VEGFR-1* and *VEGFR-2* is activated in hypoxic and ischemic tissues [34, 42–44]. Whereas *VEGFR-1* is a direct target gene for HIF-1 [45], *VEGFR-2* is activated by HIF-2 [46]. *VEGFR-2* is not induced by a short term exposure to hypoxia [34] but rather after prolonged hypoxic periods, e.g., after 48 to 72 h, such as those occurring during ischemic events [43, 47]. It has been proposed that increased VEGF levels may activate *VEGFR-2* expression through a positive feedback loop indicating a further regulatory pathway [48,

49]. The third receptor for VEGF, neuropilin-1, is upregulated in endothelial cells of cerebral blood vessels from hypoxic tissue after focal cerebral ischemia [50].

The angiopoietin/Tie2 receptor-system regulates vascular remodeling

Vascular morphogenesis not only involves endothelial cell activation and proliferation but also the subsequent maturation and remodeling of the primarily formed vascular plexus resulting in functional new vessels. Vessel maturation is characterized by the recruitment of pericytes and vascular smooth muscle cells to the endothelial cells [51]. In recent years, it became evident that two specific receptors, Tie1 and Tie2, isolated from endothelial cells, and a second family of angiogenic growth factors, the angiopoietins, play a central role for the remodeling steps of vascular morphogenesis. The angiopoietins isolated so far (Ang1–Ang4) bind exclusively to Tie2, while the ligand for Tie1 remains to be identified. Ang1 and Ang4 activate Tie2, whereas Ang2 and Ang3 behave as competitive antagonists. However, Ang2 might also be an agonist in particular microenvironments (reviewed in [52]). The discovery of a unique family of natural agonists and antagonists to coordinate the stabilizing functions of the Tie2 receptor implies that the mechanisms surrounding vessel remodeling are precisely regulated. As the coordination of angiopoietin and Tie receptor expression with hypoxia-induced activation of VEGF seems mandatory for a proper vascular development [53], it appears evident that angiopoietin and/or Tie receptor expression must be regulated by hypoxia as well.

Although none of the angiopoietins has been identified as a direct HIF target, hypoxia provides a specific signal for their distinct regulation. *In vitro*, exposure to hypoxia increased Ang2 expression in endothelial [54] and mesangial cells [55] but had no effect in bovine retinal pericytes [56]. On the other hand, Ang1 expression was induced during hypoxia in pericytes, but not in human endometrial endothelial cells [57]. Exposure to hypoxia even downregulated Ang1 expression in C6 glioma cells [58]. Recently, it was also demonstrated that Ang4 expression in human cardiac cells increases during hypoxia [59]. Concerning the Tie receptors, in human coronary, dermal and umbilical vein endothelial cells, hypoxia activated Tie1 and Tie2 expression [60, 61], although the effect of hypoxia appears to depend on the origin of endothelial cells. Indeed, other studies have shown no, or rather a downregulatory effect of hypoxia on the expression of Tie2 [54, 62]. *In vivo*, Ang2 expression in the capillary endothelium in the brain was induced during adaptation to prolonged mild hypoxia [63]. Furthermore, systemic hypoxia increased the levels of Ang2 mRNA in the cerebellum and Ang3 expression in the lung, kidney, and diaphragm. In contrast, hypoxia provoked a decline in Ang1 and Tie2 expression in the lung, liver, cerebellum and heart but not in the kidney and diaphragm [64]. Thus, expression of angiopoietins and Tie2 in response to hypoxia differs in different organs.

Also in the ischemic brain a specific temporal and spatial expression pattern of the angiopoietins and Tie receptors was observed. In a permanent model of middle cerebral artery occlusion (MCAO), Ang1 was constitutively expressed in a subset of glial and neuronal cells with no change in expression after ischemia. Ang2 expression was upregulated 6 h after MCAO and was mainly observed in endothelial cells cord tips in the penumbra and infarct area [65]. In comparison, in a transient MCAO model, Ang2 expression showed a biphasic induction, peaking at 24 h and 2 weeks after ischemia, while Ang1 expression was only increased after 7 days. Tie1 and Tie2 expression increased, starting 24 h after reperfusion and remained elevated for up to 2 weeks thereafter [66]. Also in the permanent occlusion model, Tie1 mRNA and protein expression showed a marked upregulation 3 days after MCAO, but returned to baseline levels after 7 days, whereas no ischemia-induced upregulation of Tie2 was observed at any time [65].

All these results suggest that the response of the Ang/Tie2-system to hypoxia differs in distinct cells derived from different tissues as well as in different organs. An explanation for these differences might be the presence of different cellular thresholds for activation of the hypoxic response. Alternatively, diverse transcriptional pathways mediating the hypoxic response of angiopoietins and Tie receptors might be involved. For example, NERF2 which is a member of the Ets transcription factor family found specifically in endothelial cells and a known transactivator of *Tie2* gene expression was increased during hypoxia and temporally correlated with Tie2 regulation [61]. On the other hand, there is recent evidence that the altered expression of the angiopoietins in response to hypoxia could be mediated by HIF-1. Cardiomyocytes infected with adenovirus encoding a constitutively active form of HIF-1α decreased Ang1 expression [67], while in primary human endothelial cells, infection of a constitutively stable form of HIF-1α resulted in an increased expression of Ang2 and Ang4 [59].

Platelet-derived growth factor helps in blood vessel maturation

Platelet-derived growth factor (PDGF) is a family of dimeric ligands assembled from four gene products (PDGF-A–D) that act via two receptor tyrosine kinases, PDGF receptor (PDGFR)-α and PDGFR-β [68–70]. In the brain, PDGF-B was thought to act as a neurotrophic and neuroprotective factor [68]. However, it was then shown that PDGFR-β and PDGF-B play key roles in the maturation of blood vessels. In PDGF-B deficient mice, mesenchymal cells fail to be recruited to the developing brain capillaries. The lack of covering pericytes leads to irregularly shaped, unstable blood vessels, and ultimately to edema formation [71]. Thus, the binding of PDGF-B to its receptor is crucial for the recruitment of pericytes and vascular smooth muscle cells to the immature vasculature and thus for vessel maturation [72].

As proper remodeling and vessel maturation also occurs at reduced tissue oxygenation, these results implicate PDGF in the self-adapting angiogenic

reaction during hypoxia. Indeed, it has been shown that in human umbilical vein endothelial cells and macrophages PDGF-B expression increased. On the other hand, PDGF-A levels were not affected [73, 74]. In addition, hypoxic induction of PDGF-B expression and PDGFR-β phosphorylation was also demonstrated in neural cells [75]. Furthermore, during cerebral ischemia a time- and cell type-specific induction of PDGFR-β was detected which correlated spatially and temporally with the development of new blood vessels [76]. Finally, also in human brain samples collected *post mortem* from stroke patients, increased expression of both molecules was reported [77]. Taken together, these studies clearly indicate that oxygen availability is an important regulator of PDGF-B expression and suggest the PDBF-B/PDGFR-β system is part of the concerted activation of angiogenic growth factors during hypoxia-induced vascular morphogenesis.

A bundle of growth factors assist with forming a functional vessel

The arsenal of newly discovered growth factors acting on the vascular endothelium is steadily expanding. Apart from VEGF-A, the VEGF family now includes five additional members, VEGF-B, VEGF-C, VEGF-D, VEGF-E, and placental growth factor (PlGF). The family of fibroblast growth factors (FGF), in fact the first angiogenic growth factors to be discovered, now comprises more than 20 members (reviewed in [78]. Recently, the large family of Eph receptor tyrosine kinases and their ligands, the ephrins, initially characterized in the nervous system, emerged on the angiogenesis field as they play a key role for vascular development [79]. Finally, among the many factors capable of modulating angiogenesis are transforming growth factors, interleukins and even the hematopoietic hormone erythropoietin [51, 80–82]. In the following I will focus on two important factors, the ephrins and erythropoietin. Erythropoietin is outstanding because it was the first target gene identified for HIF-1 and is one of the best studied hypoxia-inducible genes. Erythropoietin is a hematopoietic growth factor which exerts its action through its specific receptor on erythroid progenitor cells. The detection of erythropoietin receptor on various other cell types, among them neurons and endothelial cells led to the subsequent discovery of additional biological roles of erythropoietin as a neuroprotective and angiogenic factor [83]. It has been demonstrated that erythropoietin has mitogenic and chemotactic effects on endothelial cells derived from the human umbilical vein and bovine adrenal capillaries [84]. It has also been shown that vessel outgrowth of rat aortic rings was stimulated by erythropoietin [85] suggesting that erythropoietin has angiogenic properties. Indeed, neovascularization *in vivo* was stimulated in the endometrium after erythropoietin injection into the mouse uterine cavity [86] and in the chick embryo chorioallantoic membrane after erythropoietin administration [87]. The fact that brain capillary endothelial cells express two forms of erythropoietin receptor mRNA [88] implicates erythropoietin in brain angiogenesis. As a

matter of fact, erythropoietin showed a dose-dependent mitogenic activity on brain capillary endothelial cells [88]. Recent data suggest that erythropoietin can protect the blood-brain barrier against VEGF-induced permeability [89]. Thus, erythropoietin could play an important role not only for endothelial cell migration and proliferation, but also for the subsequent vessel maturation. The mechanisms underlying these effects on vascular morphogenesis have recently been reviewed in detail [82].

The ephrin ligands and Eph receptors, on the other hand, are outstanding because of their fundamental role for vessel maturation and polarity [51]. Originally identified as important factors for the formation of segmented structures and control of axon guidance in the brain, recent studies have demonstrated their essential role for the development of the vasculature in the embryo (reviewed in [90]). In particular, Ephrin-B2 and EphB4 have been shown to contribute to the molecular control of arterial differentiation [91] highlighting their crucial role for proper vascular morphogenesis. Although not much is known about hypoxic induction of ephrin ligands and Eph receptors so far, upregulation of ephrin-B2 expression was recently demonstrated in human umbilical arterial endothelial cells [92].

Taken together, hypoxia appears to regulate many angiogenic growth factors that are involved in different steps during vascular morphogenesis including endothelial cell polarity. It is tempting to speculate that tissue oxygenation might therefore also determine whether a vessel turns into an artery or a vein. It is emerging that all of these factors must be used in perfect harmony, in a complementary and coordinated manner, to form functional vessels [93]. Hypoxia may be one of the major factors regulating this coordination.

Vascular morphogenesis and oxygen

With exception of the hormonally regulated angiogenic cycles in the female reproductive system, neovascularization is virtually absent in the adult organism although the potential for making a new vascular network is maintained and can be exercised throughout adulthood. Indeed, it can be physiologically elicited in every mature tissue as a response to chronic hypoxia or an ischemic insult. Driving force of this compensatory angiogenesis is the condition of oxygen and nutrient deprivation [1]. For example, the brain with its high rate of oxidative metabolism must rely on a steady supply of oxygen. A prolonged hypoxic period will lead to a reduction of tissue oxygenation with detrimental effects on a proper brain function. In order to maintain oxygen delivery to the brain, the organism increases the vascular density in this organ, resulting in smaller intercapillary distances, which will finally restore tissue oxygenation [94, 95]. The brain is capable of structural and functional plasticity to balance its energy supply and demand [96]. Vessel growth is therefore directly regulated by availability of oxygen [30]. Five conditions seem to be crucial for a successful adaptation of the vascular systems to an altered tissue oxygenation.

1) Changes in local oxygen concentrations have to be measured fast thereby enabling an immediate response of the hypoxic gene expression machinery.
2) The spatial area of angiogenic factor action must be closely controlled.
3) Activation of angiogenic factors has to be controlled in a temporal window.
4) The concentration of locally active angiogenic factors needs to be tightly regulated.
5) Many angiogenic factors must be present in a coordinated fashion to ensure a proper vascular morphogenesis.

Instantaneous response to hypoxia

The transcription factor HIF-1 fulfills the criteria necessary for an immediate response to reduced tissue oxygenation. First, HIF-1 is ubiquitously expressed in virtually all cells of the organism [15]. Second, HIF-1 DNA-binding activity and HIF-1α protein increase exponentially as cells are subjected to decreasing oxygen concentrations, with a maximal response at 0.5% oxygen. The half-maximal response occurred at 1.5–2% oxygen (10–15 mmHg), and thus at physiologically relevant oxygen concentrations [14]. Accordingly, even slight alterations in tissue oxygenation would occur along the steep portion of the HIF-1 response curve resulting in dramatic changes of HIF activity. Finally, in cells that experience a lack of oxygen, HIF levels rise within minutes. Hypoxic exposure for less than 2 min already induced HIF-1α protein and HIF-1 DNA binding activity and maximal levels were reached after 60 min. Reoxygenation reduced HIF-1 DNA binding within 2 min, and nuclear HIF-1α protein levels within 4 to 8 min, down to a level below detection limit within 30 min. Within the same time frame, NF-κB, c-Fos, and c-Jun, transcription factors also known to be involved in hypoxic gene regulation, showed no alteration in their nuclear levels in response to hypoxia [16]. Taken together, these results highlight the predominant role of HIF-1 in short-term adaptation to changes in tissue oxygenation.

Regional control of angiogenic factor expression

Increased levels of HIFs activate transcription of e.g., VEGF and its receptors. As a result, newly growing capillaries invade the tissue and, once circulation has started, begin to supply the tissue with oxygen. While local oxygen levels start to increase, the tissue hypoxia that constituted the primary signal for gene activation of VEGF and other angiogenic factors disappears. Once oxygenation has reached normal levels, the angiogenic factors are rapidly downregulated as HIF protein levels decrease [16]. A simple negative feedback mechanism of this kind ensures a matched response leading to a complex distribution of oxygen and growth factor expression. Distant areas not yet covered by newly sprouted vessels still suffer from hypoxia. Consequently, these regions

show high levels of angiogenic factors while their expression is terminated in areas adjacent to the newly formed blood vessels. A telling example for such a distribution of oxygen and the expression of an angiogenic growth factor is the liver where a distinct oxygen and VEGF mRNA gradient can be seen. VEGF expression is low or even absent around the periportal fields which contain the arteries and thus show the best tissue oxygenation, while increased VEGF mRNA levels that are concurrent with a decrease in tissue oxygenation can be seen towards the central veins [34]. However, not all neighboring cells appear to react to a hypoxic stimulus in an identical way. In the adult brain, VEGF expression is increased by prolonged hypoxia [34, 97]. Some cells, though, appear to respond more strongly as astrocytes express high levels of VEGF, while the neighboring neurons, although experiencing the same degree of hypoxia, do not alter VEGF gene expression [98]. These findings suggest different thresholds of activation in different cell types and maybe also differences in activation within the same cell type. It will be interesting to establish the factors or conditions that determine whether or not a cell is activated by hypoxia [99].

The difference in growth factor expression between vascularized and non-vascularized tissue areas and responding and non-responding cells leads to the formation of a complex angiogenic growth factor gradient which might govern the growth direction of newly forming blood vessels. Indeed, specialized endothelial tip cells have been identified at the leading edge of the vascular network [100]. These tip cells sprout into the avascular territory, seem to read growth factor gradients and confer thereby a guidance function to the growing vessels. Such guidance function has already been described for the growth cones of axons [90].

Temporary expression control

Expression of angiogenic factors must also be controlled over time to ensure accurate vessel growth and remodeling. VEGF, when acutely administered to the adult organism, induces the formation of leaky, thin walled, tortuous and dilated vessels with limited functional properties [101, 102]. In contrast, when VEGF levels increase slowly and regulated, e.g., under control of a weak brain-specific promoter, morphologically normal and functional vessels develop [103]. The idea that time and duration of VEGF activation are crucial for normal vascular growth is supported by findings obtained in a transgenic mouse model where VEGF expression is reversibly inducible [104]. Induction of VEGF expression in the heart muscle and liver resulted in a progressive, unlimited ramification of the existing vasculature; however, abnormal vascular trees were produced and a massive, highly disruptive edema developed [105]. Premature withdrawal of VEGF resulted in vessel regression. If, however, VEGF was withdrawn after a critical transition point, remodeled and unleaky vessels persisted [105].

Taken together, these results highlight the tight temporal control necessary for proper vascular morphogenesis. It has also been suggested that the acquisition of a pericyte coating around endothelial cells [106] or a direct contact with smooth muscle cells [107], processes regulated by angiopoietins and PDGF, can terminate the endothelial responsiveness to VEGF and result in a tight and stable vasculature, thus pointing to the involvement of additional angiogenic factors (see below).

Control of angiogenic factor concentration

Not only spatial and temporal control of growth factor expression but also a tight regulation of factor concentrations is mandatory for the formation of a functional vascular network. It has been shown that VEGF dosage is a critical parameter regulating vessel density in the brain that in turn determines the architectural organization of the nervous system. Mice with intermediate levels of VEGF activity in the brain showed decreased vessel branching with an aberrant structural organization of the cortex, while severe reductions in VEGF led to decreases in vascularity resulting in degeneration of the cerebral cortex and neonatal lethality [108]. The crucial role for appropriate VEGF levels is also illustrated in VEGF deficient mice. Disruption of even a single allele of the VEGF gene resulted in embryonic lethality due to severe vascular defects [109, 110]. On the other hand, even a modest increase in VEGF expression also resulted in a disrupted embryonic development [111]. These results illustrate the immense potency of VEGF as a regulator of vascular development and explain the necessity for its stringent regulation.

Coordinated expression of angiogenic factors

Based on the aforementioned, VEGF certainly has a central role in adapting the vascular network to an altered tissue oxygenation. Furthermore, VEGF-mediated new vessel growth can be considered as a fundamental physiologically adaptive response to tissue hypoxia. However, it appears that endothelial cell proliferation induced by activated VEGF expression alone is not sufficient to produce a normal functional vascular network. In several transgenic mouse models of chronic overexpression of VEGF in the skin, using the keratin 14 promoter (K14) [112, 113] or regulatory sequences of the keratin 6 gene [114], cutaneous blood capillaries increased in density, but were tortuous and leaky resulting in skin edema formation. These data clearly show that VEGF alone can not do the job. It has been suggested, that an accompanying upregulation of Ang1 might induce leakage-resistance, as K14-Ang1 transgenic mice produced larger, more numerous and highly branched vessels without signs of vascular leakage [115]. Also, coexpression of Ang1 and VEGF had an additive effect on angiogenesis but resulted in leakage-resistant vessels [113] suggest-

ing that leakage-resistance induced by Ang1 is dominant of VEGF-induced vascular leakage. Indeed, it was recently demonstrated that Ang1 application reduced cerebral blood vessel leakage and infarction volume after cerebral ischemia [116].

In conclusion, these results implicate the requirements of several angiogenic factors in order to produce a functional vascular network. It appears that VEGF, the angiopoietins, PDGF, the ephrins, erythropoietin, and additional growth factors must act together in a coordinated manner. Although hemodynamic forces such as shear stress are fundamental for remodeling, tissue hypoxia resulting in HIF-1 activation seems to play the crucial role in the coordinated expression of these factors. Indeed, overexpression of a stabilized form of HIF-1α in the skin resulted in normal, leakage-resistant blood vessels, although VEGF was induced 6- to 9-fold in the skin of these mice [117]. This finding suggests that hypoxia induces a constellation of factors that cooperate with VEGF to produce more mature and thus tighter vessels.

Summary

Normal tissue function depends on adequate supply of oxygen through blood vessels. Reduced oxygen supply (hypoxia) induces a variety of specific adaptation mechanisms in mammals that occur at the cellular, local and systemic level. These mechanisms are in part governed by the activation of the hypoxia-inducible transcription factors HIF-1 and HIF-2. Prolyl and asparaginyl hydroxylases as recently characterized oxygen sensors allow the regulation of HIFs that in turn modulate expression of hypoxically regulated genes such as VEGF. VEGF plays a key role in the formation of a functional and integrated vascular network required during physiological processes such as embryogenesis or female reproductive cycle as well as during a variety of pathological processes such tumor growth, wound healing, retinopathy and ischemic diseases (myocardial infarction, cerebral ischemia). However, other angiogenic factors, such as angiopoietins, PDGF, ephrins and erythropoietin are additionally needed to enable the formation of a functional vascular network. Many of these factors are activated during hypoxia although no HIF binding sites have yet been identified in the regulatory sequences of theses genes. Hypoxia-induced gene products that result in new vessel growth may be part of a self-regulated physiological protection mechanism preventing cell injury, especially under conditions of chronically reduced blood blow (chronic ischemia).

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Mechanisms of Angiogenesis
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Cellular mechanisms of arteriogenesis

Matthias Heil and Wolfgang Schaper

Max-Planck-Institute for Physiological and Clinical Research, Department of Experimental Cardiology, Benekestrasse 2, 61231 Bad Nauheim, Germany

Introduction

Cardiac and peripheral vascular diseases, i.e., atherosclerosis frequently lead to organ dysfunction, death and morbidity. Although some patients may have developed the ability to escape this fatal disorder by bridging the occlusion or stenosis side with "naturally growing bypasses" [1], most patients have to undergo surgical interventions associated with a risk of side effects. For example, cardiopulmonary bypass surgery highly increases the risk for neurological complications and many of the patients undergoing percutaneous transluminal angioplasty suffer from restenosis [2, 3]. Thus, understanding the mechanism of collateral artery growth may generate options for new treatments. These therapies could lead to the enhancement of growth and remodeling processes and therefore assure the recovery of blood flow deficits caused by the arterial disorders.

Pioneering work in describing the existence of collateral blood vessels in healthy people as well as in patients undergoing chronic arterial diseases was done by Fulton and Longland [4, 5]. They also found out that in patients with coronary heart diseases these collateral vessels are often enlarged [6]. A body of evidences has been collected since this time showing that the mechanisms leading to the development of large collateral vessels with the capability to efficiently conduct blood obviously differ from the mechanisms usually summarized as angiogenesis. Therefore, the term "arteriogenesis" was established to recognize both, the pivotal differences in both processes, and the fact that the growth of collateral vessels is the most important adaptive process in the vascular system [7]. The reason why only collateral vessels and not capillaries can efficiently compensate flow deficits caused by arterial lesions, can be easily assessed from the law of Hagen-Poiseuille demonstrating that flow (Q) is proportional to the 4th power of vessel diameter (R).

$$Q = \frac{R^4 \pi \Delta P}{8 \eta l} (ml / \min)$$

Thus, thousands of capillaries would be needed to achieve the same flow capacity as from one mature collateral vessel.

Pre-requisites and triggers of arteriogenesis

For decades, one of the central questions was about the presence and the requirement of pre-existing collateral arterioles spanning two major arteries. While some denied their existence [8, 9], Fulton was propagating the concept that collateral arteries develop from pre-existing anastomoses [6, 10]. Today we know: The keys to this question are the applied methods. Due to the small size of those arteriolar connections, high-resolution methods including composition of contrast agents are essential for optimal imaging of pre-existing collateral vessels. Importantly, pre-existing collateral vessels comprise all parts of a proper artery, in particular an inner endothelium and an internal elastic lamina. Furthermore, in contrast to capillaries, they also contain a smooth muscle cell layer [11]. Based on detailed structural and functional investigations, increasing evidence was gained that the prime stimulus for collateral vessel enlargement is augmented blood flow. Blood flow changes evolve from altered blood pressure gradients after formation of arterial occlusions. The difference between high blood pressures proximally to the occlusion side and very low distal pressures, in particular at the collateral re-entry site, induces the redistribution of blood flow, now recruiting pre-existent arterioles spanning two neighboring arteries. Hence, those collateral anastomoses are subsequently subject to the exposure of physical forces, either pressure-related (longitudinal, circumferential or radial wall stresses) or the fluid shear stress (FSS). Pressure-related forces may participate in the activation of the growth process. One could assume that distension of the vessel wall, increasing the radius-to-wall thickness ratio, contributes to the induction of smooth muscle cell proliferation activity by increasing longitudinal and circumferential wall stresses [12]. However, it is becoming increasingly evident that rather FSS is the molding force of arteriogenesis. FSS at the vessel wall can be approximately estimated assuming Newtonian fluid dynamics:

$$\tau = \frac{4\eta Q}{\pi R^3}$$

This formula to calculate FSS (τ) includes blood viscosity (η), flow rate (Q) and the internal radius of a vessel (R), hence demonstrating that increased blood flow will directly result in increased FSS [13]. The validity of this formula for the arteriogenic processes was tested in a rabbit shunt model. Since FSS is difficult to manipulate over a longer period of time, an approach to this goal was developed by creating side-to-side shunts between the femoral artery and the accompanying vein [14]. This led to a relatively stable situation of chronically increased shear stress. As a side effect, circumferential stress in the collateral vessel was minimized. Hemodynamic investigation revealed that in this model collateral conductance was powerfully enhanced, being relatively close to the complete restoration of the physiological blood flow.

But how is the physical force FSS translated to a cellular level? If FSS is present for several hours, the collateral endothelium becomes activated. Endothelial cells are sensitive to biomechanical forces and a number of potential mediators have been reported [15, 16]. Typically, biomechanical stimuli are transduced on the transcriptional level via shear-stress responsive elements located in the promoter region of various genes [17–19]. In addition, a signaling cascade is initiated by the activation of ion channels which leads to cell swelling and edema, proceeds via MAP-kinases and again results in an alteration of gene expression. In a previous study, we could demonstrate the importance of volume-regulated endothelial chloride channels (VRAC) for endothelial cell activation as the first step to initiate collateral growth [20]. VRAC have been shown to be involved in the FSS signaling process. Using mibefradil, originally developed as a selective T-type calcium channel blocker, but also having inhibitory activity on VARC, collateral artery growth was decreased when tested in the mouse hind limb. Probably, blocking of VARC by mibefradil interfered with the cellular mechanisms of volume control and led to an inhibition of endothelial and smooth muscle cell proliferation.

Other potentially important mediators of endothelial cell activation are vasodilatory substances. It is evident that endothelial nitric-oxide synthase (eNOS) is upregulated by FSS [21] but it is yet not completely understood how nitric oxide (NO) influences collateral artery growth. On the one hand, NO may lead to vasodilation of peripheral resistance vessels, hence increasing blood flow through collateral arterioles, and may thereby subsequently augment flow-based remodeling processes. Furthermore, NO could support arteriogenesis by activating matrix-metalloproteinases [22] and could modulate growth factor-induced cell migration and cell proliferation [23]. On the other hand, contradictory effects of NO are also described, such as its anti-proliferative effects on endothelial and smooth muscle cells [24, 25]. Furthermore, NO may inhibit interaction of monocytes with the activated collateral endothelium (see below) by antagonizing chemokine-induced monocyte chemoattractant protein 1 (MCP-1) production in smooth muscle cells [26]. However, several reports are published suggesting that NO supports arteriogenesis [27–29]. How much other vasodilative substances are involved in arteriogenesis, is currently rather speculative. The promoter of the prostacyclin synthase seems to contain a shear stress response element [30]. Hence, prostacyclin expression could be induced in the collateral endothelium by increased FSS and could enhance arteriogenesis similarly as proposed for NO.

Cell-to-cell-interaction at the collateral vessel wall

The adhesion of circulating blood monocytes with the activated endothelium of a collateral arteriole is a pivotal step during the process of arteriogenesis. If lacking an inflammatory stimulus, monocytes and other leukocytes patrol in the

blood stream in a quiescent state with only minor adhesion tendency. But the FSS-activated collateral endothelium releases inflammatory and chemotactic cytokines which stimulate leukocyte and in particular monocyte attraction and adhesion to the collateral vessel wall. Furthermore, surface expression of adhesion molecules on endothelial cells is increased and they are clustered in so called focal adhesion complexes. Important mediators of such cell-to-cell binding processes are selectins and intercellular adhesion molecules (ICAM-1 and ICAM-2) on endothelial cells and integrins (Mac-1 and LFA-1) on monocytes. In previous studies we and others could demonstrate, that factors like VEGF being potentially released by endothelial cells have the ability to stimulate integrin expression on monocytes and increase cell adhesion [31–33]. In addition, a rapid conformation change converts the integrins into an active state. Within a short time, the anti-adhesive, quiescent status of the system is converted into a highly adhesive one, now supporting cell adhesion and invasion.

In monocytes, two integrins are mainly responsible for the interaction with endothelial cells: The heterodimeric proteins Mac-1 and LFA-1 which belong to the group of β_2-integrins. Consequently, both molecules include the β_2-subunit. The second subunit of Mac-1 is the α_M-integrin and of LFA-1 is the α_L-integrin. The monocyte interaction with the collateral endothelium is a complex multistep process. After the initial monocyte interaction with the vascular endothelium called "rolling" and being mediated by several selectins, the tight monocyte adhesion to the collateral endothelium is triggered by those integrins. The integrins interact with their corresponding adhesion molecules on the endothelial cell surface, preferably ICAM-1, ICAM-2 and VCAM-1, clustered in focal adhesion complexes. In immunohistological studies we could show that expression of ICAM-1 was markedly increased on endothelial cells of activated collateral arterioles. Furthermore, ICAM-1 was shown to be constitutively expressed on vascular smooth muscle cells [11, 34]. Hence, ICAMs not only are involved in monocyte adhesion, they also play a role in the transmigration process which finalizes monocyte invasion of the collateral vessel wall. Again, interaction between integrins on monocytes and ICAM-1/-2 and most likely VCAM-1 (see below) on endothelial cells is important to guide the monocytes into deeper regions of the vessel wall, mostly adventitia, and the perivascular space. Using monoclonal antibodies against either ICAM-1 or β_2-integrins we were able to block monocyte adhesion and transmigration *in vitro* [31] and collateral artery growth *in vivo* (unpublished data). The importance of VCAM-1 in this process is currently not completely known. Like for ICAM-1, an increased expression of VCAM-1 was detected in the rabbit hind limb model on the endothelium of collateral arterioles three days after femoral artery occlusion [11]. Upregulation of VCAM-1 was also found *in vitro* on cytokine-treated endothelial cells. In deed, VCAM-1 may participate in leukocyte-endothelium interaction by binding to its counterreceptor VLA-4 (α_4/β_1-integrin) [35]. However, in our *in vitro* experiments, both interaction steps were completely inhibited by blocking on the β_2-ICAM-1 pathway. Thus, the role of VLA-4 and VCAM-1 for monocyte adhesion to the collateral endothelium and their subse-

quent transmigration remains open and needs to be further investigated. A second possible function for VCAM-1 could evolve from recent data obtained in the rabbit shunt model where VCAM-1 was found to be strongly upregulated in the adventitia and on smooth muscle cells. This could point towards a role for VCAM-1 in the cell-to-cell communication between smooth muscle and endothelial cells or even with components of the extracellular matrix. Hence, VCAM-1 may participate in smooth muscle cell migration.

Circulating blood monocytes: critical mediators of arteriogenesis

Although the presence of adhered monocytes at the activated collateral endothelium was well described [36], the functional background of this observation needed to be addressed. Our main questions was whether the monocyte concentration in the blood may correlate with the extent or the speed of collateral artery growth. In order to investigate this we sought to manipulate monocytes numbers in the blood by pharmacological treatment [37]. About four days after bolus injection of the cytostatic agent 5-fluorouracil (5-FU) monocytes were almost completely depleted from the blood of treated mice, whereas in rabbits unfortunately no such depletion was achieved. When the femoral artery in those mice was ligated during the monocyte depletion, blood flow recovery to the foot as a mean to quantify arteriogenesis was markedly reduced. Approximately two weeks after 5-FU treatment a rebound reaction was observed leading to a several fold increase of blood monocyte numbers in both, treated rabbits and mice. When the femoral artery was ligated during the monocyte rebound phase, arteriogenesis was enhanced in both animal models demonstrating the pivotal role of blood monocyte concentration for the progress of arteriogenesis. In order to exclude that side effects of the 5-FU treatment may have interfered with the process we rescued monocytes in a group of monocyte-depleted mice by injection of monocytes isolated from blood of donor mice. In this group, blood flow recovery was more than compensated compared to the control group. The overcompensation may be due to an artificial pre-activation of the injected monocytes caused by the isolation methods. A similar and an even more definite observation had been made when rabbits were injected with liposomes containing cytotoxic bisphosponates [38]. Most likely because monocyte depletion by this treatment was more complete than after 5-FU infection, arteriogenesis was completely abolished in bisphosphonate-liposomes-treated rabbits. Postmortem angiograms taken seven days after femoral artery ligation revealed that collateral vessel network in such treated rabbits was similar to acutely ligated animals. However, when the femoral artery was ligated three days after injection of the liposomes (after monocyte blood concentrations returned to almost normal levels) no inhibition of collateral artery growth was visible (unpublished data). Hence, these studies demonstrated the pivotal role and the tight regulation of the monocyte infiltration process during the initial phases of arteriogenesis.

We were also interested in the pathway being involved in monocyte attraction. What do mediators signal to the monocytes to travel to the collateral vessel wall and where do they have to migrate to? An approach to this question was provided by observations obtained in the rabbit hind limb model by using osmotic minipumps to locally deliver test substances into the collateral system. In these experiments, the most distinct enhancement of arteriogenesis was achieved with pumps either containing a chemotactic agent for monocytes or increasing their activation. The most potent substance which we found to stimulate collateral vessel growth was the monocyte chemoattractant protein-1 (MCP-1): When MCP-1 was locally infused into the collateral network after occlusion of the femoral artery in the rabbit hind limb, a dramatic improvement of the growth process was detected seven days after occlusion [39] which was confirmed later [40]. In contrast, in MCP-1 gene deficient mice, recovery of blood flow in the hind limb was reduced which could be rescued by local delivery of MCP-1 [41]. Furthermore, the inhibition of arteriogenesis in these mice correlated with reduced monocyte accumulation around the growing collateral vessels, although still a significant number of macrophages were present. To achieve further insights into the pathway of monocyte attraction and invasion, we investigated collateral artery development in mice which were gene-deficient for the chemokine receptor-2 (CCR-2) [42]. This receptor is known as the major functional receptor for MCP-1, although most likely not the only one of biological relevance. Using these mice on both the BALB/c and the C57BL/6 background, we could impressively demonstrate the importance of the MCP-1-CCR2-pathway in arteriogenesis and show that this pathway is responsible for the recruitment of monocytes during early phases of arteriogenesis [43]. Our data displayed a dramatically reduced recovery of pedal blood flow after femoral artery ligation. This not only correlated with other physiologic parameters like the reduction in hemoglobin oxygen saturation in the foot but was also reflected by functional parameters. Due to the reduced blood supply to the distal regions of the leg, the active movement of the limb, assessed in a score, was significantly impaired. Furthermore, histological morphometry of the collateral vessel size confirmed the physiological data by showing decreased collateral vessel diameters in the CCR2 gene-deficient mice compared to controls. Finally, using well established monoclonal antibodies against monocytes/macrophages we quantified the accumulation of those cells in the perivascular space around collateral arteries and found a strong impairment in the knockout group. Taken together, our studies underlined the outstanding role of the MCP-1-CCR2-pathway for the growth of collateral blood vessels.

Growth factors et al

In the past years, the role of a large body of factors for collateral artery growth was investigated in both animal and clinical studies. One of the most well-

known growth factors in the field is the vascular endothelial growth factor (VEGF). VEGF is a very potent trigger of angiogenesis [44] and its promising ability to compensate blood flow deficits by stimulating vascular growth has been intensely investigated. It has been shown that VEGF and its homologue placenta growth factor (PlGF) can stimulate monocyte chemotaxis via the VEGF-receptor-1 (VEGFR-1, flt-1) [45]. It also has been shown *in vitro* that VEGF can stimulate monocyte adhesion to endothelial cells and the transmigration process via integrin upregulation [31]. But after local infusion in our rabbit model, only the VEGFR-1 specific PlGF was able to potently stimulate collateral artery growth, while the VEGFR-2 specific VEGF-E did not enhance arteriogenesis and VEGF-A only moderately. Furthermore, only in PlGF-treated rabbits an increase in monocyte accumulation around the collateral vessels was observed. From these results it may be concluded that the stimulation of arteriogenesis by VEGF-family members is mediated via the VEGFR-1 only and is secondary to monocyte recruitment via the VEGFR-1. In contrast, binding of VEGF-A or VEGF-E to the VEGFR-2 which is expressed on endothelial cells but not on monocytes, may induce side effects which do not support or even inhibit collateral artery growth.

A similar mechanism may also form the basis for positive effects on arteriogenesis reported for the transforming growth factor-β (TGF-β). TGF-β can induce monocyte chemotaxis [46]. Furthermore, it was shown that TGF-β can increase gene expression in a variety of cells including monocytes [47]. Indeed, we could show that treatment with TGF-β in the rabbit hind limb model enhances arteriogenesis, most likely by increasing monocyte attraction and subsequently by increasing the expression of arteriogenic genes in monocytes.

Cell proliferation and remodeling during collateral vessel growth

While monocyte invasion, their maturation into macrophages and appearance of typical signs of inflammation characterize the first phase of arteriogenesis, the subsequent growth phase is dominated by the proliferation of cells, mainly of smooth muscle and adventitial fibroblasts but also of endothelial cells on the one hand, and extensive tissue remodeling on the other hand.

Proliferation of vascular cells is initiated as early as 24 hours after experimental occlusion of the femoral artery in the rabbit model as determined by staining with the proliferation marker Ki-67. The maximal proliferation activity can be observed within the first week but cell proliferation is still present after three weeks [11]. This increase in cell mitosis coincides with a morphological change in smooth muscle cells. The appearance of a prominent rough endoplasmic reticulum and many free ribosomes indicates that smooth muscle cells are transformed from the contractile into the proliferative/synthetic phenotype. During this phase, a neointima forms comprised of smooth muscle cells in which, like in the earlier degradation of the internal elastic lamina,

matrix-metalloproteinases (MMPs) are involved [11, 48]. Several MMPs are known to be expressed by monocytes/macrophages and we could demonstrate that stimulation of arteriogenesis with MCP-1 locally augments MMP-1, MMP-2, MMP-3 and MMP-9 expression and activity [49]. Furthermore, increases in the expression of MMPs, probably produced by macrophages, were also observed in the perivascular space of growing collaterals. This could indicate that MMPs participate in the digestion of the extracellular matrix and even of skeletal muscle cells to create additional space for the growing collateral vessel. Remaining elastin fragments may attract new monocytes, which now downregulate elastin transcription in smooth muscle cells and may contribute to the development of a synthetic phenotype of these cells. In addition, it also may support smooth muscle cell migration and proliferation. The breakdown of the tension-bearing vessel wall increases the circumferential wall stress for the smooth muscle cells, a proliferative stimulus which is now increased. As a negative feedback, when finally collateral vessel radius is increased during the remodeling process and circumferential wall stress is reduced again, collateral artery growth could be terminated by this effect.

Since proteins of the fibrinolytic system, tissue-plasminogen activator (t-PA) and urokinase-plasminogen activator (u-PA) were reported to be involved in proteolysis during cell migration and tissue remodeling, these proteins were of particular interest for arteriogenesis research. Consequently, an upregulation of u-PA mRNA was found in the tissue obtained from the quadriceps muscle which correlated with increased u-PA activity [50], but surprisingly no increase in t-PA activity was observed. Hence, a second panel of experiments was performed using u-PA gene-deficient mice. In this experiment, only u-PA gene-deficient mice showed a pronounced reduction in blood flow recovery after femoral artery ligation. In contrast, when the u-PA receptor (u-PAR) gene was disrupted, no alteration in arteriogenesis was observed, indicating a u-PAR independent role of u-PA. Since u-PA was demonstrated to stimulate monocyte invasion, at least part of the inhibition effect in u-PA knockout mice could be secondary to defective monocyte recruitment [51]. But also an alternative pathway may apply. u-PA has been described to play a role in smooth muscle cell migration, a process were u-PAR is not necessarily required [52]. It is very likely, that u-PA converts the zymogen plasminogen into plasmin. Plasmin, a very potent serinprotease, in turn degrades the extracellular matrix and interstitial tissue in the microenvironment of the growing collateral arteriole, thereby creating space for migrating and proliferating smooth muscle cells. This could be enhanced by u-PA binding to u-PAR on the macrophage surface, thus accumulating additional lytic activity around the growing collateral vessel. Finally, u-PA can directly enhance smooth muscle cell proliferation, since it was reported that u-PA can act as a smooth muscle cell mitogen [52]. Finally, the integrity of the greatly expanded vascular wall of the collateral arteries is re-established which are now capable to deliver enough blood flow to maintain the integrity of the subtended tissues and to allow resumption of organ function albeit only partially.

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Mechanisms of Angiogenesis
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Balancing luminal size and smooth muscle proliferation – a key control point in atherosclerosis and arteriogenesis

Jürgen R. Sindermann[1] and Keith L. March[2]

[1] *Department of Cardiology and Angiology, and Institute for Arteriosclerosis Research, University of Münster, Albert-Schweitzer-Strasse 33, 48149 Münster, Germany*
[2] *Indiana Center for Vascular Biology and Medicine and Krannert Institute of Cardiology, 975 W. Walnut Street, IB 441, Indianapolis, IN 46202, USA*

The concept of adaptive *versus* constrictive remodeling

The response of the blood vessel wall morphology to processes such as atherosclerosis and arteriogenesis, and following angioplasty involves changes in vessel wall mass as well as controlled alterations in total vascular circumference which critically modulate the sequelae of these changes in wall mass. Although much work has focused on understanding the regulation of vascular smooth muscle proliferation and synthetic activity, relatively little is known concerning the mechanisms that control the three-dimensional arrangement of vascular smooth muscle cells (SMC) in the arterial wall. This dynamic modulation of vascular wall geometry during embryonic development as well as in response to physiologic stimuli is collectively referred to as vascular remodeling. This remodeling is a dynamic process which apparently involves both the cellular and non-cellular components of the vessel in responses which link changes in the mass and composition of the vessel wall to resulting changes in luminal area by the specification of precise wall geometry. Remodeling plays a pivotal role in the injury response mechanisms of atherosclerosis as well as after vessel trauma, like that found with balloon angioplasty. Several categories of geometric responses can be distinguished. Outward or adaptive remodeling may occur at the site of the atherosclerotic plaque, where it is understood as a reaction of the vessel wall to compensate for the luminal decrease caused by the abnormal tissue mass [1–3]. Outward remodeling can also be found after vessel trauma, in association with an aneurysmal dilation, implying an increase of luminal area achieved by a thinning of the vessel wall. Inward remodeling may be found in adaptation to a diminished flow requirement, while an inward, maladaptive remodeling may occur after vessel trauma such as that found with angioplasty [4]. This behavior results in the reduction of luminal area due to shrinkage of the vessel circumference which is superimposed on vessel wall thickening. Despite the apparent importance of this

process in determining clinical outcomes, relatively little is known about its key underlying regulatory mechanisms which comprise mechanical forces as well as inflammatory and apoptotic responses.

Vasculature and arteriogenesis

The formation of new capillaries involves the presence of endothelial cells (EC) to from new capillary sprouts. These EC are located in capillaries that degrade the basement membrane, migrate and proliferate [5]. In addition, circulating endothelial progenitor cells are also discussed of being involved in this process [6]. Sprouting is then enhanced by growth factors [7] and blood cells such as macrophages [8] and mast cells [9]. This initial step of angiogenesis on the level of microvessels is the domain of EC and is explained elsewhere in this book. Beyond this, another form of new vessel formation, the vasculogenesis or arteriogenesis in a broader sense, also involves the formation of collateral vessels from pre-existing collaterals or even the new formation of collaterals/vessels. These are vessels considerably larger than the microvessels and they do not only require the presence of EC but also the presence of vascular SMC as they are an integral part of the vessel wall.

Early studies on the remodeling of pre-existing collateral arteries have been performed in the mid-seventies on dog hearts. After coronary occlusion, monocytes were found on the endothelium and in the subintimal space of pre-existing collateral arteries in the canine coronary system [10]. It was found that the SMC change from a contractile to a proliferative phenotype and participated in neointima formation, a process well known from injury repair mechanisms after vessel trauma. This involvement of neointima into collateral vessel formation has also been shown in a rabbit hindlimb model [11]. In parallel to this trauma reaction, an inflammatory reaction may also be involved by the extravasation of blood cells around growing collateral vessels. This may contribute to the proliferative response of vascular SMC and also to apoptosis, which allows the vessel to undergo geometrical alterations, or in other words, vascular remodeling. After this remodeling response is completed the vascular SMC return to the contractile phenotype [12]. The mechanisms by which this extent of vessel wall formation and lumen formation are controlled are only partially understood. An enhanced smooth muscle cell proliferation is occasionally found and may result in a relatively thick vessel wall compared with the lumen area [13, 14]. However, it was shown in a pig model that the grown collateral vessels generally reveal a thin vessel wall with less SMC compared with regular arterioles [15].

Several animal species have been employed to study the involvement of vascular SMC in the collateral formation in the hindlimb model. In rabbits the remodeling of pre-existing collaterals in the thigh has been shown to involve neointima by smooth muscle cell proliferation [11]. In mice, the ligation of the femoral artery employs pre-existing collateral connections to be remodelled by

one to two layers of vascular SMC which form the tunica media [16]. Along with this proliferative response of SMC the collaterals are remodelled towards a bigger vessel which comprises a more than 2-fold increase in vessel diameter and an increase in vessel wall thickness. However, in this murine model a degradation of the basal membrane is found but not a neointima formation, so that the SMC are primarily recruited to form the tunica media [16]. The situation in rats is slightly different in a way that in this species the femoral occlusion employs neointima formation to recruit pre-existing collaterals to remodel into bigger vessels [17], so in this species the process of collateral remodeling is more comparable to the rabbit hindlimb model. Further studies on the arteriogenesis in the ischemic rat brain have shown that collateral formation primarily recruits pre-existing collaterals rather than the formation of new vessels [18, 19].

Apart from these descriptive observations the question arises how the remodeling response during arteriogenesis is controlled. As mentioned earlier, the alteration of flow is supposed to be one mechanism in vascular remodeling. But it is likely that changes in physical parameters are not sufficient to allow the vessel to undergo a change in geometric dimensions. The extracellular matrix as another integral part of the vessel wall is supposed of having a key role in allowing the vessel wall to perform adaptive or constrictive remodeling, thereby interacting with SMC growing in a radial or circumferential fashion. At the site of atherosclerotic plaques circumferential vessel wall stress has been proposed to influence the expression of matrix-metalloproteinase-1 (MMP-1), which might be a mechanism that renders the vessel wall to undergo remodeling [20]. We have shown in a transgenic mouse model featuring a hyperproliferative vessel wall that circumferential vessel wall stress controls the adaptive remodeling response and keeps the ratio of vessel circumference and vessel wall thickness constant across several types of arterial vessels [21]. Our data further suggest that changes in fibrilar *versus* network forming collagens provide the basis for this adaptive remodeling response. Another physiologic parameter which has to be taken into account is the shear stress which is up-regulated by endothelial nitric oxide (NO) synthase [22]. In addition to its direct effects on vasodilation, NO is also discussed in activating metalloproteinases which may play a role in growth-factor mediated cell proliferation and migration [23, 24], although NO has also been shown to exert antiproliferative effects on vascular SMC and EC [25–28]. Although several studies have focussed on the role of NO in collateral flow parameters [29–32], there is still uncertainty left as to whether an increase in collateral flow is caused by the impact of NO on vasomotor tone or on structural alterations.

Control of vessel dimensions. Circumferential *versus* radial growth of smooth muscle

Expression of the SV40 T antigen (TAg) in transgenic animals has been used to create specific models of increased proliferation and permit studies of the

range of responses to such induced growth by inactivating cell cycle negative regulators such as p53 [33, 34] and the proteins of the pRb family [35–37]. Targeted expression of TAg under control of a 2.3 kb 5' sequence of the smooth muscle myosin heavy chain (SM-MHC) gene was performed as a system to study the responses of the vascular wall to directly induced SMC proliferation in the absence of endothelial injury or inflammation [21]. The findings indicated that forced SMC proliferation in the vessel wall is associated with adaptive remodeling which conserves a strong positive correlation between vessel wall thickness and the lumen circumference (Fig. 1). The induced SMC proliferation results in both the development of a thickened vessel wall and the increase in arterial lumen area (Fig. 2). The correlation between vessel wall thickness and vessel (or lumen) circumference maintained in the transgenic animals was identical to that found in the controls. Since the lumen circumference is directly related to the internal radius, this relationship implies the conservation of circumferential vessel wall stress (σ) between the control and transgenic animals, defined as

$$\sigma = p\, r_i / t$$

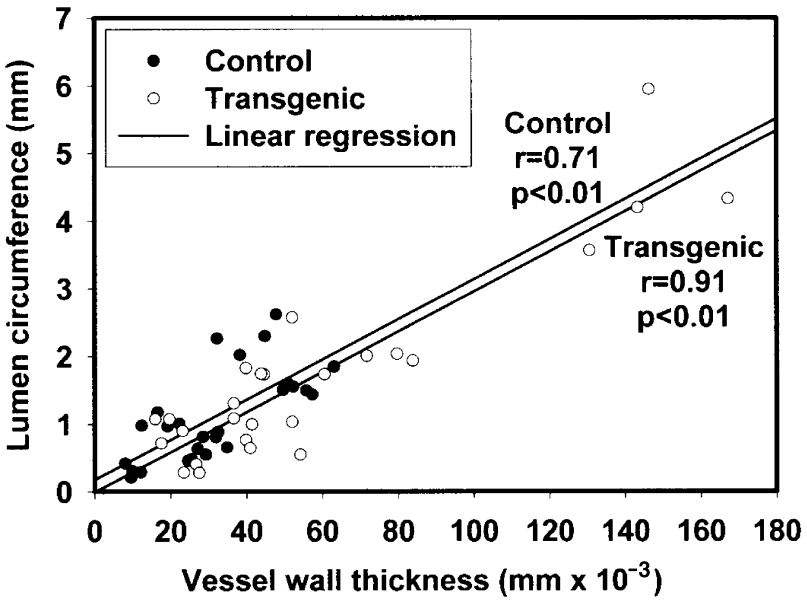

Figure 1. Correlation of vessel wall thickness and lumen circumference in transgenic mice (MHC-TAg). Transgenic mice expressing SV40 large TAg under control of a SM-MHC promoter at ages of 3–13 weeks (n = 6) and age-matched controls (n = 18) were studied with regard to lumen circumference and vessel wall thickness of various arteries (aortic segments, common carotid, femoral, and coronary arteries). Morphometry was performed on cross sections by measuring the circumference and calculating the corresponding area assuming a circular structure under *in vivo* conditions. Data are given as means of multiple sections analysed per vessel in each animal. In case of controls data reflect means of 3 age-matched animals.

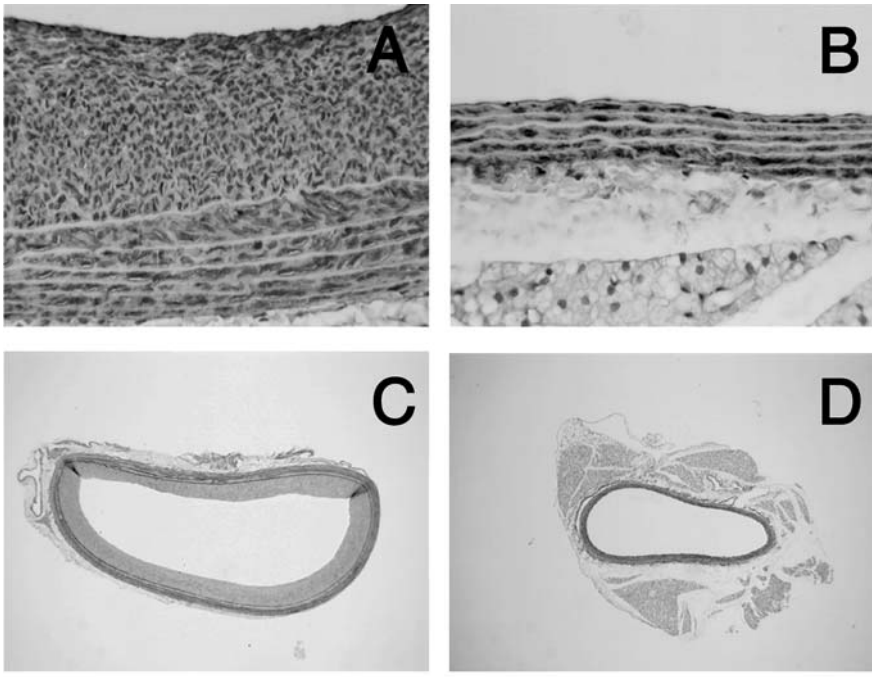

Figure 2. Vessel morphology in transgenic mice (MHC-TAg) and controls. The figure displays immunohistochemical stains for alpha-actin (panel A–B) and Verhoeff-van Gieson stains (panel C–D) of the thoracic aorta of a 13 week old transgenic mouse (panel A and C) expressing SV40 large TAg under control of a SM-MHC promoter and an age matched control (panel B and D). The panels display the proportional increase in vessel wall thickness and luminal area of transgenics. (original magnifications: A–B: ×400, C–D: ×25).

where (p) is the mean transmural pressure, (r_i) the internal radius, and (t) the vessel wall thickness [38]. As the blood pressures of transgenic and control mice were found to be equivalent [21], the parallel increases in internal vessel radius and vessel wall thickness in the transgenic animals thus provide evidence for conservation of circumferential wall stress as an important constant across multiple vessel types and between the transgenic and normal mice. Similar conservation of wall stress was reported for transgenic mice overexpressing human growth hormone, which revealed increased vessel growth in correlation with increased body weight or increased weight of the organ to be supplied, respectively. In agreement with the results obtained from MHC-TAg transgenic mice [21], the relationship between the wall and lumen were found to be similar for the various vessels studied in transgenic and control mice, again suggesting that wall stress was the controlling factor for wall thickness [39]. Such data clearly indicate that induced smooth muscle hyperproliferation and neointima formation is not necessarily accompanied by progressive vascular occlusion. In fact, increased proliferative activity of SMC is capable of

creating an enlarged vessel. These results, taken together with previous studies, suggest that the key determinants of the geometric disposition of vascular tissue are distinguishable from factors governing the smooth muscle cell cycle, indicating that (constrictive) remodeling is a process that is decoupled from SMC proliferation. These results give rise to the hypothesis that the conservation of wall stress is a primary determinant of vascular remodeling by modulating the extent of radial *versus* circumferential directionality of vascular smooth muscle tissue expansion.

Growth promoting hormones and growth inhibitors

In the past decades a variety of growth factors have been identified with different emphasis on vascular SMC or EC. The classical growth factors for the growth promotion of SMC are the platelet derived growth factor (PDGF) and the basic fibroblast growth factor (bFGF), but also epidermal growth factor (EGF), insulin like growth factor (IGF) and vascular endothelial growth factor (VEGF) function as paracrine or autocrine mitogens. All of these growth factors exert their effects via specific membrane bound receptors, which may be specific target to subunits of the growth factor. These receptors function as tyrosine kinases that catalyse the phosphorylation of tyrosine residues in proteins. The binding of a growth factor to its specific receptor activates the receptors tyrosine kinase activity that results in receptor autophosphorylation. This initiates a cascade of signal transducing proteins starting with the activation of a membrane-associated G protein Ras which induces mitogen-activated protein kinases (MAP-kinases). These kinases phosphorylate other proteins involved in cell cycle progression such as transcription factors.

PDGF may be considered a very potent mitogen for SMC. Two chains of this growth factor, the A and B chain, have been identified which are synthesized by a variety of cells including SMC, endothelium, and monocytes/macrophages. This results in three dimeric isoforms of PDGF (AA, AB, BB) which have different binding affinities to the alpha or beta PDGF receptor. [40–42]. Production of the AB and BB isoforms are involved in SMC migration and proliferation.

Another potent mitogen for SMC is bFGF, but this growth factor is also an important autocrine factor for EC [43, 44]. *In vitro* studies revealed the involvement of bFGF in the proliferation and migration of EC as well as alterations of the extracellular matrix and angiogenesis [42, 44]. bFGF is bound to heparan sulfate within the matrix which acts as a reservoir to release bFGF in response to cell injury and in response to proteases [42]. bFGF is therefore considered to have profound effects on the vascular structure.

A more complex role is attributed to IGF. The IGF-1 contributes to the control of SMC and EC proliferation, but the functions are dependent of the environment. Studies indicate that IGF may have a growth-promoting effect in microvascular EC [45]. In SMC the role of IGF depends on the presence of

other growth factors. In conjunction with PDGF or bFGF the growth factor IGF-1 enables SMC to traverse through the cell cycle [46]. In the absence of other growth factors however, SMC are induced towards hypertrophy and production of extracellular matrix [45].

The growth factor of pivotal interest for angiogenesis is VEGF, which is an endothelial cell specific growth factor without impact on other vascular cells. Studies have revealed the capacity of VEGF to promote cell migration and proliferation [47, 48] and to inhibit apoptosis in EC [49, 50]. Under hypoxic and ischemic conditions VEGF has been shown to promote arteriogenesis and angiogenesis [51, 52].

The proliferation of cells, especially SMC, is not only dependent on the presence of mitogenic factors, but also on the activity of cell cycle regulatory proteins, among which p53 and the proteins of the pRb family play pivotal roles. The proteins of the pRb family have been shown to regulate cell cycle progression and cell cycle reentry not only in SMC [53–56]. These proteins, pRb, p130 and p107, function in a cell cycle dependent manner to regulate the activity of numerous important cellular transcription factors such as the E2F family, which in turn regulate the expression of genes important for cell cycle progression and cellular differentiation. These are important features for the regulation of smooth muscle cell cycle reentry which is a key mechanism for the thickening of the vessel wall due to a traumatic stimulus. Studies employing cytostatic gene therapy with a constitutively active form of the retinoblastoma gene product and p130 gene transfer showed a significant decrease in SMC proliferation and neointima formation in a rat carotid and porcine femoral artery model of restenosis [57, 58]. Investigations on mice with targeted disruption of the p130 gene revealed a regular vessel geometry under basal conditions but an enhanced injury response to carotid artery ligation in terms of increased vessel wall area and enhanced cell proliferation. Despite this increase in wall area, the mice displayed an outward growth of the vessel by perfectly conserving the luminal area, thereby performing adaptive remodeling [59]. The results point out that the loss or the inactivation of certain cell cycle inhibitors contributes to the injury response by allowing the cells an enhanced cell cycle reentry. The data further indicate that enhanced smooth muscle cell cycle reentry is not a sufficient cause of luminal obstruction but rather capable of creating a bigger vessel by performing outward growth.

Vessel remodeling in response to pathologic stimuli

The interaction of SMC proliferation with vascular remodeling is recognized to be of utmost clinical relevance in determining the mechanisms of atherosclerosis as well as arteriogenesis. Studies have pointed out a key role of vessel remodeling in determining luminal loss or gain, as expressed by the relationship between lumen area and vessel wall thickness [60]. Adaptive remodeling displays an early physiological reaction of arterial vessels to compensate

for lumen loss in atherosclerosis [60, 61]. Following angioplasty, the impact of remodeling on luminal area increases while the impact of neointimal formation decreases over time [62]. However, several clinical studies have established a remarkable inter-individual variability in the extent and direction of remodeling [63]. Constrictive remodeling has been partially related to the presence in patients of specific environmental factors such as smoking [64, 65] and diabetes [66]. But it is clear that genetic factors as well may help determine the balance between constrictive and adaptive remodeling in each case. This divergence of findings points out that more data are needed to understand the fundamental biology of remodeling. The porcine coronary model of copper stent-induced inflammation provides an example in which neointimal thickening is accompanied by proportional lumen gain, whereas heat injury of porcine coronary arteries appears to disrupt the relationship between lumen size and neointimal thickness [67]. Mice have also been described in which the remodeling vascular response is disrupted. Studies of mice with homologous deletion of the elastin gene revealed that the loss of one allele is associated with an increase in elastic lamellae and smooth muscle [68]; while mice lacking elastin died of smooth muscle cell proliferation with vascular occlusion [69], indicating that the absence of elastin prevented the vessel from maintaining an adaptive correlation between wall mass increase and vessel circumference.

Physiologic/pathophysiologic and exercise induced remodeling

The arterial lumen size is determined by several physiologic and also pathophysiologic factors. Under basal conditions the artery size is determined by the pressure, the flow demand of the organ to be supplied, and also by the process of atherosclerosis [70]. Glagov and coworkers [71] have found that in early stages of atherosclerosis the increase in vessel wall thickness is compensated for by creating a bigger vessel. This initially adaptive process turns into a constrictive process at later stages of the disease where it becomes a manifest clinical problem. It was proposed that the narrowed lumen by increasing atheroma resulted in an increase in shear stress with resulting affection of vascular reactivity.

People with physically active professions have been shown to develop larger coronary arteries [72, 73]. Another remarkable finding was that Masai tribesmen who, despite the presence of atherosclerotic lesions, did not suffer from cardiovascular diseases due to enlarged epicardial vessels compared with American men [74]. This phenomenon may be due to physical exercise. The autopsy of “Mr. Marathon”, Clarence De Mar, in the early sixties already revealed epicardial vessels two or three times the normal size [75]. Such findings have been confirmed in animal models, where exercise training of rats [76–78], dogs [79] and monkeys [80] resulted in a significant increase in the diameter of coronary arteries. This correlation between artery size and physi-

cal exercise is also maintained in case of organ hypertrophy. O'Keefe and colleagues [81] have shown a positive correlation between the size of coronary arteries and the left ventricular mass. Similar findings have been reported from autopsy [82] and angiographic studies [83].

Influence of acute and chronic flow changes

Flow requirement is also an important parameter determining luminal size. In patients with coronary fistulas the coronary blood flow is considerably enhanced. These vessels were found to present an enlarged lumen [84]. A similar phenomenon was observed in an animal model with induced fistulas of the femoral artery [85]. Such increases in lumen diameter due to flow increase can be observed as acute response to flow changes, where it is mediated by vasodilators such as NO [86–89], and also as a chronic response to flow. The latter is primarily of interest for arteriogenesis. However, this remodeling response is possible in both, a constrictive as well as an adaptive fashion. A central role in these processes is probably attributable to NO. A chronic decrease in flow in the rabbit carotid artery was shown to reduce the vessel diameter, and this response was dependent on the endothelium as this constrictive response was abolished after the removal of the endothelium [90]. The role of NO in vessel remodeling has nicely been shown by Rudic and coworkers using endothelial NO synthase (eNOS) knock out mice [91]. In this model, a reduction in blood flow due to ligation of the external carotid artery resulted in a significant thickening of the vessel wall suggesting the eNOS as an important regulator for flow-induced constrictive remodeling. Other studies using a NO synthase inhibitor have revealed the importance of NO for the flow induced adaptive remodeling [92]. Nevertheless, despite the numerous studies showing the ultimate role of NO for vessel remodeling, the mechanisms by which NO exerts its effects are widely unknown. It may be hypothesized that NO interferes with the extracellular matrix which renders the vessel to undergo changes in vessel wall architecture.

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Molecular mechanisms of angiogenesis

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The role of VEGF in the regulation of physiological and pathological angiogenesis

Napoleone Ferrara

Department of Molecular Oncology, Genentech Inc., 1 DNA Way, South San Francisco, CA 94080, USA

Introduction

The existence of angiogenic factors was initially postulated on the basis of the strong neovascular response induced by transplanted tumors. Subsequently, it was shown that normal tissues are also a source of angiogenic activity. Many molecules have been implicated as positive regulators of angiogenesis including aFGF, bFGF, TGF-α TGF-β, HGF/SF, TNF-α, angiogenin, IL-8, the angiopoietins, etc. [1, 2].

For over a decade, the role of vascular endothelial growth factor (VEGF) in the regulation of angiogenesis had been object of intense investigation. For a historic overview of the VEGF field, see [3]. While recent evidence indicates that new vessel growth and maturation are highly complex and coordinated processes, requiring the sequential activation of a series of receptors by numerous ligands (for reviews see [2, 4, 5]), there is consensus that VEGF signaling often represents a critical rate-limiting step in physiological angiogenesis. VEGF appears to be also important in pathological angiogenesis, such as that associated with tumor growth [6]. VEGF (referred to also as VEGF-A) belongs to a gene family that includes placenta growth factor (PlGF) [7], VEGF-B [8], VEGF-C [9, 10], and VEGF-D [11]. Additionally, homologues of VEGF have been identified in the genome of the parapoxvirus *Orf* virus [12] and shown to have VEGF-like activities [13]. The main focus of this review is the biology of the prototype member, VEGF-A, a key regulator of blood vessel growth. Importantly, VEGF-C and VEGF-D regulate lymphatic angiogenesis [14], emphasizing the unique role played by this gene family in controlling growth and differentiation of multiple anatomic components of the vascular system.

Biological effects of VEGF

Mitogenesis and survival

A well-documented *in vitro* activity of VEGF is the ability to promote growth of vascular endothelial cells derived from arteries, veins and lymphatics (for

review [6]). VEGF induces a potent angiogenic response in a variety of *in vivo* models [15, 16]. Nagy et al. have recently shown that adenovirus-mediated VEGF delivery also induces lymphangiogenesis *in vivo* [17]. While endothelial cells are the primary target of VEGF, several studies have reported mitogenic effects also on certain non-endothelial cell types (for review see [18]). Recent studies have also shown that VEGF stimulates surfactant production by alveolar type II cells [19].

VEGF functions as a survival factor for endothelial cells, both *in vitro* and *in vivo* [20–24]. *In vitro*, VEGF prevents apoptosis induced by serum-starvation. Gerber et al. have shown that such survival activity is mediated by the PI 3' kinase/Akt pathway [22]. Also, VEGF induces expression of the anti-apoptotic proteins Bcl-2 and A1 in endothelial cells [21]. *In vivo*, VEGF's pro-survival effects are developmentally regulated. VEGF inhibition results in extensive apoptotic changes in the vasculature of neonatal, but not adult mice [25]. Also, a marked VEGF-dependence has been demonstrated in newly formed endothelial cells within tumors [23, 24]. Coverage by pericytes and establishment of a mature vessel wall has been proposed to be one of the key events resulting in loss of VEGF dependence [23].

Enhancement of vascular permeability and hemodynamic effects

VEGF is known also as vascular permeability factor (VPF) based on its ability to induce vascular leakage [26, 27]. It is now well established that such permeability-enhancing activity underlies important roles of this molecule in inflammation and in other pathological circustances. Bates et al. have shown that VEGF induces an increase in hydraulic conductivity of isolated microvessels [28] and that such effect is mediated by increased calcium influx [29]. Consistent, with a permeability-enhancing role, VEGF induces endothelial fenestration in specific vascular beds [30] and in cultured adrenal endothelial cells [31].

An issue that has been long debated is whether increased vascular permeability is a necessary step for angiogenesis [32]. Eliceiri et al. have shown that members of the Src family are differentially involved in mediating VEGF-dependent permeability and angiogenesis [33]. According to these studies, the permeability-enhancing activity of VEGF specifically depends on Src, or Yes [33]. Mice lacking *Src* and *Yes* display a normal angiogenic response to VEGF and do not display overt defects in the vasculature, suggesting that enhanced vascular permeability is not a requirement for VEGF-dependent angiogenesis, at least in the circumstances examined.

VEGF induces vasodilatation *in vitro* in a dose-dependent fashion [34, 35] and produces transient tachicardia, hypotension and a decrease in cardiac output when injected intravenously in conscious, instrumented rats [35]. Such effects appear to be caused by a decrease in venous return, mediated primarily by endothelial cell-derived nitric oxide (NO) [35]. Hypotension was a dose-

limiting side effect in human trials in which VEGF was systemically administered [36].

Effects of VEGF on bone marrow cells and hematopoiesis

The earliest evidence that VEGF may affect blood cells came from a report by Clauss et al., describing the ability of VEGF to promote monocyte chemotaxis [37]. Subsequently, Broxmeyer et al. reported that VEGF may have hematopoietic effects, inducing colony formation by mature subsets of granulocyte-macrophage progenitor cells [38]. Long-term, continuous infusion experiments with recombinant VEGF or by administration of adenoviral vectors encoding VEGF to adult mice, inhibited dendritic cell development [39], and increased the production of B cells and the generation of immature myeloid cells [40]. Conditional gene-knock out technology has been employed to achieve selective VEGF gene ablation in bone marrow cell isolates and HSCs [41]. VEGF deficient heamatopoietic stem cells (HSCs) and bone marrow mononuclear cells failed to repopulate lethally irradiated hosts, despite the co-administration of a large excess of wild-type cells. These studies also elucidated an internal autocrine loop, not blocked by extracellular inhibitors like antibodies, by which VEGF controls HSC survival during hematopoietic repopulation [41].

The VEGF isoforms

The human VEGF-A gene is organized in eight exons, separated by seven introns. [42, 43]. Alternative exon splicing was initially shown to result in the generation of four different isoforms, having respectively 121, 165, 189 and 206 amino acids following signal sequence cleavage ($VEGF_{121}$, $VEGF_{165}$, $VEGF_{189}$, $VEGF_{206}$) [42, 43]. $VEGF_{165}$, the predominant isoform, lacks the residues encoded by exon 6, while $VEGF_{121}$ lacks the residues encoded by exons 6 and 7. Less frequent splice variants have been also reported, including $VEGF_{145}$ [44], $VEGF_{183}$ [45] and VEGF165b, a variant reported to have paradoxically an inhibitory effect on VEGF-induced mitogenesis [46].

Native VEGF is a basic, heparin-binding, homodimeric glycoprotein of 45 kDa [47]. The properties of native VEGF closely correspond to those of $VEGF_{165}$ [48]. The crystal structure of VEGF has been solved at a resolution of 2.5 Å [49]. The VEGF monomer resembles that of PDGF, but its N-terminal segment is helical rather than extended. [49]. $VEGF_{121}$ is an acidic polypeptide that fails to bind to heparin [48]. $VEGF_{189}$ and $VEGF_{206}$ are highly basic and bind to heparin with high affinity [48]. $VEGF_{121}$ is a freely diffusible protein. In contrast, $VEGF_{189}$ and $VEGF_{206}$ are almost completely sequestered in the ECM. $VEGF_{165}$ has intermediary properties, as it is secreted, but a significant fraction remains bound to the cell surface and ECM [50]. However, the ECM-bound isoforms may be released in a diffusible form by

heparin or heparinase, which displaces them from their binding to heparin-like moieties. Additionally, plasmin cleavage at the COOH terminus generates a soluble bioactive fragment having molecular weight of ~34 kDa [48]. Importantly, loss of the heparin-binding domain results in a substantial loss of VEGF's mitogenic activity for endothelial cells [51]. These findings suggested that $VEGF_{165}$ has optimal characteristics of bioavailability and biological potency. In agreement with such conclusions, only $VEGF_{164}$ (murine VEGF is shorter by one amino acid) is able to fully rescue a tumorigenic phenotype in mouse *VEGF*$^{-/-}$ cells [52]. The key role of the heparin-binding VEGF isoform(s) is also emphasized by the finding that 50% of the mice expressing exclusively $VEGF_{120}$ ($VEGF^{120/120}$) die shortly after delivery, while the remainder die within two weeks [53]. The survivors demonstrate impaired myocardial contractility and defective angiogenesis leading to ischemic cardiomyopathy. Recent studies have also evidenced a deficit in the distribution of endothelial cells and impaired filopodia extension in $VEGF^{120/120}$ mice, suggesting that the heparin-binding VEGF isoforms provide essential, spatially restricted, stimulatory cues that direct sprouting endothelial cells to initiate vascular branch formation [54].

Regulation of VEGF gene expression

Oxygen tension

Extensive evidence has shown that oxygen tension plays a key role in regulating the expression of a variety of genes, including VEGF. It is now well established that HIF-1 is a key mediator of hypoxic responses [55]. HIF-1 is a basic, heterodimeric, helix-loop-helix protein consisting of two subunits, HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT), known also as HIF-1β [56]. In response to hypoxia, HIF-1 binds to specific enhancer elements, resulting in increased gene transcription. Recent studies have uncovered the critical role of the product of the von Hippel-Lindau (VHL) tumor suppressor gene in the HIF-1-dependent hypoxic responses (for review see [57]). The *VHL* gene is inactivated in patients with von Hippel-Lindau disease, a condition characterized by capillary hemangioblastomas in the retina and cerebellum, and in most sporadic clear cell renal carcinomas. Renal cell carcinoma cells either lacking endogenous wild-type *VHL* gene or expressing an inactive mutant form demonstrated altered regulation of VEGF gene expression [58]. Most of the endothelial cells mitogenic activity released by such cells was neutralized by anti-VEGF antibodies [58]. Although the spectrum of functions of the VHL protein remains to be fully elucidated, it is now established that such protein interacts with a series of proteins including elongins B and C and CUL2 [59]. A function of the VHL protein is to provide a negative regulation of a series of hypoxia-inducible genes, including the VEGF, platelet derived growth factor B chain and the glucose transporter GLUT1 genes [60]. In the

presence of a mutant VHL, mRNAs for such genes were produced both under normoxic and hypoxic conditions. Reintroduction of wild-type VHL resulted in inhibition of mRNA production under normoxic conditions and restored the characteristic hypoxia-inducibility of those genes [60]. HIF-1 was shown to be constitutively activated in VHL-deficient renal cell carcinoma cell lines [61]. Other studies demonstrated that that one of the functions of VHL is to be part of a ubiquitin ligase complex that targets HIF subunits for proteasomal degradation following covalent attachment of a polyubiquitin chain (for review see [62]). Oxygen promotes the hydroxylation of HIF at a proline residue, a requirement for the association with VHL. Recently, a family of prolyl hydroxylases related to *Egl-9 C. elegans* gene product were identified as HIF prolyl hydroxylases [62].

Growth factors, hormones and oncogenes

Several major growth factors, including EGF, TGF-α, TGF-β, KGF, IGF-1, FGF and PDGF, up-regulate VEGF mRNA expression [63, 64], suggesting that paracrine or autocrine release of such factors cooperates with local hypoxia in regulating VEGF release in the microenvironment. Also, inflammatory cytokines such as IL-1-α IL-6 induce expression of VEGF in several cell types, including synovial fibroblasts, suggesting that VEGF may be a mediator of angiogenesis/permeability in inflamamtory disorders [65, 66]. Thyroid stimulating hormone has been shown to induce VEGF expression in several thyroid carcinoma cell lines [67]. Specific transforming events also result in induction of VEGF gene expression. Oncogenic mutations or amplification of ras lead to VEGF up-regulation [68, 69]. These studies indicate that mutant *ras* dependent VEGF expression is necessary, albeit not sufficient, for progressive tumor growth *in vivo*.

The VEGF receptors

Initially, VEGF binding sites were identified on the cell surface of vascular endothelial cells, *in vitro* [70] and *in vivo* [71]. Subsequently, it became apparent that receptors for VEGF are abundant also on bone marrow-derived cells. There is a considerable complexity in VEGF signaling. VEGF binds two highly related receptor tyrosine kinases (RTK), VEGFR-1 and VEGFR-2. Both VEGFR-1 and VEGFR-2 have seven immunoglobulin (Ig) like domains in the extracellular domain (ECD), a single transmembrane region and a consensus tyrosine kinase sequence which is interrupted by a kinase-insert domain [72, 73]. A member of the same family of RTKs is VEGFR-3 (Flt-4) which, however, is not a receptor for VEGF, but instead binds VEGF-C and VEGF-D [14]. In addition to signaling receptors, VEGF interact with a family of co-receptors, the neuropilins.

VEGFR-1 (Flt-1)

Although Flt-1 (fms-like-tyrosine kinase) was the first RTK to be identified as a VEGF receptor over a decade ago [74], the precise function of this molecule is still object of debate**.** Recent evidence indicates that the conflicting reports may be due, at least in part, to the fact that VEGFR-1 functions and signaling properties can be different depending on the developmental stage and the cell type, e.g., endothelial *versus* hematopoietic cells. Similar to VEGF, the expression of the VEGFR-1 gene is directly upregulated by hypoxia via a HIF-1 dependent mechanism [75]. VEGFR-1 binds not only VEGF-A but also PlGF [76] and VEGF-B [77], which fail to bind VEGFR-2**.** An alternatively spliced soluble form of VEGFR-1 (sFlt-1) has also been proposed to be an inhibitor of angiogenesis [78]. The binding site for VEGF (and PlGF) has been mapped primarily to the second Ig-like domain [79]. Wiesman et al. have solved the crystal structure of a VEGF-Flt-1 domain 2 complex and shown the poles of the VEGF dimer to be in a predominantly hydrophobic interaction with domain 2 [80]. Flt-1 reveals a weak tyrosine autophosphorylation in response to VEGF [74, 81, 82]. Park et al. initially proposed that VEGFR-1 may be not primarily a receptor transmitting a mitogenic signal, but rather a "decoy" receptor, able to regulate in a negative fashion the activity of VEGF on the vascular endothelium, by sequestering and rendering this factor less available to VEGFR-2 [76]. According to this hypothesis, the observed potentiation of the action of VEGF by PlGF can be explained, at least in part, by displacement of VEGF from VEGFR-1 binding [76]. Not only the full-length membrane bound form of VEGFR-1, but also sFlt-1 could perform such a "decoy" function [83]. Recent studies have shown that indeed a synergism exists between VEGF and PlGF *in vivo*, especially during pathological situations, as evidenced by impaired tumorigenesis and vascular leakage in $Plgf^{-/-}$ mice [83]. Gille et al. have identified a repressor motif in the juxtamembrane region of VEGFR-1 that impairs PI-3 kinase activation and endothelial cell migration in response to VEGF [84]. However, other studies indicated that VEGFR-1 is able to interact with various signal transducing proteins and generate in some circumstances a mitogenic signal [85, 86].

In spite of the uncertain role of VEGFR-1 as a signaling receptor, knockout studies have demonstrated its essential role during embryogenesis. $Flt\text{-}1^{-/-}$ mice die *in utero* between day 8.5 and 9.5 [87, 88]. Endothelial cells develop but fail to organize in normal vascular channels. Excessive proliferation of angioblasts has been reported to be responsible for such disorganization and lethality [88], lending support to the hypothesis that, at least during early development, VEGFR-1 is a negative regulator of VEGF action. More compelling evidence in support of this view stems from the report that a targeted mutation resulting in a VEGFR-1 lacking the TK domain, but able to bind VEGF, does not result in lethality or any overt defect in vascular development [89]. Nevertheless, at least one biological response, the migration of monocytes in response to VEGF (or PlGF), was clearly shown to be mediated by

Flt-1 [90]. VEGF-induced macrophage migration is suppressed in Flt-1($TK^{-/-}$) [89].

Several recent studies have emphasized the effects of VEGFR-1 in hematopoiesis and recruitment of endothelial progenitors. Hattori et al. have shown that VEGFR-1 activation by PlGF is able to reconstitute hematopoiesis by recruiting VEGFR-1^{+} HSC [91]. In addition, Gerber et al. have shown that VEGFR-1 activation by enforced expression of PlGF rescues survival and ability to repopulate in *VEGF*$^{-/-}$ HSC [41]. Furthermore, Luttun et al. have shown that delivery of PlGF promotes collateral vessel growth in a model of myocardial ischemia through the recruitment of bone marrow-derived cells, such as monocytes [92].

LeCouter et al. provided evidence for a novel function of VEGFR-1 in liver sinusoidal endothelial cells (LSEC). VEGFR-1 activation achieved with a receptor-selective VEGF mutant resulted in the paracrine release of HGF, Il-6 and other hepatotrophic molecules by LSEC, such that hepatocytes were stimulated to proliferate when co-coltured with LSECs [93]. Such a mechanism was sufficient to protect the liver from CCl4-induced damage, in spite of the inability of the VEGFR-1 agonist to induce LSEC proliferation, both *in vitro* and *in vivo*. These findings suggest that a key function of VEGFR-1 signaling in the vascular endothelium is not the regulation of angiogenesis but, rather, the paracrine release of tissue-specific growth/survival factors, possibly in a vascular bed-specific fashion [93]

VEGFR-2 (KDR/Flk-1)

KDR (kinase domain receptor) binds VEGF with lower affinity than VEGFR-1, with K_d of approximately 75–125 pM [94]. The key role of this receptor in developmental hematopoiesis and angiogenesis is evidenced by lack of vasculogenesis and failure to develop blood islands and organized blood vessels in null mice, resulting in death *in utero* between day 8.5 and 9.5 [95]. VEGFR-2 has been identified on a subset of multi-potent human HSCs [96]. There is now general agreement that VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF.

The binding site for VEGF has been mapped to the second and third Ig-like domains [97]. VEGFR-2 undergoes dimerization and strong ligand-dependent tyrosine phosphorylation in intact cells and results in a mitogenic, chemotactic and and pro-survival signal. Several tyrosine residues have been shown to be phosphorylated (for review see [18]). Also, VEGF has been shown to induce the phosphorylation of at least 11 proteins in bovine aortic endothelial cells [98]. Among these, VEGF induces phosphorylation of PLCγ, PI3-kinase, ras GTPase activating protein (GAP) [98], src family [33] and several other signal transduction molecules [18]. VEGF induces endothelial cell growth by activating the Raf-Mek-Erk pathway. Compared to other growth factor systems, an unusual feature of VEGFR-2 activation of this pathway is the requirement

for PKC but not ras [99, 100]. VEGF mutants which bind selectively to VEGFR-2 are fully active endothelial cell mitogens and permeability-enhancing agents, whereas mutants specific for VEGFR-1 are devoid of both activities [101]. Furthermore, VEGFR-2, but not VEGFR-1, activation has been shown to be required for the anti-apoptotic effects of VEGF for HUVE cells [22]. As previously noted, such a pro-survival effect of VEGF is mediated by the PI3 kinase/Akt pathway [22]. Recent studies suggest, however, that at least in some circumstances, VEGFR-1 may transmit a pro-survival signal in endothelial cells, possibly mediated by induction of the anti-apoptotic gene survivin [102].

Neuropilin (NP1 and NP2)

Earlier studies indicated that certain tumor and endothelial cells express cell surface VEGF binding sites distinct in affinity and molecular mass from the two known VEGF RTKs [103]. Interestingly, $VEGF_{121}$ failed to bind these sites, indicating that exon-7 encoded basic sequences are required for binding to this putative receptor [103]. Subsequently, Soker et al. [104] identified such isoform-specific VEGF receptor as NP1, a molecule that had been previously shown to bind the collapsin/semaphorin family and was implicated in neuronal guidance (for review see [105]. When co-expressed in cells with VEGFR-2, NP1 enhanced the binding of $VEGF_{165}$ to VEGFR-2 and $VEGF_{165}$-mediated chemotaxis [104]. It has been proposed that NP1 presents $VEGF_{165}$ to the VEGFR-2 in a manner that enhances the effectiveness of VEGFR-2-mediated signal transduction [104]. Fuh et al. have shown that NP1 is able to directly bind VEGFR-1, suggesting that one of the mechanisms by which this receptor functions as a negative regulator of VEGF activity is competing for NP-1 binding [106]. Importantly, binding to NP1 may contribute to explain the greater mitogenic potency of $VEGF_{165}$ relative to $VEGF_{121}$. So far there is no evidence that NP1 or the related NP2 signals following VEGF binding [105]. In contrast, in response to semaphorin binding, NP1 and NP2 mediate a signal that results in axon repulsion. Recent evidence indicates that the formation of complexes with plexins is a requirement for NP signaling in neurons [105]. The role of NP1 in the development of the vascular system has been demonstrated by gene targeting studies, documenting embryonic lethality in null mice [107]. Furthermore, Lee et al. have shown that, in the zebrafish, NP1 is required for vascular development and mediates VEGF-dependent angiogenesis [108]. Interestingly, recent studies have linked NP2 to lymphatic vessel development [109].

Role of VEGF in physiological angiogenesis

Embryonic and early postnatal development

In 1996, two studies demonstrated an essential role played by VEGF in embryonic vasculogenesis and angiogenesis [110, 111]. Inactivation of a single VEGF allele in mice resulted in embryonic lethality between day 11 and 12. The *VEGF*$^{+/-}$ embryos exhibited a number of developmental anomalies, defective vascularization in several organs and a markedly reduced number of nucleated red blood cells within the blood islands in the yolk sac. In contrast, inactivation of PlGF [83] or VEGF-B genes did not result in any obvious development abnormalities, although, VEGF-B inactivation in mice results in reduced heart size and impaired recovery from experimentally induced myocardial ischemia [112].

To determine the role of VEGF in early postnatal life different strategies have been employed [25]. Partial inhibition of VEGF achieved by Cre-*LoxP*-mediated gene targeting resulted in increased mortality, stunted body growth and impaired organ development, most notably of liver and kidney. Administration of mFlt (1–3)-IgG, which achieves a higher degree of VEGF inhibition, resulted in nearly complete growth arrest when the treatment was initiated at day 1 or day 8 postnatally. Such treatment was also accompanied by rapid lethality, primarily due to kidney failure [25]. The pivotal role of VEGF in kidney development is also demonstrated by very recent studies showing that selective VEGF deletion in podocytes leads to glomerular disease in a gene dosage-dependent fashion [113]. Heterozygous mice developed renal disease by 2.5 weeks of age, characterized by proteinuria and endotheliosis. Homozygosity resulted in perinatal lethality [113]

Skeletal growth and endochondral bone formation

Endochondral bone formation is a fundamental mechanism for longitudinal bone growth during vertebrate development [114]. Cartilage, an avascular tissue, is replaced by bone in a process named endochondral ossification. VEGF mRNA is expressed by hypertrophic chondrocytes in the epiphyseal growth plate, suggesting that a VEGF gradient is needed for the correct directional growth and cartilage invasion by metaphyseal blood vessels [115]. Following VEGF blockade with a soluble VEGFR-1 chimeric protein, blood vessel invasion is almost completely suppressed, concomitant with impaired trabecular bone formation [115]. Although proliferation, differentiation and maturation of chondrocytes were apparently normal, resorption of hypertrophic chondrocytes was inhibited, resulting in a marked expansion of the hypertrophic chondrocyte zone. These findings indicate that VEGF-dependent blood vessel recruitment is essential for coupling cartilage resorption with bone formation. importantly, cessation of the anti-VEGF treatment is followed by capillary

invasion, restoration of bone growth, resorption of the hypertrophic cartilage and normalization of the growth plate architecture. A similar, although less dramatic phenotype was obtained, when VEGF was deleted in the cartilage of developing mice by means of Cre-*LoxP*- mediated, tissue specific gene ablation [116]. Furthermore, examination of $VEGF^{120/120}$ mice revealed not only a delayed recruitment of blood vessels into the perichondrium but also show delayed invasion of vessels into the primary ossification center, demonstrating a significant role of heparin-binding VEGF isoforms at both an early and last stage of cartilage vascularization [117].

Ovarian angiogenesis

Angiogenesis is a key aspect of normal cyclical ovarian function. Follicular growth and the development of the corpus luteum (CL) are dependent on the proliferation of new capillary vessels [118]. The process of selection of a dominant follicle in monovular species has been also associated with angiogenesis, as the selected follicles possess a more elaborate microvascular network than other follicles [119]. Subsequently, the blood vessels regress, suggesting the coordinated action of inducers and inhibitors of angiogenesis in the course of the ovarian cycle [120]. Previous studies have shown that the VEGF mRNA expression is temporally and spatially related to the proliferation of blood vessels in the ovary, suggesting that VEGF may be a mediator of the cyclical growth of blood vessels which occurs in the female reproductive tract [121, 122]. Administration of VEGF inhibitors suppresses luteal angiogenesis [123, 124] and delays follicular development [125] in rodents and primates. More recent studies have indicated that EG-VEGF, a novel selective angiogenic factor, plays a cooperative role with VEGF in the regulation of angiogenesis in the human ovary [126]. EG-VEGF is not structurally related to VEGF but belongs to a unique gene family [127]. A sequential activation of the two genes occurs in the CL [128]. While VEGF is strongly expressed in early stage CL, coincident with the initial development of a capillary plexus, its expression is markedly reduced by mid-luteal phase. In contrast, EG-VEGF starts being expressed later than VEGF but persists throughout mid- and early-late luteal phase. Therefore, EG-VEGF may be especially important for the formation of a more mature vascular bed that includes arterioles and thus for the persistence and adequacy of luteal function.

Role of VEGF in pathologic conditions

Solid tumors

In situ hybridization studies have demonstrated that the VEGF mRNA is markedly up-regulated in the vast majority of human tumors so far examined

(for review see [6]. In 1993 Kim et al. reported that anti-VEGF blocking antibodies exert a potent inhibitory effect on the growth of several tumor cell lines in nude mice [129]. Subsequently, many other tumor cell lines were found to be inhibited *in vivo* by this as well as other anti-VEGF treatments, including small molecule inhibitors of VEGFR signaling, antisense oligonucleotides and anti-VEGFR-2 antibodies (for review see [6]). While these studies have shown that tumor cells represent the major source of VEGF, recent studies have indicated that tumor-associated stroma is also an important site of VEGF production and complete VEGF blockade requires suppression of both compartments [130, 131]. Recently, Cre-LoxP-mediated gene targeting has been used to show that VEGF inactivation suppresses tumour angiogenesis in the Rip-Tag model, a well-established genetic model of insulinoma [132]. Furthermore, at least in the Rip-Tag model, matrix metalloproteinase 9-mediated proteolytic events have been shown to result in enhancement of the activity of low, constitutive, levels of VEGF, by making it available to bind VEGFR-2, thus triggering the angiogenic switch [133].

Several studies have shown that combining anti-VEGF with chemotherapy [134] or radiation therapy [135] results in a greater anti-tumor effects that either treatment alone. Clinical trials in cancer patients are ongoing with several VEGF inhibitors, including a humanized anti-VEGF monoclonal antibody (rhuMab VEGF) [136], an anti-KDR antibody [137] and various small molecules inhibiting KDR signal transduction [138, 139]. Phase II clinical data have provided initial evidence that rhuMab VEGF, in combination with conventional chemotherapy, results in increase in time to progression and even survival in patients with metastatic colorectal carcinoma [140]. Furthermore, a double blind placebo-controlled phase II trial has shown a significant increase in time to progression in renal cell carcinoma patients treated with an rhuMab VEGF as a single agent [168]. In light of the fact that many renal cell carcinoma patients harbor mutations in the VHL gene, which result in altered regulation of VEGF, this finding is particularly intriguing. Phase III studies are currently underway to confirm and fully assess the benefit of these anti-VEGF treatments in patients with advanced cancer. Most recently, Hurwitz et al. [169] have presented the resulted of a large randomized placebo-controlled phase III in metastatic colorectal cancer. Survival was significantly increased in patients in the chemotherapy (irinotecan, 5-fluorouracil, leucovorin) plus rhuMab VEGF arm relative to chemotherapy alone. Interestingly, the increased incidence of thrombosis and proteinuria which was observed in phase II was not observed in this phase III study. Thus, the prolongation of survival and improvement in other markers of clinical benefit observed with the addition of rhuMab VEGF to standard chemotherapy confirms the importance of angiogenesis in the clinical outcome of patients with colorectal cancer.

Hematologic malignancies

VEGF is expressed in a variety of cell lines derived from various hematologic malignancies, including T-cell lymphoma, acute lymphoblastic leukemia, Burkitt's lymphoma, acute lymphocytic leukemia, histiocytic lumphoma, promyelocytic leukemia, chronic myelocytic leukemia, plasma cell leukemia and multiple myeloma (for review see [141]). Expression of both VEGF receptors has been detected in some, but not all, leukemia cell lines and VEGFR-1 was found to be more frequently expressed than VEGFR-2. These findings suggest that the production of VEGF by malignant myeloid precursors might serve both as an autocrine growth stimulus and a diffusible, paracrine, signal mediating angiogenesis within the bone marrow. Several reports have documented inhibitory effects by small molecule inhibitors targeting VEGFR-1 and VEGFR-2 on the growth of human myeloid leukemia cell line and in acute myeloid leukemia blasts [142, 143]. Further evidence for a functional role of VEGFR-2 in leukemic cell growth was provided by experiments showing that an anti-VEGFR-2 antibody inhibits proliferation of xenotransplanted human leukemia cells and significantly increased survival of nude mice [144]. Taken together, these findings suggest that inhibition of VEGF or VEGFR signaling may be effective in the treatment of hematological malignancies and currently several clinical trials are testing this hypothesis.

Intraocular neovascular syndromes

Diabetes mellitus, occlusion of central retinal vein or prematurity with subsequent exposure to oxygen can all be associated with intraocular neovascularization, which may result in vitreous hemorrhages, retinal detachment, neovascular glucoma and blindness [145]. All of these conditions are known to be associated with retinal ischemia [146]. Elevations of VEGF levels in the aqueous and vitreous of eyes with proliferative retinopathy secondary to diabetes and other conditions have been previously described [147–149]. Subsequent animal studies, using various VEGF inhibitors, have directly demonstrated the role of VEGF as a mediator of ischemia-induced intraocular neovascularization [150, 151].

Neovascularization and vascular leakage are a major cause of visual loss also in age-related macular degeneration (AMD), the overall leading cause of blindness [145]. Earlier studies have demonstrated the immunohistochemical localization of VEGF in surgically resected choroidal neovascular membranes from AMD patients [152], suggesting a role for VEGF in the progression of AMD-related choroidal neovascularization. Currently, anti-VEGF strategies are being explored in clinical trials in AMD patients, using either a recombinant humanized anti-VEGF Fab (rhuFab VEGF) [153] or 2'-Fluoropyrimidine RNA oligonucleotide ligand (aptamers) [154]. rhuFab VEGF has been recently found to reduce angiogenesis and vascular leakage

in a primate model of AMD [155]. Both the aptamer and rhuFab VEGF are currently in phase III trials.

Inflammation and brain edema

Several studies have suggested that VEGF is implicated in various inflammatiory disorders (for review see [27]). VEGF is strongly expressed by epidermal keratinocytes in wound healing and psoriasis, conditions that are characterized by increased microvascular permeability and angiogenesis [156]. Transgenic overexpression of VEGF in the skin, driven by the keratin 14 promoter, led to increased density of tortuous cutaneous blood capillaries. Highly increased leukocyte rolling and adhesion in postcapillary skin venules were noted. These studies suggest that chronic orthotopic overexpression of VEGF in the epidermis is sufficient to induce cardinal features of chronic skin inflammation. Interesting, no changes in lymphatic vessels were detected in this model [157]. Very recent studies have shown, however, that myeloid cell activation and infiltration, a key aspect of acute inflammatory responses, requires HIF-1α but is largely independent on VEGF [158].

VEGF up-regulation has been implicetd the development of brain edema. As previously noted, hypoxia is a major trigger for VEGF expression and enhanced levels of VEGF together with its receptors, Flt-1 and Flk-1/KDR, have been reported by several groups in the rat brain following the induction of focal cerebral ischemia [159]. van Bruggen et al. have tested the hypothesis that VEGF antagonism achieved by systemic administration of a soluble VEGFR-1 chimeric protein may have beneficial effects in a murine model of cortical ischemia [160]. A significant reduction in the volume of the edematous tissue was observed shortly after the onset of ischemia and the infarct size measured several weeks later revealed a significant sparing of cortical tissue in the treated group. As previously, noted, it has been proposed that members of the Scr family mediates VEGF-dependent vascular permability [33]. Paul et al. have reported that $Src^{-/-}$ mice have a reduced brain damage following induction of cortical ischemai. A Src inhibitor was also shown to have protective effect in a stroke model [161].

Pathology of the female reproductive tract

Hyperplasia and hypervascularity are features of the polycystic ovary syndrome, a leading cause of infertility. Recent studies suggest that VEGF and EG-VEGF may cooperate in the induction of angiogenesis in this condition [128]. Angiogenesis is also important in the pathogenesis of endometriosis, a condition characterized by ectopic endometrium implants in the peritoneal cavity. High levels of VEGF have been measured in the peritoneal fluid of patients with endometriosis [162, 163].

Therapeutic implications and perspectives

There is now little doubt that the VEGF family plays an essential role in the regulation of embryonic and postnatal physiologic angiogenesis processes, such as normal growth processes [25, 115] and cyclical ovarian function [123]. Furthermore, VEGF inhibition have been shown to inhibit pathological angiogenesis in a wide variety of models, including genetic models of cancer, leading to the clinical development of a variety of VEGF inhibitors. Clearly, a major question is what impact VEGF inhibition will have in human patients, especially those with highly advanced malignancies. This question will be answered by the several phase III clinical trials that are currently underway, targeting colorectal, lung and renal cell carcinomas. Initial results indicate that there is at least some reason for optimism. As mentioned, phase II clinical trials with VEGF inhibitors have provided evidence of clinical efficacy [140]. Most recently, however, a large phase III study has provided clear evidence that VEGF inhibition provides a clinical benefit. However, progression eventually occurs in many patients, raising the issue that pathways mediating angiogenic escape after VEGF inhibition exist. Different angiogenic mechanisms might be differentially important at various stages of the neoplastic progression. However, progression eventually occurs in many patients, raising the issue that pathways mediating angiogenic escape after VEGF inhibition exist. Different angiogenic mechanisms might be differentially important at various stages of the neoplastic progression and several evidences indicate that VEGF may be especially critical in the initial stages [164]. Such a notion may be crucial for the design of further clinical trials. Furthermore, the identification of reliable markers that can predict which patients are more likely to respond to anti-VEGF therapy (or other anti-angiogenic treatments) is of utmost importance.

The potential clinical utility of VEGF inhibition is not limited to cancer. Trials in AMD patients are already in phase III. Furthermore, as already noted, gynecologic conditions such as endometriosis or the polycystic ovary syndrome might benefit from this treatment.

The ability of VEGF and other angiogenic factors to promote collateral vessel growth in various animals model of ischemia [165] generated a large amount of enthusiasm and led to several clinical trials in patients with coronary or limb ischemia [4]. So far, the clinical results with VEGF (or bFGF) have been either inconclusive, due to the small sample size, or frankly disappointing [36]. As previously discussed, several factors may account for the discrepancy between the remarkable efficacy of both molecules in various animal models and the clinical results [4]. Currently, several laboratories are exploring the possibility that a more persistent exposure than that achieved in the earlier clinical trials, when only bolus or brief infusions were administered [36], may achieve better results. In this context, recent studies using a conditional VEGF switching have shown that early cessation of the VEGF stimulus results in regression of newly formed vessels in the heart or in the liver. However,

after a critical duration of exposure, the vessels persisted for months after VEGF withdrawal and resulted in an improvement in organ perfusion [166]. Also, a greater understanding of the differential role of the VEGF receptors may open additional avenues. In particular, recent studies have emphasized that VEGFR-1, a molecule with highly complex and apparently conflicting roles, possesses important activities in hematopoiesis and in the recruitment of mononuclear cells. The fact that VEGFR-1 activation is associated with fewer side effects relative to VEGF makes it a particularly attractive target. Furthermore, the recent report that VEGFR-1 activation may protect the liver from toxic damage, by instructing the quiescent endothelium to produce a series of tissue-specific growth factors extends the potential clinical applications of VEGFR-1 agonists [93].

Other activities of VEGF may have interesting clinical implications. For example, on the basis of the key role played by VEGF in bone angiogenesis and endochondral bone formation, it is conceivable that application of this factor may be useful to enhance revascularization in orthopedic conditions like non-healing fractures. A recent study has shown that VEGF administration leads to enhanced blood vessel formation and ossification in models of bone damage [167].

These findings raise hope that a return to human trials with a better molecular and biological understanding of blood vessel growth and differentiation may be more rewarding than the early attempts.

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Mechanisms of Angiogenesis
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The anti-inflammatory actions of angiopoietin-1

Gavin Thurston, John S. Rudge, Ella Ioffe, Nicholas Papadopoulos, Christopher Daly, Srilatha Vuthoori, Thomas Daly, Stanley J. Wiegand, and George D. Yancopoulos

Regeneron Pharmaceuticals, 777 Old Saw Mill River Road, Tarrytown, NY 10591, USA

Introduction

Endothelial cells are central players in inflammation. Because the endothelium is the primary structure to maintain fluid and macromolecules in the bloodstream, it is a key regulator of the plasma leakage that is one of the hallmarks of inflammation. In addition, endothelial cells upregulate adhesion proteins that participate in leukocyte adhesion and extravasation during inflammation. Pharmacologic agents that specifically limit the endothelial cell responses in inflammation might be useful for treating inflammatory diseases such as acute lung injury, rheumatoid arthritis, and psoriasis. However, identifying the molecular pathways in endothelial cells that can specifically suppress their involvement in inflammation has been a challenge.

Angiopoietin-1 (Ang1) is a ligand for the endothelial-specific tyrosine kinase receptor Tie2. Ang1 is necessary for blood vessel development, as revealed by genetic experiments in which deletion of the angiopoietin-1 gene in mice results in embryonic lethality and produces a cardiovascular phenotype similar to the deletion of Tie2 gene. The function of Ang1, as gleaned from these genetic experiments, seems to be to promote vessel maturation during the post-sprouting phase of blood vessel development. As such, Ang1 has distinct and complementary actions to those of the prototypic angiogenic factor VEGF.

During the study of transgenic mice that overexpress Ang1, we found that Ang1 appears to make the vasculature less leaky in response to some inflammatory mediators. This observation was followed by studies showing that Ang1 given to adult mice can also make blood vessels less leaky in response to inflammatory stimuli. Since that time, exogenous addition of Ang1 has been tested in various models of inflammation *in vivo* and *in vitro*. As used to date, *Ang1* has shown anti-leakage or anti-inflammatory effects in a variety of models of inflammation, but not in all models. The reasons for the seemingly different responses to Ang1 are not understood, nor are the mechanisms of the anti-leak action. In this chapter, we summarize studies on the anti-leak and anti-inflammatory actions of Ang1, and point to directions needed to under-

stand the mechanism of action. We have focused on models where Ang1 has been added exogenously to manipulate components of the inflammation, and do not discuss many other studies in which endogenous levels of Angiopetin/Tie2 were measured.

Angiopoietin-1: Properties, signaling, and protein structure

Tie2 roamed as an orphan receptor for several years, until Davis and co-workers, using a novel secretion-trap cloning approach [1], identified a secreted protein that bound to Tie2. Angiopoietin-1 was found to bind to Tie2 receptor, but not to a very similar receptor Tie1, and to induce Tie2 phosphorylation. Interestingly, and unlike VEGF, Ang1 does not directly induce endothelial cell proliferation in culture. Ang1 consists of a fibrinogen-like domain that binds to Tie2, a coiled-coil domain that serves to dimerize angiopoietin monomers, and an N-terminal domain that helps form higher order oligomers that are necessary for Tie2 activation [1–3]. Studies with recombinant forms of Ang1 have shown that tetrameric forms are necessary for activation of Tie2 in endothelial cells, whereas Ang1 dimers appear to be sufficient for activation of ectopically expressed Tie2 [2].

Angiopoietin-1 is expressed by myocardium in early development and by perivascular cells later in development and in adult tissue [1]. In general, Ang1 is expressed constitutively and does not appear to be strongly increased or decreased in inflammation, although some studies have found decreased expression of Ang1 [4]. Of potential interest, high concentrations of Ang1 are found in platelets, likely in the α-granules [5, 6] (Papadopoulos et al., unpublished results). Angiopoietin-1 mediates its effects by inducing autophosphorylation of the Tie2 tyrosine kinase receptor, which results in binding of several signaling proteins to phosphorylated tyrosine residues within the Tie2 cytoplasmic domain [7–9]. Perhaps most important among the signaling pathways that are subsequently activated is the PI-3 kinase/Akt pathway, which appears to provide the cell survival action of Ang1-Tie2 interaction [10–13]. The caspase inhibitor survivin has been implicated as a downstream effector [11, 12].

Other Angiopoietins (Ang2, Ang3, and Ang4) were subsequently found by homology [14, 15]. These angiopoietins also bind to Tie2. Angiopoietin-2, 3, 4 have similar structural and oligomerization properties as Ang1 [2], although Ang2 and Ang4 do not obligately act as phosphorylating agonists for Tie2. The anti-inflammatory actions of these other angiopoietins have not been fully explored.

Gene targeted mice: Tie2, Ang1, and vessel maturation

Initial evidence for the functions of Angiopoietins and Tie receptors came from gene-targeted mice. Genetic deletion of the *Tie2* gene results in embryonic

lethality at approximately embryonic day 10.5 (E10.5), associated with cardiac failure, hemorrhage, and other vascular defects. The initial phases of angiogenesis and blood vessel formation, including sprouting, are able to proceed. The heart and major blood vessels of the embryo are able to form, but the heart fails to undergo normal development and trabeculation [16, 17]. In addition, blood vessels throughout the embryo apparently do not remodel or form normal hierarchical networks. Furthermore, the aortas of E9.5 *Tie2* null mice have fewer endothelial cells [16] and a profound lack of smooth muscle cells, and those smooth muscle cells that are present appear to be tenuously attached to the blood vessels.

Mice genetically deficient for Ang1 have a similar phenotype as Tie2 deficient mice. Gene targeting of Ang1 results in embryonic lethality by E12.5 [18], with defects in heart and vascular development. In the hearts of Ang1 deficient mice, the ventricular trabeculae are poorly developed and the endocardium is tenuously attached to the underlying matrix. In addition, Ang1 deficient mice have a poorly formed vascular network, with less complex vascular trees and poor organization into hierarchical networks. By electron microscopy, endothelial cells in Ang1 deficient mice appear to be poorly associated with the basement membrane and with underlying perivascular cells [18]. Although the phenotype of the two knockout mice is very similar, Ang1 deficient mice die 1 to 2 days later than those lacking Tie2, which may be due to compensation by other Angiopoietins.

The conclusion from the gene deletion studies is that the Ang1/Tie2 system plays a key role in the normal development of the heart and vascular systems. Ang1 produced by cardiac cells and perivascular cells appears to promote the cell-cell interactions that takes place between perivascular cells and endothelial cells during vessel maturation, or, alternatively, it may provide a survival signal to endocardial cells and endothelial cells. Indeed, these two roles may be two faces of the same coin, particularly in the immature cardiovascular system of early development.

Transgenic overexpression of Ang1: Vessel morphology and leakage

In an effort to further elucidate the role of Ang1 in blood vessel function, mice that overexpress *Ang1* under the skin-specific *K14* promoter [19] were produced. These mice are notably reddened in appearance, particularly in the ears, snout and paws. The reddening is associated with a dramatic increase in the diameter of the dermal blood vessels compared to those in control mice. The enlarged vessels are in the position of capillaries subjacent to the epidermis and surrounding the hair follicles, and these vessels contain an increased number of endothelial cells [20]. The epidermis and dermis appear otherwise normal.

While studying the function of the skin blood vessels in the *K14-Ang1* mice, it was learned that the vessels are not leaky under baseline conditions,

and, surprisingly, are resistant to plasma leakage induced by inflammatory mediators such as histamine, serotonin, and mustard oil [20]. When these inflammatory mediators are applied to the ear skin of normal mice, Evans blue tracer leaks readily from the blood vessels. In contrast, when the agents are applied to the skin of *K14-Ang1* mice, very little leakage occurs. The route of plasma leakage induced by mustard oil is via increased numbers of endothelial cell gaps, which are openings up to 1 μm in diameter between the junctions of adjacent endothelial cells. *K14-Ang1* mice had decreased numbers of endothelial cell gaps compared to wild-type mice following treatment with mustard oil [20].

Transgenic overexpression of Ang1 and VEGF: vessel morphology and leakage

Mice that overexpress VEGF-A in the skin, under either *K14* or *K5* promoters [20–22], emphasize the dual pro-inflammatory and angiogenic aspects of VEGF, and serve to underscore the differences between Ang1 and VEGF. In one line of *K14-VEGF*$_{164}$ transgenic mice [20], the mice has mild reddening of the ear skin and snout. The dermis of these *K14-VEGF* mice has an increased density of blood vessels, evident by increased numbers of tortuous capillary-sized vessels near the epidermis and surrounding the hair follicles. These tortuous vessels leak the plasma tracer Evans blue under baseline conditions [20]. In addition, the basement membrane of these vessels can be labeled by intravascular perfusion of Ricin lectin, indicating defects in the endothelial barrier. Applying inflammatory stimuli to the ear skin of *K14-VEGF* mice results in even larger amounts of plasma leakage. The epidermis of the *K14-VEGF* mice is thickened under baseline conditions, and the dermis contains infiltrating leukocytes. As the mice age, red lesions appear in the ear skin [20, 23].

To test the effects of combined expression of VEGF and Ang1, *K14-Ang1* mice were bred to *K14-VEGF* mice [20] to produce double transgenic *K14-Ang1/VEGF* mice. The skin of the resultant double transgenic mice is dramatically reddened, and the vascularity of the skin is higher than that of either *K14-VEGF* or *K14-Ang1* mice. The morphology of the dermal vessels appears to be a combination of the *Ang1* and VEGF effects. In particular, numerous small vessels and enlarged vessels are both present [20]. The dermis of young adult *K14-Ang1/VEGF* mice is normal in thickness and does not contain infiltrating leukocytes. Furthermore, the skin vessels in *K14-Ang1/VEGF* mice are not leaky to Evans blue or Ricin lectin under baseline conditions [20]. Thus, transgenic expression of Ang1 seems to inhibit much of the leakage and inflammatory actions of transgenic VEGF; however, Ang1 and VEGF appear to act on distinct signaling pathways for vessel growth.

Extending anti-leak action of Ang1 to non-transgenic mice: adenoviruses and proteins

In an effort to extend the findings of *K14-Ang1* mice into a non-transgenic setting, we developed adenoviruses encoding a recombinant form of *Ang1* [24]. Normal adult mice injected iv with adeno-*Ang1* appear healthy and remained active. Similar to the *K14-Ang1* mice, the blood vessels in the skin of mice given adeno-*Ang1* become resistant to the plasma leakage normally induced by local injection of VEGF or topical application of mustard oil [24]. The resistance to leakage develops by 1 day after iv injection of adenovirus. However, unlike the *K14-Ang1* mice, adult mice given adeno-*Ang1* do not appear reddened, and the morphology of the skin blood vessels is normal for at least 7 days after adenoviral injection [24]. Subsequent experiments have shown that the anti-leakage action can also be produced by intraperitoneal injection of different recombinant Angiopoetin-1 proteins (E. Ioffe, G. Thurston et al., unpublished results), thus this effect is not due to the adenoviral production of Ang1. Thus, systemic administration of Ang1 to adult mice can induce anti-leakage effects, showing that Ang1 can induce responses in seemingly mature blood vessels.

In vitro studies on Ang1 and inflammation: pathways and mechanisms

The effects of Ang1 on the barrier properties of endothelial cells in culture have been tested, using human umbilical vein endothelial cells (HUVEC) and human lung microvascular endothelial cells [25, 26]. Endothelial cell monolayers exposed to thrombin or VEGF become less effective as a barrier to macromolecular solutes and tracers. However, co-treatment of endothelial monolayers with Ang1 significantly reduces the thrombin-induced and VEGF-induced decrease in the endothelial cell barrier [25, 26]. Interestingly, treatment with Ang1 alone seems to tighten the baseline barrier of endothelial cell monolayers [25]. In addition, Ang1 reduces the TNF-α induced transmigration of neutrophils across endothelial cell monolayers, and the thrombin-induced adhesion of PMNs. Pretreatment with Ang1 also suppresses the thrombin-induced production of the cytokine IL-8 [26]. In one study, the authors link the anti-leak action of Ang1 to the endothelial cell-cell junctions, and conclude that by this mechanism Ang1 may exert a major action on the integrity of blood vessels [25].

Angiopietin-1 has been reported to suppress the upregulation of cell surface activation proteins in endothelial cells by pro-inflammatory mediators such as VEGF and INF-α [27, 28]. In one set of studies, treatment of HUVEC with Ang1 suppressed the VEGF-mediated induction of the leukocyte adhesion molecules ICAM-1, VCAM-1, and E-selectin [27]. This suppression of the adhesion molecules by Ang1 is associated with decreased adhesion of leukocytes to HUVEC monolayers. In another study, treatment with Ang1 suppressed the VEGF- and TNF-α mediated induction of the pro-coagulant mol-

ecule tissue factor [28]. In both studies, the suppressive actions of Ang1 are reportedly through Tie2-induced activation of the PI-3 kinase/Akt pathway.

Is Ang1 a general anti-inflammatory agent? Tests in other models

Following the studies on the anti-inflammatory actions of Ang1 in simple model systems, various workers sought to extend the findings to more complex and relevant disease models that involve plasma leakage. One group investigated the effects of Ang1 on the retinal vasculature in rat and mouse models of diabetes [29]. Diabetic retinopathy is often associated with plasma leakage from the retinal microvessels, and may also be associated with endothelial cell activation. The group induced diabetes in mice and rats by treatment with streptozotocin, and the animals were treated by either intraocular injections of Ang1 protein, or systemic iv injection of adeno-Ang1. Intraocular Ang1 treatment reduced the amount of leakage from the retinal vessels in diabetic rats, as well as reducing expression of the inflammatory adhesion molecule ICAM-1 and reducing leukocyte adhesion to the endothelium of retinal vessels [29]. Ang1 treatment also seemed to reduce endothelial cell damage in the retinal vessels. Similarly, systemic treatment of diabetic mice using adeno-Ang1 resulted in decreased numbers of adherent leukocytes and decreased endothelial cell damage. The authors conclude that Ang1 may be useful to protect the retinal vasculature from the pathological complications of diabetes [29].

Another group investigated the effect of Ang1 in a model of tissue transplantation. Normally, arteriosclerosis develops in cardiac allografts, associated with chronic graft rejection. In a recent study, heterotypic cardiac syngrafts and allografts were performed in rats [30]. The process of graft rejection was associated with increased immunoreactivity for Tie2 and Ang2, but not Ang1. Adeno-Ang1 infused into the heart vasculature decreased fibrosis in the graft, and showed a tendency to improve graft survival. In addition, Ang1 decreased the incidence of arteries with arteriosclerotic lesions and occluded vessels [30]. Significantly, Ang1 caused a reduction in the number of infiltrating leukocytes, particularly CD4+ T lymphocytes and macrophages. The authors suggest that the anti-inflammatory action of Ang1 may provide a therapeutic approach to reduce complications associated with transplant rejection [30].

In acute myocardial infarction, providing Ang1 to the region near the infarct may reduce the size of the infarct tissue. In a study of a rat model of acute myocardial infarction [31], Ang1 was delivered to the region of the infarct by gene therapy with adenoviruses. Adeno-Ang1, but not control adenovirus, reduced the infarct size, increased the density of capillaries, and improved cardiac performance [31]. It is not clear in this study whether the exogenous Ang1, in conjunction with the endogenously high VEGF, provided a pro-angiogenic signal and thereby improved cardiac performance, or whether it acted as an anti-inflammatory agent.

Ang1 is not effective in some models of inflammation

Other studies of disease models have failed to show anti-leakage or anti-inflammatory effects of Ang1. Lung edema is a serious clinical problem in several clinical settings including sepsis and ventilator-induced lung injury. The effects of Angiopoietin-1 have been tested in several *in vivo* models of acute lung injury. In one set of experiments, mice were exposed to hyperoxia for 3 days following administration of adenovirus encoding angiopoietin-1 or control reagent (S. Albelda, M. Christofidou-Solomidou et al., unpublished data). The mice given Adeno-Ang1 and hyperoxia had the same levels of protein in their bronchiolar lavage fluid (BAL) as the control group given hyperoxia alone. Similarly, Ang1 did not seem to reduce infiltrating neutrophils or subjective scores of acute lung injury (S. Albelda, M. Christofidou-Solomidou et al., unpublished data). Thus, Ang1 did not have a protective effect in this model.

Delayed type hypersensitivity (DTH) reaction is a local T cell dependent response to a sensitizing antigen. The elicitation phase of DTH response is characterized by inflammation and tissue injury associated with elevated cytokine production, vascular endothelial cell activation, leukocyte extravasation, and tissue edema. In an experimental mouse model of allergic contact dermatitis, cutaneous DTH response is induced by topical application of reactive haptens to the ear skin. The response can be evaluated by measuring ear swelling at various times after the elicitation. To analyze the effect of Ang1 on the inflammatory processes associated with DTH, contact dermatitis was induced in K14-Ang1 transgenic mice and in mice treated systemically with adenoviral or recombinant forms of Ang1 (E. Ioffe et al., unpublished data). In all experiments to date, transgenic or systemic Ang1 had no effect on the magnitude of leakage, as assessed by ear swelling. Thus, Ang1 does not appear to suppress vascular leakage associated with DTH inflammatory response.

Summary, perspectives, and future directions

Results from numerous labs over the past several years have shown that exogenously provided Angiopoietin-1 can have anti-leak or anti-inflammatory actions in various *in vivo* and *in vitro* experimental models (summarized in Tab. 1). The reported anti-inflammatory actions of Ang1 have included: suppression of leak, inhibition of leukocyte adhesion or endothelial cell expression of adhesion molecules, inhibition of leukocyte transmigration, and decreased graft *versus* host reaction. However, Ang1 has not been effective in all models; in this review we cite two studies in which Ang1 did not reduce inflammatory indices, and undoubtedly more negative studies are out there but have not been published. Thus, Ang1 appears to be able to inhibit various aspects of inflammation in particular settings, but does not appear to be a potent, general anti-inflammatory agent that can broadly suppress the many faces of inflammation. Ang1 is not a patentable dexamethasone!

Table 1. Models of inflammation in which the effects of exogenous Ang1 have been tested

Model of inflammation	Species	Method of Ang1 delivery	Form of Ang1 protein delivered	Endpoints	Ang1 effects	Reference
Acute skin inflammation (Mustard oil)	Mouse	Transgenic skin overexpression of Ang1	Ang1	Leakage in ear skin	Reduced leakage of Evans blue	Thurston et al., 1999 [20]
Acute skin inflammation (serotonin, other)	Mouse	Transgenic skin overexpression of Ang1	Ang1	Leakage	Reduced leakage	Thurston et al., 1999 [20]
Transgenic overexpression of VEGF in skin	Mouse	Transgenic skin overexpression of Ang1	Ang1	Leakage	Reduced leakage, and ear thickness	Thurston et al., 1999 [20]
Local injection of VEGF in skin	Mouse	Adenoviral systemic	Ang1*	Leakage	Reduced leakage	Thurston et al., 2000 [24]
Acute skin inflammation (Mustard oil)	Mouse, rat	Adenoviral systemic; protein systemic	Ang1*; Ang1FD4	Leakage	Reduced leakage	Thurston et al., 2000 [24]
Endothelial cell barrier in culture	Human	Protein to culture media	Ang1*	Flux of tracer	Decreased flux, baseline and after thrombin	Gamble et al., 2000 [25]
Endothelial cell activation in culture	Human	Protein to culture media	Ang1*	Expression of ICAM-1, VCAM-1, E-selectin, tissue factor	Decreased induction of ICAM-1 etc after VEGF, TNF-alpha	Kim et al. 2001, 2002 [27, 28]
Endothelial cell barrier and activation in culture	Human	Protein to culture media	Ang1	Flux of tracer, PMN adnesion, IL-8 production	Decreased flux and after thrombin, mixed effect on adhesion, decreased IL-8 production	Pizurki et al., 2003 [26]

(Continued on next page)

Table 1. (Continued)

Model of inflammation	Species	Method of Ang1 delivery	Form of Ang1 protein delivered	Endpoints	Ang1 effects	Reference
Diabetic (STZ) retinopathy	Mouse, rat	Local intraocular protein; adenoviral systemic	Ang1*	Retinal inflammation	Reduced leakage, ICAM1, and leukocyte sticking	Joussen et al., 2002 [29]
Cardiac transplant	Rat	Adenoviral local	Ang1	Histology, graft rejection, occluded arteries	Reduced fibrosis and infiltrating cells, increased graft acceptance, reduced occluded arteries	Nykanen et al., 2003 [30]
Myocardial infarct	Rat	Adenoviral local	Ang1	Capillary density, infarct size, echocardiography	Increased capillary density, reduced infarct size, improved cardiac performance	Takahashi et al., 2003 [31]
Hyperoxia	Mouse	Adenoviral systemic	Ang1*	Lung leakage, Evans blue tracer	No effect on leakage	S. Albelda et al., unpublished
DTH reaction in skin	Mouse	Adenoviral systemic; transgenic, protein systemic	Ang1*; Ang1; Ang1FD4	Ear skin swelling	No effect on ear thickness	E. Ioffe et al., unpublished

In the table, Ang1 refers to native mouse or human Ang1, Ang1* is a recombinant form of Ang1 that is easier to produce and purify and contains the fibrinogen domain from Ang1 and the coiled-coil domain of Ang2, and Ang1FD4 is a recombinant form of Ang1 that contains 4 fibrinogen domains of Ang1 linked to 2 Fc domains from human IgG [2]. These recombinant versions of Ang1 bind to and activate Tie2 receptor.

Endothelial cells play a major role in regulating the movement of fluid, macromolecules, and leukocytes across the vessel wall in normal physiology and in inflammation. As such, the endothelium is an attractive target for anti-inflammatory therapy. The mediators of inflammation use a diverse set of endothelial cell surface receptors and several key intracellular signaling pathways, including increased intracellular Ca^{2+} concentration and activation of NF-κB. More information is required on where these pathways may converge on the downstream effector molecules, and how other pathways, such as those activated by Ang1/Tie2, might interact with these activation pathways to suppress the inflammatory signal cascade in the endothelium. One possible explanation for the different effects of Ang1 in different models of inflammation is that Ang1 is only able to suppress inflammatory reactions that invoke a particular intracellular signaling pathway. Although the studies of Ang1/Tie2 signaling have proven to be complex [8], further studies with cultured endothelial cells and other readouts of inflammation may help to determine which inflammatory situations are most amenable to suppression by Ang1.

An alternative explanation for the different effects is that Ang1 can only suppress forms of inflammation in which the endothelial cells are essential active participants, whereas Ang1 may not effectively suppress forms in which leukocytes, or other cell types, have a driving role. For example, in the hyperoxia model cited above, the airway epithelium is the primary site of injury; these cells lack Tie2 receptor and are thus not likely to respond directly to Ang1 treatment. An extension of this idea, which is rather speculative, is that inflammation can assume forms that are either largely endothelial-dependent or endothelial-independent [32, 33], and Ang1 can significantly suppress only the former. More understanding of the actions of Ang1, or other agents that act specifically on the endothelium, may help determine whether two such forms are indeed distinguishable.

In all of the cases cited above, Ang1 was added exogenously. In many cases, Ang1 was given systemically or globally, thus acting in an endocrine manner on Tie2 receptors present on the luminal surface of the target endothelial cells. In contrast, in other cases cited above (e.g., skin transgenic mice), exogenous Ang1 acts in a paracrine manner on the abluminal surface of blood vessels near the cellular source. However, in contrast to these experimental manipulations, endogenous Ang1 secreted by perivascular cells acts in a closely paracrine manner on the abluminal surface of endothelial cells. It is not known whether the cellular source or the directionality of Ang1 are important for Ang1/Tie2 signaling, although these points are clearly worth keeping in mind, particularly as Ang1 may play a role in the cell-cell communication between endothelial cells and closely-associated perivascular cells.

Various different forms of Ang1 protein, including native Ang1 and multiple recombinant versions of Ang1, have produced an anti-leakage effect. In addition, various different delivery systems, including transgenic and adenoviral, have been used to provide increased amounts of Ang1 in inflammation

models. Thus, the anti-leak action of Ang1 does not seem to depend upon a particular form of Ang1 or a particular method for its delivery. However, because Ang1 is often not efficiently produced by transfected or transduced cells, and is also a difficult protein to purify in a non-aggregated form, it is important to critically evaluate whether Ang1 is being effectively provided in a given experimental model. Some of the variable results cited above may be due in part to different efficiencies of protein delivery.

Does endogenous Ang1 act to tighten blood vessels or to regulate the inflammation response? Inflammatory mediators such as histamine and serotonin can rapidly induce plasma leakage in a phenotypically specialized subset of the vessels, namely the postcapillary venules [34]. In addition, the inflammation-induced increase of leukocyte adhesion molecules such as P-selectin, ICAM-1, and E-selectin, also occurs specifically in the venules. Although many of the inflammatory mediators that induce inflammation in postcapillary venules have been identified, the factors that regulate endothelial cell phenotype, including those that specify the phenotype of the venular endothelium, are not understood. The Ang1/Tie2 system may play a role in specifying vessel phenotype by making vessels less able to respond to inflammatory mediators, or it may modulate the response of vessels that are specified by other mediators. To address these key issues, inhibitors of Ang1/Tie2 are required, particularly for use *in vivo*. Only then will we further unravel the role of Ang1 in normal vessel function.

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Mechanisms of Angiogenesis
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The hemostatic system in angiogenesis

Victor W.M. van Hinsbergh[1], Pieter Koolwijk[3] and Klaas Hoekman[2]

[1] *Laboratory for Physiology, and* [2]*Department of Clinical Oncology, VU University Medical Center, Van der Boechorststraat 7, 1081BT Amsterdam, The Netherland*
[3] *Department of Biomedical Research, Gaubius Laboratory TNO-PG, Leiden, The Netherlands*

Introduction

In the adult organism the vascular tree is fully developed and angiogenesis is limited to the endometrium and ovaries [1, 2]. The formation of new blood vessels becomes essential in adults during tissue repair after severe wounding or tissue damage by inflammation. Furthermore, angiogenesis is associated with a number of pathological conditions, such as cancer, diabetic retinopathy and rheumatoid arthritis [1–3]. Tumors largely use existing mechanisms of angiogenesis induction, which normally act during development or tissue repair. Many processes and factors that play a role in angiogenesis during the development of the embryo occur similarly in angiogenesis during tissue repair. In addition, the formation of new blood vessels in repair-associated and pathological angiogenesis is usually accompanied and influenced by vascular leakage; the deposition of fibrin and vitronectin in the clot matrix or fibrinous exudate, which acts as scaffolding for invading cells; the occurrence of inflammatory cells; and often platelet activation and the release of angiogenesis-modulating factors [4–9]. Thus the clotting system not only limits blood loss, but it also contributes to angiogenesis and the repair process as a whole by generating a fibrinous repair matrix and angiogenesis-modulating factors. These factors derived from the coagulation pathway and released from activated platelets provide cellular signals, which influence the initiation of angiogenesis and stabilization of newly formed vessels [7–10].

In this survey we shall summarize several aspects of the interaction between the hemostatic system and angiogenesis: the fibrin matrix as the scaffolding for cell invasion and tube formation; signaling factors which are generated during clotting; degradation products of the clotting system that act as negative regulators of angiogenesis; and the direct contribution of several coagulation factors to the formation of stable vessels in the embryo. Better understanding of the interaction of hemostatic factors and fragments derived from it will help us to find interventions that improve angiogenesis in ischemic areas devoid of the appropriate neovascularization, and in adapting the neovascularization process in tumors in order to reduce tumor growth.

The fibrinous matrix as a scaffolding for cell invasion and angiogenesis

Fibrin structure and angiogenesis

The temporary fibrin matrix enforced by other plasma proteins such as vitronectin and fibronectin provides excellent scaffolding for the invasion of cells [11–13]. Immediately after the formation of a clot, leukocytes migrate into it. One or two days later the endothelial cells also migrate into the clot as the start of angiogenesis and the recanalization of the clot, subsequently followed by other tissue cells [14]. The composition of the clot determines the rate of cell invasion and angiogenesis. On the one hand, factors associated with the clot, such as growth factors and protease inhibitors, affect the response of the invading cells, on the other hand, the matrix structure of the fibrin network is an important determinant of the extent to which capillary tubules form [15–17]. The rate of polymerization and the degree of cross-linking of the fibrin matrix determine the architecture of the fibrin network and its fibrinolytic sensitivity [18–20]. In addition, the carboxyl-terminal part of the Aα-chain of fibrinogen is an important determinant of the thickness of the fibrin bundles formed, and as such the structure of the fibrin network [21–23]. Thin fibrin bundles will associate into a tight and rigid network, while thick bundles associate into a porous and malleable network [24]. Furthermore, the incorporation of vitronectin and plasma fibronectin, proteins with important adhesive properties towards cells further contribute to the suitability of the clot to act as a substrate for endothelial migration and tube formation.

Information regarding the role of the structure of the fibrin matrix on endothelial tube formation has mainly been obtained from *in vitro* experiments, in which endothelial cells were allowed to form capillary-like tubular structures after stimulation with angiogenic growth factors and cytokines. In such models the tube formation is largely determined by the urokinase-type plasminogen activator (u-PA) and plasmin system [16, 25, 26], while also membrane-type-1-matrix-metalloproteinase (MT1-MMP) contributes to this process [27–30]. It was shown that endothelial migration and the morphogenesis of capillary tubes was determined by the structure of fibrin [15–17]. Malleable fibrin matrices, which have a higher turbidity, allowed a considerably faster ingrowth of tubular structures than rigid fibrin matrices [15, 16]. This was accompanied by a higher fibrinolytic sensitivity as reflected by the generation of fibrin degradation products. Cell-bound u-PA activity, which facilitates cell movement, was required, while the fibrinolytic activity induced by tissue-type plasminogen activator (t-PA) did not stimulate capillary-like tube formation and eventually caused lysis of the fibrin matrix [16]. Thus the fibrin structure appears to be an important determinant of the extent of tube formation and the stability of the matrix, on which the newly formed vessels depend until sufficient new extracellular matrix has been formed. Stabilization of the fibrin matrix is also achieved by the cross-linking of the fibrin matrix, either by plasma factor XIII or by tissue transglutaminase [31]. *In vivo* the

addition of tissue transglutaminase and the subsequent cross-linking of fibrin improves wound healing [32].

Additional experiments underlined the importance of the fibrin structure for capillary tube formation [31, 33]. When the effects of unfractionated and low molecular weight (LMW) heparins on parameters of angiogenesis were determined, it was observed that the rigidity of the fibrin matrix was differentially affected by the two heparins. While the presence of LMW-heparin made the fibrin coagulate into a rigid structure, the fibrin matrix, which coagulated in the presence of unfractioned heparin, was much more malleable. Only the fibrin prepared with LMW-heparin showed a reduced endothelial ingrowth and tube formation [33]. The two heparins did not differ in their effects on bFGF or VEGF-stimulated endothelial proliferation. The finding that LMW-heparin reduced the extent of capillary-tube formation may help to explain the clinical observation that the survival of tumor patients treated with LMW-heparin is higher than that of patients treated with unfractioned heparin [34].

Proteases involved in endothelial tube formation in a fibrinous matrix

Proteases play an important role in several aspects of the angiogenesis process [35–37]. They are needed to degrade the basal membrane to allow endothelial cells to expand. They are involved in cell migration both of endothelial cells, which form the core of the new vessel, and of pericytes that contribute to vessel stabilization in the final state of new tube formation. Finally, the generation of the lumen of the new vessel probably requires proteolytic activity to create sufficient space.

Two protease systems are currently thought to play a crucial role in the migration and invasion of endothelial cells in a fibrinous matrix. On the one hand, the u-PA/plasmin system is involved, which is localized on the membrane surface by cellular receptors, in particular the u-PA receptor [25, 26, 30, 38–40]. The u-PA bound to its receptor is activated and partakes shortly in local proteolysis, after which it is rapidly inhibited by plasminogen activator inhibitor type-1 (PAI-1) and the whole complex of u-PAR, uPA and PAI-1 is internalized [41]. The u-PA and PAI-1 are degraded in the lysosomes, while the u-PA receptor dissociates from the complex in the endosomes and is shuttled back to the cell surface. In this way it can contribute to a continuous process of detachment and re-attachment that is required for a cell to move [42]. It is tempting to speculate that the focally generated proteolytic activity of u-PA is involved in altering focal attachment sites. As pointed out by Chapman et al. for monocytes the u-PA receptor interacts with integrins, such as the $\alpha 3\beta 1$-integrin, and thus forms a multiprotein complex in lipid rafts [43]. In endothelial cells such an interaction also occurs between the u-PA receptor and the $\alpha v\beta 5$- and $\alpha 5\beta 1$-integrins [44, 45]. Whether these integrins primarily transfer cellular signals from the interaction between u-PA and u-PAR, or also become themselves proteolytic substrates for the enzymatic activity of u-PA and plasmin during cell migration, is not known.

The second proteolytic system that contributes to the formation of endothelial tubes and angiogenesis is that of the matrix metalloproteinases (MMPs) [27, 35, 46–52]. In particular, membrane-type MMPs (MT-MMPs) contribute to the formation of endothelial tubes. Hiroaka et al. [27] have shown that MT1-MMP has fibrinolytic activity and enhances angiogenesis in plasminogen-deficient animals. The involvement of MT1-MMP in endothelial migration and tube formation *in vitro* has now been shown by a number of groups [28, 29, 53, 54]. MT1-MMP is activated intracellularly by furin-like enzymes [55, 56]. It is present as an active enzyme on the cell surface, where it localizes matrix degradation close to the cell membrane. MT1-MMP has a direct activity against different extracellular matrix proteins including laminin, fibronectin, vitronectin, collagens and fibrin [48, 56]. MT1-MMP can activate pro-MMP-2 and pro-MMP-13 at the cell surface. This surface-linked action, which is comparable to that of u-PA activation at the u-PA receptor, localizes MMP activity to the pericellular area. Interestingly, Prager et al. showed that the MT1-MMP/MMP-2 complex could also activate u-PA on the u-PA receptor of endothelial cells *in vitro* and induce endothelial cell migration [30]. This further underlines the importance of the assembly and activity of localized multiprotein complexes in the regulation of cell migration. The activity of MMPs is regulated by various tissue inhibitors of metalloproteinases (TIMPs), but MT1-MMP is poorly inhibited by TIMP-1 [46]. Six types of MT-MMPs are presently known of which four have a transmembrane domain (MT1-, MT2-, MT3- and MT5-MMP) and two (MT4- and MT6-MMP) are anchored to the membrane by a glycosylphosphoinositol (GPI) group [46]. Overexpression of MT1-, MT2- or MT3-MMP enhanced tube formation by endothelial cells *in vitro* in a fibrin matrix, while MT4-MMP was unable to do so [57]. Whether the other transmembrane MT-MMPs play a similar role in neovascularization to that of MT1-MMP requires further investigation, but one may speculate that they may contribute to tissue-specific reactions

In vivo a fibrinous matrix is not encountered as a pure fibrin matrix, but as a plasma clot or fibrinous exudate, which also contains vitronectin, fibronectin and other proteins. As part of the fibrinous exudate in an inflamed tissue or tumor stroma, it is often intermingled with collagen fibers and other proteins of the interstitial matrix. It is likely that this will shift the specific proteolytic activities that are required for the various aspects of angiogenesis into the direction of the MMPs. In addition to MT1-MMP, the gelatinases MMP-2 and MMP-9, which degrade denatured collagen, have been reported to play a pivotal role in the onset of angiogenesis, the so-called angiogenic switch [47, 48]. MMP-2 interacts with MT1-MMP by which it is activated and localized on the cell surface. The role of MMP-9 in angiogenesis is complicated. It regulates the onset of angiogenesis in spontaneous pancreatic tumors in the mouse [48, 58]. Interestingly Hamano et al. [59] reported that, when MMP-9 deficiency was overcome temporarily so that the initial neovascularization - the angiogenic switch - proceeded in tumors, the subsequent withdrawal of MMP-9 caused an increased tumor growth and vascularization. This paradoxical effect

was due to the fact that MMP-9 could generate a collagen fragment of the α3-chain of type IV collagen, named tumstatin, with a strong anti-angiogenic activity (see below).

The signaling clot

Activation of the clotting system not only results in the formation of a sealing fibrin meshwork, but also generates activated coagulation factors and release products of platelet, which are liberated during platelet activation. These products contribute to the cell activation and tissue repair process that follows the initial sealing of the wound. Thrombin and activated coagulation factor X directly activate endothelial cells, and thrombin has been indicated as an angiogenesis-stimulating factor [60–63]. Thrombin also can act as a growth factor for smooth muscle cells [64]. The number of the thrombin receptors (PAR-1) in these cells can increase in vascular disease [65]. One may wonder whether the thrombin receptor also contributes to the recruitment of pericytes during repair-associated angiogenesis. Apparently it plays a role in angiogenesis in the embryo, because half of the murine embryos that are deficient in PAR-1 die due to vascular instability and hemorrhage [66–69]. Similarly, several coagulation factors, including tissue factor, factor V and prothrombin contributed directly to the development of stabile vessels in the embryo as became clear from studies in mice that were deficient in these factors [70] (see below). These factors acted on neovascularization in a manner independent of fibrin coagulation. It seems plausible that these factors derived from the coagulation cascade also may have an effect in repair-associated angiogenesis. In addition to the generation of activated coagulation factors during clotting, activated platelets release various factors, which are known to contribute to the initiation and regulation of angiogenesis [8, 9, 71].

Platelets contribute to angiogenesis

The platelet represents a cell fragment that is entirely devoted to tissue repair. Platelets are pinched off from megakaryocytes and released in the circulating blood. They are the building blocks of the hemostatic plug, which prevents the loss of blood immediately after wounding. Platelet adhesion and activation is triggered by the exposed collagen. The activated platelets provide a large negatively charged surface on which the coagulation pathway proceeds so that the hemostatic plug subsequently is enforced by a fibrin network that weaves around and between the platelets in the plug. When platelets become activated, they release many products from their α-granules, which are involved in clot stabilization and in the onset of tissue repair [71]. In these α-granules they have stored many factors that affect angiogenesis. Partly, they have been stored during maturation of the megakaryocytes, cells from which platelets are

derived; partly they accumulated later during the presence of platelets in the blood. These factors include vascular endothelial growth factor-A (VEGF-A) [72–75], thymidine phosphorylase (synonymous with platelet-derived endothelial cell growth factor) [76, 77], platelet-derived growth factor-B (PDGF-B) [78, 79], transforming growth factor-β (TGF-β) [80], hepatocyte growth factor (HGF) [81], and the angiogenesis-enhancing agent sphingosine-1-phosphate [82] (Tab. 1). The lymphangiogenic factor VEGF-C is also released by platelets.

Table 1. Platelet products contributing to angiogenesis

Angiogenesis inducers released from α-granula.
VEGF (incl. fibronectin)
Thymidine phosphorylase (PD-ECGF)
HGF
TGF-β
PDGF
CD40L
Sphingosine-1-phosphate
Released inhibitors of angiogenesis.
PF4
TGF-β
Proteases inhibitors [PAI-1 and TIMPs]
Thrombospondin-1

See Anitua et al. [71] for an overview of platelet α-granule content.

The VEGF-A, which is found in the serum derived from the blood of healthy humans, is entirely from the platelets which released their contents during the clotting process. Changes in serum VEGF-A levels during chemotherapy in breast cancer patients appeared to be directly related to changes in the platelet content of the blood [73]. The VEGF-A released from platelets can bind to fibronectin, by which it forms a highly effective angiogenic factor [83]. Thymidine phosphorylase is also a potent angiogenic factor. PDGF does not influence endothelial cells directly, but it is an important growth factor for pericytes and smooth muscle cells and thus plays a crucial role in stabilizing newly formed vessels. Mice lacking PDGF-B or PDGF-B receptors die around birth of cranial hemorrhages, due to impaired pericyte recruitment [84]. TGF-β acts by recruiting inflammatory cells and activating endothelial cells and pericytes and thereby changing their production of matrix remodeling proteases, such as u-PA and PAI-1, and other matrix proteins. Usually TGF-β has a pro-angiogenic effect, but depending on environmental conditions it may become anti-angiogenic, possibly by shifting the balance between proteases, protease inhibitors and matrix proteins to conditions which are less favorable for cell migration and invasion. Together, it is established that the platelet contributes to the onset of repair-associated angiogenesis. In

addition, it also contains factors that can modulate or counteract the angiogenic process.

Platelet products as inhibitors of angiogenesis

Several products of platelets can counteract angiogenesis. Inhibitors of urokinase and matrix metalloproteinases, such as PAI-1 and TIMPs, can attenuate the outgrowth of new vascular structures. These inhibitors are released by platelets and can also be induced by other cells after stimulation of the cells with platelet-derived TGF-β. Two other proteins which are released from platelets received much attention as potential angiogenesis inhibitors, platelet factor-4 and thrombospondin.

Platelet factor-4 (PF-4) is a basic protein which is released from platelet α-granules. It has a high tendency to bind to heparins and heparin sulfates via its C-terminus. PF-4 can inhibit angiogenesis *in vitro* and in tumor models *in vivo* [85, 86]. One may anticipate that displacement of growth factors from binding sites on heparan sulfates may contribute to its mode of action, a suggestion that is strengthened by the finding that a PF4-derived fragment, that contains the heparin-binding site, also inhibits angiogenesis *in vitro* [86]. However, a PF-4 analogue without heparin binding ability is also able to suppress angiogenesis *in vivo*, suggesting that the anti-angiogenic effect of PF-4 is more complex [87]. Mayo et al. [88] used the backbone of PF4 to develop new anti-angiogenic compounds. They have suggested that the three anti-parallel β-sheets in the molecule contribute to the anti-angiogenic nature of these compounds.

Thrombospondin-1 released by platelets inhibits angiogenesis *in vivo* and *in vitro* [89–91]. Several domains have been recognized in the thrombospondin molecule influencing angiogenesis. The effect of thrombospondin-1 on angiogenesis depends on its local concentration and its interaction with other matrix components in the affected tissue. It is particularly active on bFGF stimulated angiogenesis. The thrombospondin-1 molecule consists of several domains, which have different effects on angiogenesis. The aminoterminal heparin-binding 25 kDa part of thrombospondin has pro-angiogenic properties *in vitro* and in bFGF-induced angiogenesis in the rabbit cornea [92]. The remainder of the molecule has anti-angiogenic properties, which have been attributed largely to two domains called the second and third type-1 repeat [93, 94]. Calzada et al. [95] pointed out an interesting interaction between thrombospondin-mediated modulation of angiogenesis and the α4β1-integrin present on endothelial cells. Soluble α4β1-integrin antagonists inhibit neovascularization of mouse muscle explants in a thrombospondin-1-dependent manner. This inhibition is not observed in explants from thrombospondin-1-deficient mice. Other studies have indicated the importance of the interaction of thrombospondin-1 with CD36, which causes a rapid and transient activation of p59 fyn kinase, caspase-3 and c-Jun terminal kinase, and eventually endothelial apoptosis [96, 97].

Degradation products as regulators of angiogenesis

The inhibitory activity of the thrombospondin fragment is an example of a much larger group of angiogenesis regulators. A considerable number of angiogenesis inhibitors have now been recognized that represent degradation products derived from extracellular matrix proteins or from proteases of the hemostatic system (Tab. 2). The earliest recognized were angiostatin and endostatin, in addition to thrombospondin itself. In a search to find out whether primary tumors may produce inhibiting agents that can suppress the growth of the tumor and its metastases, Folkman, O'Reilly and co-workers isolated angiostatin and endostatin from the urine and tumor tissue of mice that had received grafts of tumors [98, 99]. When these compounds were administrated to

Table 2. Proteolytic degradation products as endogenous inhibitors of angiogenesis

Proteolytic fragments of hemostasis factors
Plasminogen fragments:
Angiostatin
Kringle 5
HMW-kininogen fragment:
Kininostatin (kringle 5 of HMWK)
Prothrombin fragments:
Kringle 2
Fragments of kringles 1 and 2
Antithrombin III fragment.
Fibrinogen fragments:
Fibrinogen degradation peptide E
Alphastatin: N-terminal 24 aminoacids of the α-chain of fibrinogen
Urokinase fragment
Proteolytic fragments of platelet products/matrix
Thrombospondin-1
Proteolytic fragments of the extracellular matrix.
Collagen type XVIII fragment:
Endostatin
Collagen type IV fragments:
Arresten,
Canstatin,
Tumstatin,
Metastatin
Laminin fragments
Nidogen fragment
Histidine-rich glycoprotein (HGRP) fragment
Fragments of growth factors
Prolactin fragment

See text for references.

tumor-bearing mice, these investigators observed growth arrest, regression and dormancy of the tumor grafts [99, 100]. Angiostatin is a fragment of several kringles of plasminogen, which is formed by the hydrolysis of the parent molecule by various proteases, in particular MMP-3, MMP-7, MMP-9 and u-PA [101]. Its anti-angiogenic activity is thought to be exerted by its ability to induce endothelial apoptosis [102, 103]. Endostatin is the C-terminal part of collagen type XVIII, which is generated by cathepsin-L and a protease with elastase activity [99, 104, 105]. It inhibits VEGF-induced migration and induces apoptosis [102, 103, 105, 106]. The mechanism of action was only recently in part recognized [105–109]. In its soluble state, endostatin binds to endothelial heparin sulphates and subsequently to integrins, in particular $\alpha 5\beta 1$-integrin [106–108]. The complex moves into the caveoli and affects cellular signaling through caveolin and Src and disturbs the proper organization of the F-actin cytoskeleton and probably other processes associated with it [109]. In addition it may also affect other pathways in the cell, which finally cause endothelial apoptosis. Kranenburg et al. [110] suggested an additional mechanism of action. Endostatin has the tendency to form cross-β sheets and forms aggregates. It was proposed that these cross-β sheets may be a common motif for angiogenesis-inhibiting agents [110]. This hypothesis requires further verification.

At present a considerable number of extracellular matrix fragments have been reported (summarized in Tab. 2) [111]. The collagen type IV fragment tumstatin inhibits focal adhesion kinase (FAK) through interaction with $\alpha v\beta 3$-integrin interaction [112]. It inhibits activation of phosphatidylinositol 3-kinase (PI3-kinase), protein kinase B (PKB/Akt), and mammalian target of rapamycin (mTOR) and causes endothelium-specific protein inhibition through the dissociation of eukaryotic initiation factor 4E protein (eIF4E) from 4E-binding protein 1 [113, 114]. The collagen type-IV fragment arresten acts as a competitive inhibitor for endothelial $\alpha 1\beta 1$-integrin binding to matrix collagen [111]. A fragment of histidine-rich glycoprotein also had anti-angiogenic properties [115]. It is likely that many additional extracellular matrix fragments will be recognized that act as angiogenesis modulators. Furthermore, fragments of growth factors and growth factor receptors may be recognized as having anti-angiogenic activity, including a fragment of prolactin [116].

Proteolytic fragments of factors involved in the hemostatic system represent a special group of anti-angiogenic regulatory molecules (Tab. 2). In addition to thrombospondin-1 fragments, fragments from fibrinogen [117, 118], plasminogen [98, 101, 119], prothrombin [120–122], high-molecular-weight kininogen [123, 124] and urokinase-type plasminogen activator [125] have been reported to have anti-angiogenic activity *in vitro* as well *in vivo*. The N-terminal 24 amino acid peptide of the α-chain of human fibrinogen has been recognized to harbor the anti-angiogenic activity that was originally observed to be present in fibrinogen degradation product E [118]. Similarly peptide stretches have been identified in the kringle-1 and -2 domains of prothrombin that have anti-angiogenic properties on endothelial cells *in vitro* and in the

angiogenesis of the chorioallantoic membrane of chick embryos *in vivo*. While angiostatin (kringles 1–4 of plasminogen) was the first degradation product recognized to have anti-angiogenic properties, the kringle-5 domain of human plasminogen has been shown to possess the most powerful anti-angiogenic activity of the plasminogen fragments [101, 119]. The kringle-5 domain of HMW kininogen has a similar action [123, 124]. Both have been reported to have anti-angiogenic activity in specific tumors in mice. The targets of these molecules are not yet known, but one may speculate that they may act on or interfere with the binding of specific ligands to cellular receptors.

Coagulation factors involved in developmental angiogenesis

An intriguing observation was made when the effects of the deletion of single coagulation factors were studied in mice. One would expect bleeding or thrombotic complications, depending on the nature of the deficiency. However, in addition to these complications, which often had already occurred as early as in the embryo, other abnormalities were found, which indicated that several of these factors were involved the development and stability of the vascular system in the embryo [10, 70]. In this group of coagulation factor deficiencies, defects in the yolk sac and embryo vasculature, accompanied by mid-gestational bleeding occurred in all or some of the murine embryos (Tab. 3). They were observed in animals with a deficiency of tissue factor (80–100%) [126–128], factor V (50%) [129], prothrombin (50%) [66, 67] or the thrombin receptor protease activated receptor-1 (PAR-1) [68, 69, 129]. The effects of factor V deficiency were independent of those caused by prothrombin and PAR-1 deficiency, as the combined deficiency of factor V and PAR-1 caused an additive effect of their single deficiencies and a nearly complete lethality [130]. The defects in the yolk sac and embryo and the subsequent mid-gestational bleeding were also found in 60% of the animals with a deficiency in tissue factor pathway inhibitor (TFPI), a defect that again was not related to coagulopathy [131]. Fibrinogen deficiency did not reduce the percentage of TFPI- or PAR-1- deficient animals that developed vascular abnormalities and mid-gestational bleeding [130, 131]. These data indicate that tissue factor/TFPI, factor V and the (pro)thrombin/PAR-1 interaction are involved in the development of stable vascular structures rather than acting only on hemostasis in the embryo.

The prothrombin and factor V deficient animals, that survived mid-gestation, as well mice deficient in the coagulation factors VII and X, developed fatal bleeding around birth or within a few days after birth [70, 132, 133]. Some of the fibrinogen-deficient animals, on the average 30%, died from the same cause. Depending on the genetic background this percentage may vary between 10% to 70% (see [134]).

In contrast to the previous group, animals that were deficient in factors that reduce coagulation, i.e., antithrombin III, protein C and thrombomodulin and

Table 3. Vascular and hemostatic defects caused by deficiencies of coagulation factors in mice during development

Defects in the yolk sac and embryo vasculature accompanied by mid-gestational bleeding and death of the embryo.
Tissue factor (80–100%)
TFPI (60%)
Factor V (50%)
Prothrombin (50%)
PAR-1 (50%)
Factor V and PAR-1 (96%)
Factor X (33%, only mid-gestational bleeding reported)
Progressive development of fibrin deposits before birth causing death.
Antithrombin III (100%)
Thrombomodulin (100%)
Protein C (100%)
TFPI (100% of the remaining embryos after mid-gestational period)
Fatal bleeding perinatally or several days after birth.
Factor VII (100%)
Factor V (100% of embryos surviving mid-gestational period)
Factor X (90–100%)
Prothrombin (100% of embryos surviving mid-gestational period)
Fibrinogen (30%)
Adult hemostatic disorders upon challenge.
Factor IX, VIII, XI, fibrinogen (surviving perinatal period)

See text for references

those deficient in TFPI that survived mid-gestation, died before birth through the progressive development of fibrin deposits [131, 135–138]. These phenotypes can be rescued by simultaneous fibrinogen deficiency, as has been shown for TFPI-deficient mice that survived the mid-gestational period. The fact that the absence of fibrinogen completely prevents these complications, further underpins the idea that coagulopathy is the cause of death. No hemostatic complications have been observed in unchallenged animals deficient in various other coagulation factors and PAR-2, PAR-3 and PAR-4 [70, 139]. However, when deficiency of fibrinogen (coagulation) and that of PAR-4 (required for thrombin-mediated platelet activation in mice) were combined, these animals developed fetal bleeding around birth comparable to the prothrombin-deficient mice that survived mid-gestation [140]. This emphasizes the idea that the coagulation cascade contributes both to the formation of a fibrin meshwork and also to the activation of thrombin that can activate platelets via protease-activated receptors (PARs).

Animals deficient in one of the fibrinolysis factors plasminogen, u-PA, t-PA, PAI-1, u-PAR and α2-antiplasmin (α2-AP), or of both u-PA and t-PA, survive and have a normal vessel development [141–146]. However, upon chal-

lenge (wounding) and in tumors the formation of new vessels can be retarded [147]. Animals deficient in u-PA or plasminogen had a reduced neovascularization in ischemic heart tissue [50], and in bFGF-stimulated angiogenesis in the cornea [148]. Deficiency of plasminogen also reduced VEGF-stimulated neovascularization in the cornea [148] and vascularization of subcutaneously fibrin-filled sponges (our unpublished observation). The role of plasminogen inhibitor type-1 (PAI-1) in angiogenesis is more complex. As it is the prime inhibitor of plasminogen activators one would expect that the deficiency in PAI-1 increases angiogenesis and tumor growth. However, this is often not the case [149, 150]. Excess of proteolytic activity generated in the absence of PAI-1 may reduce the suitability of the matrix for cell invasion, angiogenesis and tumor growth. Administration of PAI-1 to PAI-1-deficient mice can both stimulate and inhibit angiogenesis depending on the dose. At high concentrations PAI-1 prevents activation of urokinase and plasminogen; at limited concentrations it stimulates angiogenesis primarily through its anti-proteolytic activity, by which it limits excessive proteolysis of matrix proteins required for angiogenesis [151].

Conclusion and prospects

From this survey it is clear that the hemostatic system exerts a number of effects on the regulation of angiogenesis. It not only offers a structural scaffolding for invading cells by the fibrin matrix, but also factors that directly and indirectly act on angiogenesis: VEGF-A from platelets; thrombin that acts via its receptor PAR-1; and other factors from the coagulation cascade. Furthermore, the recently recognized proteolytic degradation products that exert angiogenesis-inhibiting properties probably only represent the tip of the iceberg of factors that will be recognized in the coming decade. This underlines the importance of the hemostatic system as a source of factors that facilitate or are even needed for tissue repair. A better understanding of this process could be used to improve wound healing. This not only concerns the generation of angiogenesis-stimulating factors from the clotting system, but also the prevention of the local generation of anti-angiogenic products e.g., in ulcerating wounds that heal poorly or not at all.

Unfortunately, tumors can misappropriate the hemostatic system. The generation of activated coagulation factors and the release of platelet products in the stroma of the tumor can be used by the tumor to stimulate its nourishing blood supply and to grow. Dvorak's visionary statement "tumors are wounds that do not heal" [4], could be paraphrased by "tumors mimic wounds that do not heal to guarantee their access to healing factors which support their growth". The labile hemostatic balance in cancer patients reflects the active interaction between the tumor and the hemostatic system.

In addition to role for the hemostatic system in supporting angiogenesis by sustaining the invasion of endothelial cells derived from the vicinity of the

wound, the fibrin scaffolding and locally generated factors can facilitate the homing of circulating CD34-positive cells to the area in which angiogenesis has to occur [152, 153]. These cells provide a mechanism for enforcing angiogenesis. Although it remains to be established whether they contribute significantly to the vascular lining themselves, or play an orchestrating role in endothelial proliferation and the tube forming and branching process that accompanies angiogenesis, their contribution is well appreciated.

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Mechanisms of Angiogenesis
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Signal transduction in angiogenesis

Ian Zachary

Department of Medicine, University College London, 5 University Street, London WC1E 6JJ, UK

Introduction

The formation of a mature vasculature requires coordinated signalling between multiple extracellular factors and their respective cell surface receptors. The discovery of vascular endothelial growth factor (VEGF) or VEGF-A, the first endothelial-specific angiogenic factor to be identified, and its receptors, VEGFR2 and VEGFR1, were major milestones in understanding the molecular basis of blood vessel formation. Subsequent work has demonstrated that VEGF and its major signalling receptor VEGFR2 are essential for two key processes in early vascular development, vasculogenesis, defined as the differentiation of endothelial cell progenitors and their assembly into the primary capillary plexus, and angiogenesis, or the sprouting of new capillaries from pre-existing vessels [1]. The early vasculature consists of a system of endothelial tubules that subsequently become stabilized through the recruitment of periendothelial cells such as pericytes and vascular smooth muscle cells (VSMC). Ensuing steps result in the maturation of blood vessels through vessel growth, the deposition of extracellular matrix, innervation, and vessel patterning and homing. Concomitantly, blood vessels are also progressively differentiated to become either veins or arteries and to form organ-specific and functionally distinct vascular beds. Several other families of angiogenic factors and their receptors are required for many of these later stages. Angiopoietins (Ang) and Tie receptors have been identified as critical for the stabilization of early vessels and periendothelial cell recruitment. Members of the ephrin family of transmembrane ligands and their tyrosine kinase receptors, the ephs, have been implicated in the demarcation of arteries and veins and interactions between endothelial cells and VSMC. In addition, a variety of other factors, such as fibroblast growth factors (FGFs), PDGF and TGF-β, that either have non-endothelial target cells or, like fibroblast growth factor-2 (FGF-2), are not restricted to endothelial cells, participate in angiogenesis and/or vessel maturation due to effects on pericyte and VSMC recruitment [2].

The functional specialization of angiogenic factors and the ordering of multiple cellular processes during blood vessel formation implies not only that individual ligand-receptor interactions initiate distinct arrays of signalling events, but that information from different angiogenic factors is integrated,

coordinated and compartmentalized to ensure the spatio-temporal organization of cellular processes necessary for development of a mature vascular system. Accordingly, the identification of signalling cascades triggered by individual receptors, and an understanding of how they are ordered and integrated, are key prerequisites for understanding the molecular basis of vascular development.

This chapter focusses on the role of endothelial signalling in angiogenesis. Signalling by other factors, such as PDGF, acting primarily on non-endothelial vascular cells, though essential for vessel maturation, is therefore omitted. Because most work on endothelial and angiogenic signalling has concentrated on VEGF and its receptors, VEGF signalling is considered in detail, followed by a discussion of biologically relevant signalling cascades organized according to different angiogenic cellular functions, such as survival, proliferation and migration [3]. This is followed by more condensed overviews of the signalling pathways and associated biological functions initiated by two other important families of angiogenic factors, the FGFs and angiopoietins. A later section will consider the emerging area of angiogenic genomic analysis. Finally, it will be discussed how progress in delineating angiogenic signalling can help to develop a framework for understanding the molecular basis for functional specialization, integration and coordination among distinct angiogenic ligand/receptor systems during blood vessel formation.

VEGF ligands

VEGF (VEGF-A) is the prototypical member of a family of related growth factors, which includes placenta growth factor (PlGF), VEGFs B, C, and D, and the *orf* virus-encoded factor, VEGF-E [1, 4–8]. Alternative splicing of human VEGF mRNA from a single gene containing 8 exons gives rise to at least 5 different isoforms of 121 (mouse $VEGF_{120}$), 145, 165 (mouse $VEGF_{164}$), 189 and 206 amino acid residues [9, 10]. Exon 6 is absent in $VEGF_{121}$ and $VEGF_{165}$ and exon 7 is absent from $VEGF_{121}$ and $VEGF_{145}$. $VEGF_{121}$, $VEGF_{145}$ and $VEGF_{165}$ are secreted and form dimeric proteins whereas $VEGF_{189}$ and $VEGF_{206}$ are thought to be secreted, but are not readily diffusible and probably remain sequestered in the pericellular matrix. All isoforms, excepting $VEGF_{121}$, bind heparin via a region rich in charged basic amino acid residues encoded by exon 6 and a domain encoded by exon 7. Human $VEGF_{165}$, the most abundant and biologically active form [10, 11], is glycosylated at Asn75 and is typically expressed as a 46 kDa homodimer of 23 kDa monomers [9]. $VEGF_{121}$ and $VEGF_{145}$ also exhibit biological activity in endothelial cells, but have lower potency than $VEGF_{165}$ [10, 11]. The crystal structure of $VEGF_{9-108}$ shows that VEGF consists of two monomers each containing a core cysteine knot motif held together by three intrachain disulphide bonds similar to the structure of PDGF, arranged head-to-tail in a homodimer with two interchain disulphide bridges [12].

Striking confirmation of the central role of VEGF in embryonic blood vessel development came from the discovery that targeted inactivation of only a single allele of the VEGF gene in mice causes a lethal impairment of angiogenesis [13, 14]. The importance of larger VEGF isoforms, including $VEGF_{165}$, was demonstrated by the finding that mice expressing only $VEGF_{120}$ and lacking the longer heparin-binding isoforms, die shortly after birth due to haemorrhage and ischaemic cardiomyopathy [15].

Other VEGF family ligands can influence angiogenesis, but their biological importance is, in most cases, less clear. VEGF-C and its receptor, VEGFR3 (Flt-4), are strongly implicated in formation of the lymphatic endothelium (lymphangiogenesis). Robust lymphatic vessel development and weak angiogenesis was induced by VEGF-C in the chicken chorioallantoic membrane [16], and transgenic mice overexpressing VEGF-C in keratinocytes of the skin epidermis develop enlarged lymphatic vessels, while mice overexpressing $VEGF_{164}$ in the same location showed only blood vessel hyperplasia [17]. However, ectopic application of VEGF-C has a potent angiogenic effect in the mouse cornea [18] and rabbit ischaemic hindlimb models [19]. VEGF-D also induces lymphangiogenesis and angiogenesis [20]. PlGF alone appears to be weakly angiogenic, but VEGF/PlGF heterodimers are able to bind to VEGFR2, are mitogenic for endothelial cells and stimulate angiogenesis *in vivo* [21]. Though mice lacking PlGF develop normally and are viable, they exhibit reduced angiogenesis in pathophysiological situations such as ischaemia [22]. PlGF-deficient mice also exhibit a delay in collateral artery growth following arterial occlusion [23], and administration of PlGF stimulates collateral vessel growth [24].

VEGF receptors

Two distinct receptor tyrosine kinases (RTKs) have been identified for VEGF, VEGF Receptor(R)1 (known as Flt-1) and VEGFR2 (human KDR/mouse Flk-1) which share approximately 44% amino acid homology [1, 3]. VEGF-C and -D also bind VEGFR2 and also bind to a third receptor, VEGFR3 (Flt-4), which does not recognize VEGF-A [6, 7]. PlGF and VEGF-B bind with high-affinity only to VEGFR1 [25], and VEGF-E binds with high affinity only to VEGFR2 [8]. The specificities of VEGF family members for their receptors are illustrated in Figure 1.

VEGFR2 and VEGFR1 are structurally related to the PDGF family (class III) of RTKs and have a similar domain structure characterized by cytoplasmic regions with an insert sequence within the catalytic domain, a single hydrophobic transmembrane domain and seven immunoglobulin (Ig)-like domains in the extracellular regions (Fig. 1). Analysis of mutant and chimeric forms of VEGFR1 and R2 indicate that Ig-like domains 2 and 3 are the most important for ligand binding [26–29]. The reported affinities of VEGF for VEGFR1 and VEGFR2 are, respectively, 16–114 pM and 0.4–1 nM [30–33].

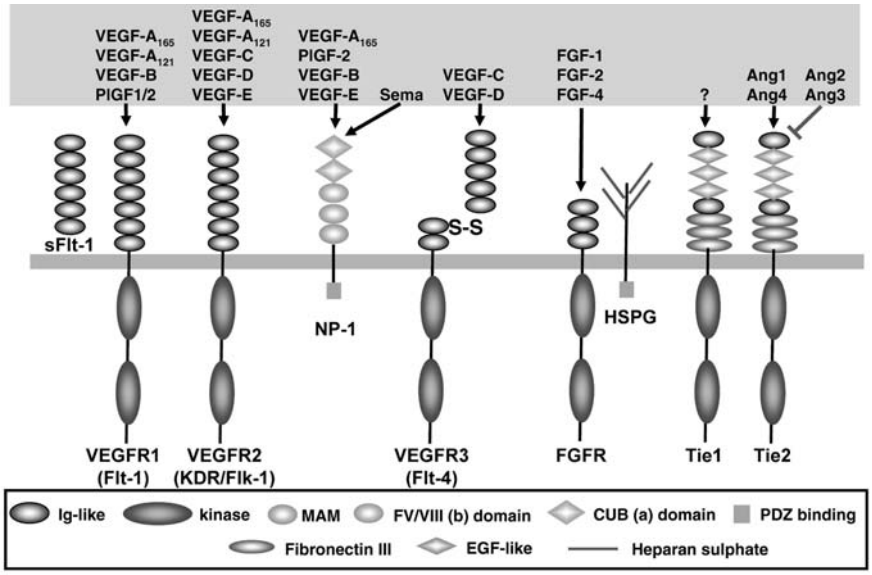

Figure 1. Angiogenic factors and receptors. VEGF (VEGF-A) binds to two related tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), each possessing an extracellular domain containing seven Ig-like loops, a single hydrophobic membrane-spanning domain, and a large cytoplasmic domain comprising a single catalytic domain containing all the conserved motifs found in other RTKs which is interrupted by a non-catalytic region, called the kinase insert. The extracellular domain of VEGFR1 is also independently expressed as a soluble protein (sFlt-1). VEGFR3 (Flt-4), a receptor for VEGF-C and VEGF-D, undergoes proteolytic processing to yield disulphide-linked 120 and 75 kDa polypeptides. Neuropilin-1 (NP-1) is a non-tyrosine kinase receptor for $VEGF_{165}$, PlGF-2, VEGF-B and VEGF-E, but does not bind to $VEGF_{121}$. NP-1 has a short cytoplasmic domain containing a carboxyl-terminal PDZ domain-binding motif, a single transmembrane domain and a large extracellular region comprising a membrane proximal C or MAM (meprin, A5, μ) domain, tandem b1 and b2 domains related to the C1 and C2 domains of coagulation factors VIII and V, and tandem CUB domains (a1 and a2), homologous to regions of complement components C1r and C1s. NP-1 is also a receptor for semaphorins (sema). FGFs binds to a family of related RTKs, FGFRs1–4, consisting of an extracellular domain containing three Ig-like regions, a single hydrophobic membrane-spanning domain, and a split cytoplasmic tyrosine kinase domain. FGFs require heparan sulphate proteoglycans (HSPGs) for receptor dimerisation and activation. One HSPG co-receptor for FGF-2, syndecan-4, contains a carboxyl-terminal PDZ domain-binding motif. Angiopoietins bind to Tie2 receptors comprising an extracellular domain with three fibronectin type III homology domains, and one complete and one partial Ig-like domain separated by three epidermal growth factor-like cysteine-rich motifs, a single transmembrane domain, and an intracellular bipartite tyrosine kinase domain. No ligand for Tie1 has been identified. (For colored picture see color plate 8)

VEGFR3 is synthesized as a glycosylated 195 kDa molecule which undergoes cleavage in its extracellular domain to generate 120 and 75 kDa chains which are linked by disulphide bridges [1].

Targeted disruption of VEGFR1 and R2 in mice prevents normal vascularization and embryonic development, although the two knockouts have distinct phenotypes. VEGFR2-deficient mice die between embryonic day E8.5 and E9.5, produce neither differentiated endothelial cells nor organized blood vessels, and have severely reduced haematopoietic precursors [34]. In contrast,

the primary defect in VEGFR1-null mice is over-production of endothelial progenitors leading to embryonic death at E8.5 to E9.5 [35, 36].

Disruption of the VEGFR3 gene in mice does not prevent vasculogenesis or angiogenesis but causes defects in normal vascular development antecedent to the emergence of lymphatic vessels leading to embryonic death by E9.5. This indicates that VEGFR3 is important for angiogenesis and is not restricted to mediating lymphatic vessel growth and development [37]. However, though VEGFR3 expression starts during E8 in developing blood vessels, it is subsequently restricted largely to the lymphatic vasculature. Furthermore, lymphangiogenesis is inhibited with resulting lymphoedema in transgenic mice expressing soluble VEGFR3, and the rare autosomal dominant disorder, primary lymphoedema, is associated with missense mutations in the VEGFR3 gene resulting in an inactive kinase.

VEGF receptor signalling

VEGFR2 is activated through ligand-stimulated receptor dimerization and trans(auto)phosphorylation of tyrosine residues in the cytoplasmic domain. Monomeric receptors have 100-fold less affinity for VEGF, which preferentially binds predimerized receptors [3]. Heterodimeric receptors with one functional and one non-functional unit are unable to transduce signals and antagonize VEGF activity. Six autophosphorylation sites have so far been identified in VEGFR2, Y951 and Y996 in the kinase insert, Y1054 and Y1059 in the kinase domain, and Y1175 and Y1214 in the carboxyl terminal tail [38, 39]. The function of most of these sites has not been defined, but Y951 associates with the src homology (SH)2 domain protein, VEGF receptor-associated protein (VRAP) [40], and Y1175 is a major binding site for phospholipase C(PLC)-γ [39]. VEGFR2 has been shown to associate with other SH2 domain proteins, including growth factor receptor-bound protein-2 (GRB2), NCK and SHC and the protein tyrosine phosphatases SHP-1, SHP-2 and human cellular protein tyrosine phosphatase A (HCPTPA) [41–43]. Tyrosine phosphatase-mediated inactivation of VEGFR2 may provide a mechanism for regulation of its function. In support of this concept, TNF-α induced heterologous downregulation of VEGF biological activities in human umbilical vein endothelial cells (HUVEC) was linked with increased association between SHP-1 and VEGFR2 [44].

Most biologically relevant VEGF signalling in endothelial cells is mediated via VEGFR2 [33, 41, 45–48], and this receptor is thought to be primarily responsible for endothelial functions of VEGF, vasculogenesis and angiogenesis (Fig. 2). Studies utilizing either porcine aortic endothelial cells (PAE) expressing VEGFR2, or receptor-specific VEGF family ligands, indicate that VEGFR2 mediates proliferation and migration [33], tyrosine phosphorylation of downstream targets [41] Ca^{2+} mobilization [46], prostacyclin (PGI_2) production and ERK activation [47], nitric oxide (NO) production [48], and survival and phosphatidylinositol 3'-kinase (PI3K)/Akt activity [49].

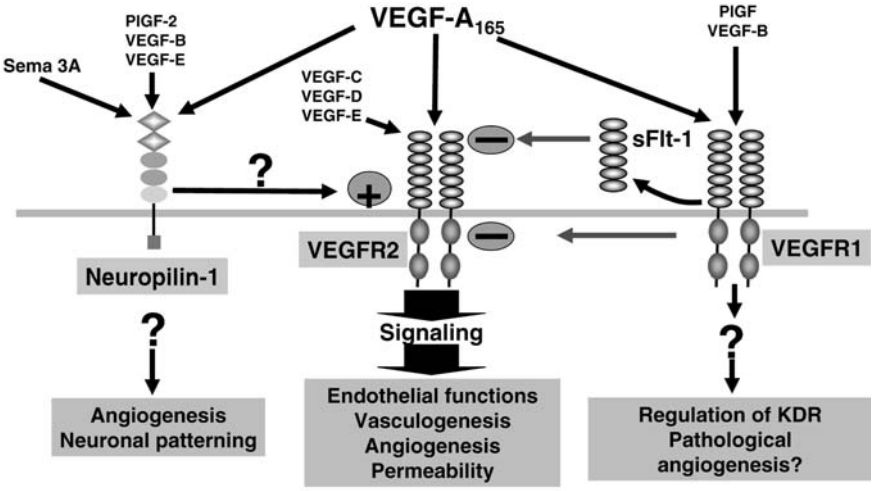

Figure 2. Biological roles of VEGF receptors. VEGFR2 is the major receptor responsible for VEGF signalling and biological functions in endothelial cells, and plays a key role in mediating vasculogenesis, endothelial cell differentiation, and angiogenesis in development, and disease-associated neovascularisation. VEGFR1 is thought to act as a "decoy" receptor during embryogenesis, by regulating functions of VEGF mediated via VEGFR2, either by direct inhibition of VEGFR2, or by inhibition of VEGF by sFlt-1. VEGFR1 may play a role in pathophysiological angiogenesis in the adult. NP-1 is essential for embryogenic angiogenesis, but the precise role of this receptor in the function of VEGF is unclear. NP-1 has no defined signalling role as yet. (For colored picture see color plate 9)

Survival signalling

A major pathway through which VEGF promotes endothelial cell survival is PI3K-dependent activation of the anti-apoptotic kinase, Akt/PKB ([49, 50]; Fig. 3). Akt in turn phosphorylates and inhibits the pro-apoptotic proteins Bad and caspase-9. Long-term survival effects of VEGF may be mediated through upregulation of anti-apoptotic proteins, including Bcl-2 and A1 [51], and the IAP (inhibitors of apoptosis) proteins, survivin and X-chromosome-linked IAP (XIAP) [52], which act by inhibiting upstream caspases, and terminal effector caspases, respectively (Fig. 3).

Akt mediates VEGF-induced nitric oxide (NO) production via endothelial constitutive nitric oxide synthase (eNOS) phosphorylation at serine 1179 [53, 54] and Akt survival signalling is also critically dependent on VE-cadherin, the major component of endothelial adherens junctions [55]. Either deficiency or cytosolic truncation of VE-cadherin lethally impairs remodeling and maturation of embryonic blood vessels, and these effects are associated with increased endothelial cell death, loss of VEGFR2 associations with VE-cadherin, β-catenin, and PI3K, reduced Akt signalling and decreased Bcl-2 expression [55]. The role of VE-cadherin was specific for VEGF, and responses to FGF-2 were not affected. These findings suggest that VEGFR2 survival

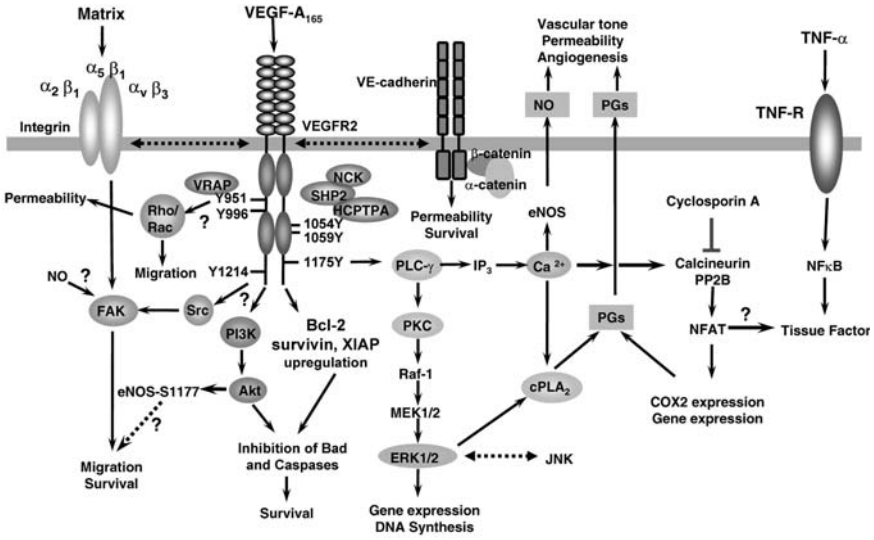

Figure 3. Functional VEGF signalling in endothelial cells. Most biologically relevant VEGF signalling is mediated via VEGFR2. Activation of VEGFR2 occurs through ligand-induced dimerisation and receptor autophosphorylation at multiple tyrosine residues in the intracellular domain. VRAP and PLC-γ associate with Y951 and Y1175, respectively, but the role of other sites is yet to be defined, though VEGFR2 associates with several other SH2 domain proteins including Nck and the tyrosine phosphateses SHP2 and HCPTPA. VEGF-dependent endothelial cell survival is mediated in part via PI3K-mediated activation of the anti-apoptotic kinase Akt, though the mechanism of PI3K activation is unclear. Akt phosphorylates and inhibits the pro-apoptotic protein Bad, leading to inhibition of caspase activity. Upregulation of the anti-apoptotic proteins Bcl2, survivin and XIAP may also mediate inhibition of terminal effector caspases. Akt also causes Ca^{2+}-independent eNOS activation through phosphorylation at ser 1177, and this pathway may also be essential for migration. Increased tyrosine phosphorylation of FAK mediated in part through Src contributes to VEGF-dependent survival and migration signalling, but the mechanism responsible for VEGFR2-mediated activation of this pathway is unclear. Direct interactions (indicated by bidirectional dotted arrow) and convergent FAK signalling between VEGFR2 and integrin receptors for collagen, fibronectin and vitronectin, may also play a role in survival functions of VEGF. VEGFR2 residue Tyr 951 mediates activation of Rho and Rac via an unknown mechanism and this pathway is also implicated in migration signalling. Rac activation may mediate VEGF-induced fenestration contributing to the permeability-increasing effect of VEGF. Interaction between VEGFR2 and VE-cadherin is important for survival signaling and may also regulate permeability. A major mitogenic signalling mechanism for VEGF is the PLC-γ pathway resulting in hydrolysis of phosphatidylinositol 4,5-bisphosphate, generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol and subsequent mobilization of intracellular Ca^{2+} and PKC activation. PKC mediates activation of ERKs 1/2 via Raf-1 and MEK, and this pathway is a major mediator of both mitogenesis and $cPLA_2$ activation leading to generation of COX-derived prostanoids (PGs), including PGI_2 and PGE_2. Ca^{2+} signalling is also important for eNOS activation and NO generation, and activates the serine/threonine phosphatase, calcineurin (PP2B), a target for the immunosuppressant, cyclosporin A, leading to activation of the transcription factor, nuclear factor of activated T-cells (NFAT) and induction of COX-2 and tissue factor gene expression. VEGF also interacts synergistically with TNFα to promote tissue factor expression. Question marks and dotted lines indicate areas where the mechanism involved in unclear, or where a link is inferred but not yet established. (For colored picture see color plate 10)

signalling is tightly coupled with the functional integrity of cell-cell endothelial adherens junction components once the early stages of blood vessel assembly are complete.

VEGF-dependent survival signalling is also probably directly linked to integrins [56]. VEGFR2 has been reported to associate with $\alpha_v\beta_3$ and VEGF mitogenicity and receptor activity were enhanced by endothelial adhesion to the $\alpha_v\beta_3$ ligand, vitronectin [57]. Since VEGF increases tyrosine phosphorylation of focal adhesion kinase (FAK) [58], and FAK is a major component of β_3 and β_1 integrin signalling pathways, activation of FAK might also contribute to VEGF survival signalling (Fig. 3). Other signalling pathways may contribute to survival functions of VEGF. For example, the PKC activator phorbol myristate acetate, was shown to promote survival of HUVECs and endothelial cell tube formation in 3-D collagen gels [59].

Mitogenic signalling

VEGF is a strong activator of extracellular signal-regulated protein kinases (ERKs) 1 and 2 via VEGFR2 and the presumption that this pathway plays a central role in angiogenesis is supported by the finding that specific inhibitors of MEK1/2, the kinase responsible for ERK activation, impair endothelial tubulogenesis *in vitro* [60]. The canonical pathway through which RTKs activate ERKs involves GRB2 tyrosine phosphorylation, subsequent stimulation of the guanine nucleotide exchange protein, SOS, and ras activation, leading to activation of the Raf-1/MEK/ERK cascade. There is strong evidence, however, that VEGFR2 activates ERK via a ras-independent pathway mediated through PLC-γ and PKC ([39, 61, 62]; see Fig. 3). VEGF induces strong PLC-γ tyrosine phosphorylation and activation leading to generation of diacylglycerol and inositol 1,4,5-trisphosphate and subsequent activation of PKC and Ca^{2+} mobilization. Activation of c-Src was reported to mediate VEGF signalling through PLC-γ [63], though it is unclear how c-Src couples with and activates PLC-γ. VEGF induces ras-independent induction of the ERK pathway whereas PKC inhibitors block VEGF-induced activation of ERK1/2 and MEK [61, 62]. Furthermore, VEGFR2 residue Y1175 is essential for receptor association with PLC-γ, and VEGF-induced PLC-γ tyrosine phosphorylation, ERK activation and mitogenesis [39]. Two reports provide evidence that VEGF-induced ERK activation occurs via NO-mediated Raf-1 activation, but the mechanism involved is unclear [64, 65] and VEGF-induced ERK activation has also been shown to be independent of NO [61].

Several studies also show that PKC inhibition using either pharmacological inhibitors or antisense oligonucleotides to PKC-α and PKC-ζ isoforms blocks VEGF mitogenic signalling [66–68], and diverse PKC inhibitors block VEGF-induced angiogenesis *in vitro* [69, 70]. VEGF, but neither FGF-2 nor serum, was reported to induce endothelial migration and proliferation via an NO-dependent reduction in PKCδ activity at 8–24 h [71].

There is some evidence for cross-talk between the ERK and c-Jun N-terminal protein kinase (JNK) pathways in VEGF mitogenic signalling. VEGF activates JNK and expression of a dominant negative JNK-1 mutant inhibited

VEGF-induced thymidine incorporation, while the dominant negative Y185F ERK2 mutant blocked JNK activation [72].

As Figure 3 shows, the bifurcating PLC-γ pathway also plays a crucial role in endothelial production of NO and PGI_2. ERK mediates VEGF-induced activation of cytosolic phospholipase A_2 ($cPLA_2$) [47], the key rate-limiting step in release of arachidonic acid and its subsequent conversion to prostanoids via cyclooxygenase (COX), and PKC inhibitors block VEGF-induced and ERK-mediated $cPLA_2$ activation and PGI_2 production [61]. Ca^{2+} mobilization is essential for VEGF-induced PGI_2 production probably because of its role in exocytotic release of cellular PGI_2 [61]. Ca^{2+} signalling also mediates short-term NO production through activation of the constitutive eNOS isoform. Ca^{2+}-independent NO production is mediated via Akt, and may also involve VEGF-induced upregulation of eNOS mRNA [73], while long-term prostanoid production, is mediated through VEGF-induced expression of the inducible COX-2 isoform via Ca^{2+}/calcineurin-dependent activation of the transcriptional regulator, nuclear factor of activated T cells (NFAT) [74]. As discussed below, NO and PGI_2 have been implicated in several biological effects of VEGF including angiogenesis, increased vasopermeability, hypotension and local arterioprotection [75].

Chemotactic signalling

The extracellular matrix (ECM) plays an essential role in maintaining the integrity and stability of nascent vessels through interactions with integrin receptors expressed on the surface of endothelial cells and VSMC. In stable vessels, endothelial and other vascular cells are sheathed in a basement membrane composed largely of collagen type IV and laminin, and breakdown of this basement membrane through the action of matrix-degrading metalloproteinases (MMPs) is a prerequisite for endothelial cell migration and the sprouting of new vessels during development and in disease [2]. VEGF induces the expression of several proteinases, including plasminogen activators and MMP-2 [76, 77]. A natural inhibitor of MMPs, tissue inhibitor of metalloproteinase-3, inhibits angiogenesis by preventing VEGF binding to VEGFR2 [78]. A pivotal role seems to be played by MMP-9 in promoting angiogenesis. In a model of tumour angiogenesis, MMP-2, MMP-9, VEGF and VEGFR2 were all prominently expressed in angiogenic lesions, and both VEGFR2 inhibition and loss of MMP-9, but not MMP-2, inhibited angiogenic switching and tumour growth [79]. Exposure of a cryptic epitope in collagen IV has been shown to function as an important endothelial migration control site during retinal neovascularisation, and exposure of this cryptic site was inhibited in MMP-9-deficient mice [80]. Despite the cogent evidence that MMP-9 is crucial for promoting endothelial cell migration during VEGF-dependent angiogenesis, little is known regarding the mechanisms linking MMP expression to VEGF receptor signalling.

Focal adhesion kinase (FAK) is a key transducer of signals converging from integrins and RTKs important for both survival and migration ([3, 81]; see Fig. 3). VEGF induces tyrosine phosphorylation of FAK and the focal adhesion-associated protein paxillin to promote recruitment of FAK to new focal adhesions in HUVECs [58, 82]. VEGF also stimulates tyrosine phosphorylation of the FAK-related tyrosine kinase, Pyk2, in a bone marrow endothelial cell line [83]. VEGF has been shown to increase FAK phosphorylation at Y397 and Y861, but selectively stimulate Y861 phosphorylation via a Src-dependent pathway. Src inhibition also impairs VEGF-induced cell migration and survival [82]. A dominant negative Src construct selectively inhibited VEGF-induced angiogenesis and reduced VEGF-dependent survival in the chick CAM model, without affecting the angiogenic response to FGF-2 [84]. Src is also required for VEGF-stimulated formation of a complex between FAK and integrin $\alpha_v\beta_5$ [85]. Though angiogenesis is normal in mice lacking individual Src family kinases, these findings indicate important roles for Src-dependent FAK signalling in VEGF-induced angiogenesis and endothelial cell migration and survival. Redundancy and compensatory mechanisms between Src family kinases may help to account for the absence of vascular defects in individual Src kinase knock-out mice.

Key roles for the integrins $\alpha_v\beta_3$ and $\alpha_v\beta_5$ in endothelial cell migration and survival and angiogenesis *in vivo* have been widely inferred from the anti-angiogenic effects of antagonists of these integrins based on the peptide motif RGD [56, 86]. Paradoxically, mice deficient in either β_3 [87] or β_3 and β_5 [88], are viable and exhibit no defects in vascularisation, but instead show enhanced tumour angiogenesis and increased VEGFR2 expression [88]. These findings provocatively challenge the assumption that α_v integrins play a pro-angiogenic role and have led to the alternative hypothesis that these integrins may act to inhibit angiogenesis, at least in some situations [89]. An essential role of other integrins in angiogenesis is more clearly established. Mice deficient in the fibronectin receptor, $\alpha_5\beta_1$, die during embryogenesis and exhibit major vascular abnormalities [90], while antibodies against the collagen receptors, $\alpha_1\beta_1$ and $\alpha_2\beta_1$, inhibit VEGF-driven angiogenesis [91].

A role for VEGF-induced p38 MAP kinase activation in endothelial cell migration is suggested by the finding that the p38 kinase inhibitor SB203580 inhibited actin reorganization and cell migration whereas the MEK inhibitor PD98059 had no effect on these biological effects [92]. NO may also play a role in VEGF-induced endothelial cell migration (Fig. 3). NO regulates non-chemotactic movement (podokinesis) of endothelial cells and VEGF-induced endothelial cell migration [93], and has been shown to regulate focal adhesion integrity and FAK tyrosine phosphorylation in endothelial cells [94]. Akt-dependent phosphorylation of eNOS at serine 1177 was also shown to be required for VEGF-induced cell migration [95].

Small GTPases of the Rho family play important roles in cell migration and a variety of other cellular functions. VEGFR2 mediates activation of RhoA and Rac1, and dominant negative RhoA and Rac1 mutants inhibit the migration of

HUVECs in response to VEGF. The mechanism involved in VEGFR2-mediated Rho/Rac activation is unclear, but VEGF-induced Rho A activation and migration of HUVECs required residue Tyr 951 in VEGFR2 and were inhibited by antisense oligonucleotides to the heterotrimeric G protein Gq/11 [96].

PLC-γ is also strongly implicated in mediating cellular responses linked to cell migration, including changes in actin organization and focal adhesion turnover [3], though its role in the endothelial chemotactic response to VEGF has not yet been ascertained. VEGF also induced activation of phospholipase D in HUVECs via a pathway sensitive to the PKC inhibitor Ro 31-8220 [97]. Given that a role has been proposed for PLD in cytoskeletal rearrangement, these findings suggest that PLD could mediate cell migratory responses to VEGF.

Role of NO and PGI_2 signalling in VEGF biological function

NO is strongly implicated in mediating several biological effects of VEGF. VEGF induces NO production [98, 99], and disruption of the mechanisms governing NO generation selectively impairs VEGF-dependent endothelial functions and angiogenesis *in vivo*. Inhibitors of eNOS selectively inhibited VEGF-induced neovascularisation in the rabbit corneal implant model but did not affect FGF2-induced angiogenesis [100]. Inhibition of eNOS also attenuated VEGF-stimulated proliferation of coronary post-capillary venule endothelial cells [101]. As already discussed, NO may be a permissive factor in VEGF-induced endothelial cell migration [93, 94], and Akt-dependent phosphorylation of eNOS at serine 1177 is required for VEGF-induced cell migration [95].

NO is an important regulator of vascular tone, and administration of VEGF causes relaxation of coronary arteries and hypotension mediated at least in part via increased NO production and VEGFR2 [102–104]. Mice deficient in the constitutive or endothelial NOS isoform exhibit sustained pulomonary hypertension [105], but otherwise have no overt vascular phenotype, though this may reflect redundancy and compensation by other NOS isoforms. It seems likely that NO plays a local regulatory role in VEGF-dependent neovascularisation or other vascular functions which may be significant during development and in disease-associated angiogenesis in the adult.

There is also evidence for an involvement of cyclooxygenases and their products in angiogenesis. VEGF is a strong inducer of endothelial PGI_2 and PGE_2 production [47, 61, 74], and upregulates COX-2 expression [74]. Non-specific COX inhibitors and isoform-specific COX inhibition suppressed *in vitro* endothelial cell angiogenesis. Colon tumours are frequently associated with elevated expression of the inducible COX-2 isoform, and non-steroidal anti-inflammatory drugs (NSAIDS) such as aspirin, which are known to inhibit COX, exhibit striking anti-neoplastic activity in experimental animal tumour models and inhibit endothelial cell tubule formation induced by colon cancer cells in co-culture [106]. COX-2-deficient female mice are infertile due to

abnormalities in ovulation, ferltilisation, implantation and decidualisation. These defects are partly due to down-regulation of blastocyst $VEGF_{164}$ expression and a failure of COX-2-derived PGI_2 to promote vascular permeability and angiogenesis [107].

Permeability signalling

A role for Src in regulating vascular permeability is indicated by the finding that mice deficient in either $pp60^{c\text{-}src}$ or the β_5 integrin subunit, but not β_3-deficient mice, display a reduced vascular permeability response to VEGF [85]. Roles for NO and PGI_2 in regulating vascular permeability are supported by the observations that COX and eNOS inhibitors reduced permeability changes induced by VEGF *in vivo* [108]. PLCγ tyrosine phosphorylation, mobilization of intracellular Ca^{2+} and PKC activation are also implicated as effectors of VEGF-induced vascular permeability in an assay of venular permeability [109].

Cellular mechanisms that have been proposed to mediate VEGF-induced permeability include increased formation of caveolae, small vesicles called vesicular-vacuolar organelles, and fenestrae, specialized regions of the plasma membrane highly permeable to small solutes and abundantly present in the endothelia of kidney glomeruli and a few other specialized vascular beds [110, 111]. A dominant negative Rac mutant prevented VEGF-induced endothelial fenestration and converted leaky vascular plexi into well-defined vascular networks [112]. Otherwise, the signalling pathways mediating these processes are poorly understood.

The integrity of intercellular junctions plays an important role in regulating endothelial paracellular permeability. Phosphorylation of endothelial intercellular junctional components may be a mechanism through which cell-cell adhesions are weakened, leading to increased vasopermeability. Adherens junctions comprise VE-cadherin associated via its intracellular domain with β and γ catenin and $p120^{cas}$ to form a complex that is linked to the actin cytoskeleton via interactions with α catenin [113]. VEGF increases phosphorylation of VE-cadherin [114], and β-catenin [115], and of the tight junction proteins, occludin and zona occludens protein 1 [116].

VEGFR1 signalling

Despite the critical role of VEGFR1 in angiogenesis revealed by VEGFR1-null mice, it has proved extremely difficult to establish unequivocally that VEGFR1 or its ligands mediate biological effects in endothelial cells (Fig. 2). Earlier studies largely failed to find evidence for PlGF-stimulated cellular functions such as migration or proliferation in endothelial cells naturally expressing VEGFR1, and VEGF has relatively weak biological effects in

PAE/VEGFR1 cells. The best-characterized biological responses mediated by VEGFR1 are the stimulation of migration and increased tissue factor expression in monocytes [117], though these have not yet been associated with any intracellular signalling events, or biological role *in vivo*. VEGFR1 has also been implicated in the differentiation of osteoclasts from haematopoietic cells of the monocyte-macrophage lineage. VEGFR1 is expressed in blood monocytes in the absence of VEGFR2 and VEGFR1-positive monocytic cells differentiate into multinuclear osteoclasts when stimulated with macrophage-colony stimulating factor and the osteoclast differentiation factor, RANK ligand [118]. Furthermore, VEGFR1 mediates FAK tyrosine phosphorylation and chemotaxis in the Raw 264.7 osteoclast precursor cell line [119]. VEGFR1 also mediates VEGF induction of MMP-1, -3 and -9 expression in VSMC [120].

Determining whether VEGFR1 is able to transduce a biologically meaningful signal has proved an equally elusive goal. VEGFR1 is a weaker kinase than VEGFR2 [30, 33], and though specific phosphorylation sites have been identified at tyrosines 1169, 1213, 1242, 1327 and 1333, their biological functions are unclear [121]. Tyrosines 1169 and 1333 in the carboxyl terminal tail region of VEGFR1 have both been reported to mediate binding of PLC-γ, and VEGFR1 can mediate tyrosine phosphorylation of PLC-γ and activation of ERK [121, 122]. However, several studies have yielded varying and sometimes conflicting results regarding activation of signalling pathways in VEGFR1-expressing cells. Whereas VEGF caused activation of PLC-γ but weak activation of ERK in VEGFR1-expressing NIH3T3 cells [45], another investigation in VEGFR1-expressing PAEC showed ERK activation but no effect on PLC-γ activity in response to PlGF [123]. VEGFR1 supports Ca^{2+} fluxes in *Xenopus laevis* oocytes [30] and VEGFR1 has been reported to mediate Ca^{2+} mobilization in trophoblast cells [124], but PlGF and VEGFR1 did not mediate Ca^{2+} mobilization in HUVECs [46]. VEGFR1 was also reported to interact with the p85 subunit of PI3K in a yeast two-hybrid system [125], but so far this has not been associated with a biological activity. A study of VEGF mutants with high specificity for either VEGFR2 or VEGFR1 concluded that stimulation of VEGFR2 was sufficient for mitogenesis, signalling via PLC-γ and PI3K, and for increased permeability [126].

Overall, it can be concluded tentatively that VEGFR1 is able to mediate limited signalling initiated by VEGF, particularly via PLC-γ, but does so more weakly than VEGFR2, probably as a result of a weaker intrinsic kinase activity in VEGFR1. The specific VEGFR1 ligand, PlGF, also seems to be noticeably weaker than VEGF in stimulating signalling pathways, and defined physiological roles for either PlGF or VEGFR1 signalling have not been unambiguously established.

While it remains controversial whether VEGFR1 can independently transduce a biological signal, current thinking favours the view that, at least during embryogenesis, VEGFR1 functions primarily as a “decoy” receptor that negatively regulates VEGFR2-mediated actions of VEGF [1]. Whereas

VEGFR2-deficient mice produce neither differentiated endothelial cells nor haematopoietic precursors [34], the primary defect in VEGFR1 null mice is an over-production of endothelial progenitors [35, 36], resulting in a lethal disorganisation of vessels and consistent with VEGFR1-mediated dampening of signalling leading to progenitor production. Surprisingly, mice expressing only the extracellular region of VEGFR1 and lacking its kinase domain develop normally [127], indicating that VEGFR1 intracellular signalling is entirely dispensible for embryonic angiogenesis. VEGFR1-mediated signalling and biological function is constitutively suppressed by a motif within the juxtamembrane domain [128], and VEGFR1 may also directly modulate VEGFR2-mediated biological responses [129]. Furthermore, activation of a VEGFR1 kinase domain chimera with colony-stimulating factor 1 (CSF-1) suppressed ERK activation and proliferation mediated via a VEGFR2/CSF-1 chimera [130]. The extracellular domain of VEGFR1 is also expressed naturally in soluble form, sFlt1, and inhibits angiogenesis by forming an inactive complex with VEGF [131].

New insights into how VEGFR1 and PlGF may function in the adult animal have come from *in vivo* studies pointing to positive roles for PlGF and VEGFR1 signalling in pathophysiological angiogenesis and recruitment of endothelial progenitors. Earlier findings provided evidence for the formation of biologically active VEGF/PlGF heterodimers [21]. Angiogenesis in experimental tumours was higher, and Lewis lung carcinoma cells overexpressing PlGF-2 grew much faster, in wild-type mice compared to mice lacking the VEGFR1 kinase domain [132]. PlGF can induce revascularisation of ischaemic heart and limbs, and anti-VEGFR1 antibody inhibits neovascularisation in the ischemic retina and tumour growth in nude mice [22]. Cooperation between VEGF and PlGF in pathophysiological angiogenesis is supported by the finding that PlGF-deficient mice display impaired neovascularisation specifically induced by VEGF during ischaemia, inflammation, wound healing and cancer [133]. Combinations of VEGF and PlGF also synergistically evoke biological responses in PlGF-deficient cells, including survival, migration and proliferation, that are not evinced by wild-type cells [133]. PlGF may enhance VEGF-driven angiogenesis via cross-talk between VEGFR1 and VEGFR2 involving either direct or indirect intermolecular phosphorylation of VEGFR2 [134]. These interactions are manifested only in PlGF-deficient endothelial cells, and appear to be unmasked by the lack of endogenous PlGF, since $PlGF^{+/+}$ cells are less responsive to exogenous PlGF. PlGF-activated VEGFR1 also induced Akt activation and a distinctive pattern of gene expression in $PlGF^{-/-}$ cells [134]. PlGF also reconstitutes haematopoiesis by recruiting $VEGFR1^{+}$ cells from the bone marrow [135]. These findings provide the strongest evidence yet that PlGF can signal via VEGFR1 and raise the intriguing possibility that activation of VEGFR1 by VEGF or PlGF may have distinct biological consequences.

Neuropilin

Neuropilin-1 (NP-1) is a non-tyrosine-kinase receptor for the $VEGF_{165}$ isoform (K_d 0.3 nM) [136, 137], and also binds the heparin-binding PlGF-2 isoform, VEGF-B, and VEGF-E [138, 139], but does not recognize $VEGF_{121}$ or other VEGF family members. NP-1 is expressed in endothelial cells, several tumour cell types and in certain neurons and was originally identified as a receptor for semaphorin 3A, a member of a family of polypeptides involved in axonal guidance and patterning [140, 141]. NP-1 consists of a short cytoplasmic domain, a single transmembrane domain and a large extracellular region comprising a membrane proximal C domain which shares homology with MAM (meprin, A5, μ) domains found in meprin metalloproteinases and the amino-terminal domain of the receptor tyrosine phosphatase μ, tandem b1 and b2 domains related to the C1 and C2 domains of coagulation factors VIII and V, and tandem CUB domains (a1 and a2) homologous to the non-catalytic regions of complement components C1r and C1s (Fig. 1). A related molecule, NP-2, has the same domain structure and shares 44% overall amino acid identity with NP-1 [141].

Overexpression of NP-1 in mice results in increased capillary formation, vasodilatation and malformation of the heart [142], whereas mice deficient in NP-1 exhibit defects in embryonic axonal patterning and vascular abnormalities including defective development of large vessels and impaired neural and yolk sac vascularisation [143]. Inactivation of both NP-1 and NP-2 causes a more severe failure of embryonic vascularisation resulting in death at E8.5 [144]. Co-expression of NP-1 with VEGFR2 in endothelial cells increased binding of $VEGF_{165}$ 4-fold and enhanced chemotaxis and mitogenicity of $VEGF_{165}$ [137]. However, despite the evidence that NP-1 is essential for normal vascular development, the underlying mechanisms remain obscure, and the role of NP-1 in the endothelial functions of VEGF are not fully understood.

The small size of the NP-1 cytoplasmic domain and the lack of any associated signalling function suggests that NP-1 by itself is not a functional receptor but acts as a co-receptor. However, a conserved carboxy-terminal PDZ-binding motif in the NP-1 cytosolic domain associates with Neuropilin-1-interacting Protein-1 (NIP-1), a PDZ domain protein identical to RGS-GAIP-interacting protein (GPIC), synectin and the semaphorin F-binding protein Semcap1 [145]. Whether interactions between synectin, or other PDZ domain proteins, and NP-1, transduce biological signalling is as yet unclear.

Heparan sulphate proteoglycans

Heparan sulphate proteoglycans (HSPGs) may act as additional low-affinity binding sites for VEGF, which are able to modulate binding of VEGF to its receptors. Low concentrations of heparin have been reported to augment the binding of VEGF to HUVECs, while higher concentrations reduced binding [146]. The glycosylphosphatidylinositol anchored HSPG, Glypican-1, has also

been reported to restore VEGF binding to heparinase-treated cells [147]. Evidence for an essential role of HSPGs in VEGF receptor signalling and biological function similar to that for FGF-2 discussed below, is so far lacking.

FGFs and their receptors

The FGF family consists of at least 20 structurally related polypeptides, of which FGF-2 (basic FGF), FGF-1 (acidic FGF) and FGF-4 are angiogenic and stimulate arteriogenic collateral vessel growth *in vivo* [2]. The best-studied FGF, FGF-2, can directly stimulate angiogenesis in a variety of experimental animal models and elicits diverse biological effects in cultured endothelial cells [148, 149]. Uncertainty has persisted regarding a crucial physiological role for FGFs in angiogenesis because of the absence of a classical secretory signal sequence in FGF-1 and FGF-2 and the unambiguous demonstration of a vascular phenotype in knock-outs of either FGF-2 or its major receptor, FGFR1. FGF-2-deficient mice are viable and phenotypically normal but display an unexpected defect in vascular tone characterized by decreased vascular smooth muscle contractility, low blood pressure and thrombocytosis [150]. The absence of a stronger phenotype in these mice may be due to redundancy and compensation among the different members of the FGF family. Targeted gene disruption of FGFR1 casuses embryonic death prior to gastrulation and in advance of the earliest stages of vascular development making it difficult to assign more specific roles for this receptor in angiogenesis [151]. However, embryonic overexpression of a dominant negative FGFR1 mutant prevents normal development and maintenance of the vasculature, suggestive of a role for signalling through this receptor in blood vessel formation [152].

The four structurally-related FGF receptors so far identified, FGFRs 1–4, comprise an extracellular ligand-binding region containing three Ig-like domains, a single transmembrane domain and an intracellular kinase domain divided into two parts by a kinase insert ([153]; see Fig. 1). FGFR1 binds FGF-1 (acidic FGF) and FGF-2 with high affinity and FGF-4 with lower affinity, FGFR2 binds all three with equal affinity, FGFR3 binds FGF-1 with high affinity and FGF-2 with reduced affinity, and FGFR4 binds FGF-1 and FGF-4 with high and FGF-2 with lower affinity. An important difference between FGFs and VEGF is that FGFs are monomeric and require low-affinity heparin or HSPG receptors to promote FGFR dimerisation and activation [154, 155]. The HSPG, syndecan-4, acts as a co-receptor for FGF-2 and enhances FGF-2 stimulation of endothelial cell growth and migration [156].

FGF-2 and FGFR1 signalling

The major autophosphorylation sites in FGFR1 are Y463 located in the juxtamembrane region, Y583/Y585 in the kinase insert region, Y653/654 in the

kinase domain, Y730 and Y766 in the carboxyl-terminal tail [149]. Y463 mediates binding to the SH2 domain adapter protein Crk [157], Y653/Y654 are critical for kinase activity and Y766 is responsible for binding to PLC-γ [158].

Like VEGF, FGF-2 is a strong activator of ERKs1 and 2. The mechanism of FGF-2-induced ERK activation differs from that of other RTKs in that it is not mediated by a direct association between FGFR1 and GRB2, but by phosphotyrosine-independent binding of the phosphotyrosine binding (PTB) domain of a 90 kDa protein called FGFR substrate (FRS)2 to the juxtamembrane domain of FGFR1 (Fig. 4). Subsequent tyrosine phosphorylation of FRS2 in turn results in binding of GRB2 and SHP-2, and the formation of this complex is necessary for ERK activation [159–161]. The SH2 domain adapter protein, Shb, binds to Y766 in FGFR1 and this association appears to regulate FRS2 tyrosine phosphorylation and is essential for FGFR1-mediated ERK activation [162]. In contrast to VEGF, activation of PKC appears to play no role in FGF-2-induced ERK activation [61]. The finding that FGF-2-mediated neovascularisation in the chicken chorioallantoic membrane (CAM) assay is blocked by the MEK inhibitor PD98059, indicates an essential role for ERK activation in the angiogenic response to FGF-2 [163]. FGFR1-mediated acti-

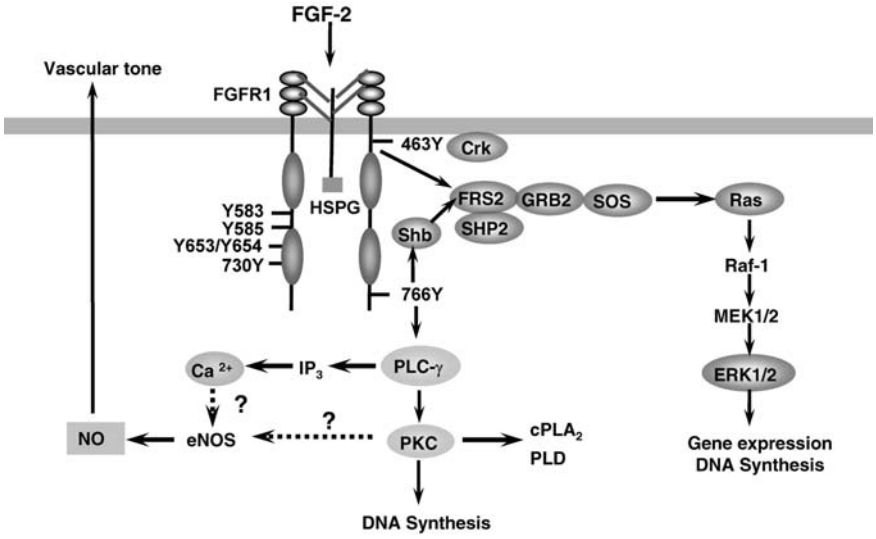

Figure 4. FGF-2/FGFR1 endothelial signalling. Dimerisation and activation of FGFR1 by FGF-2 requires HSPGs. FGFR1 is phosphorylated at multiple sites, most of which do not have a defined function. The SH2 domain adapter protein Crk associates with the juxtamembrane tyrosine 463. Tyr 766 mediates binding to PLC-γ. Binding of the adapter protein, FRS2, to the juxtamembrane region of FGFR1 via a phosphotyrosine-binding (PTB) domain in FRS2, mediates recruitment of GRB2 and ras-dependent activation of ERKs. Binding of the SH2 domain adapter protein, Shb, to Tyr 766 regulates FRS2 tyrosine phosphorylation and ERK activation. FGF-2 induces NO generation, but the mechanism involved is unclear, though PLC-γ mediated PKC activation and increased intracellular Ca^{2+} are implicated. (For colored picture see color plate 11)

vation of p70 S6 kinase has been implicated in regulation of endothelial proliferation [164].

Binding of PLC-γ to Y766 results in its tyrosine phosphorylation and activation leading to PKC activation and production of inositol 1,4,5-trisphosphate (Fig. 4). Several studies show that PLC-γ activation is not required for FGF-2-induced mitogenesis or chemotaxis [165, 166]. Nevertheless, a requirement for PKC activation in FGF-2-induced endothelial cell proliferation has been demonstrated [167]. Furthermore, Y766 is required for stimulation of PLD, PLA_2 and PI3K activity, and mutation of this residue dramatically alters the effect of FGF-2 on cytoskeletal organization in endothelial cells [168] and reduces ERK activation as a result of a reduced association with FRS2 [160].

In addition to those already highlighted, a comparison of FGF-2 and VEGF signalling reveals several differences. A dominant negative Src cytoplasmic tyrosine kinase had no effect on FGF-2-induced angiogenesis in the CAM model system, but inhibited VEGF-induced angiogenesis [85]. Though FGF-2 has been reported to cause $cPLA_2$ and PLD activation, it is a weak inducer of endothelial PGI_2 generation in HUVECs compared with VEGF [61]. It is also unclear whether FGF-2 and FGFR1 can activate the PI3K/Akt pathway, which plays key roles in the anti-apoptotic survival functions of VEGF and the control of eNOS activity. FGF-2 is able to stimulate endothelial NO production [169], and cells from mice transgenically expressing syndecan-4 display enhanced NO generation in response to FGF-2, but not $VEGF_{165}$ [170].

Angiopoietins and Tie receptors

The angiopoietin (Ang) family of angiogenic factors comprises Ang1 to Ang4 [171]. Angiopoietins bind specifically to the Tie (also known as Tek) tyrosine kinase receptor family, but with different effects. Ang1 and Ang4 are agonistic ligands for Tie2, whereas Ang2 and Ang3 are antagonists for this receptor [172–174]. Despite intensive efforts, no ligand has yet been identified for Tie1. Tie receptors have an extracellular domain comprising three fibronectin type III homology domains, and one complete and one partial Ig-like domain separated by three epidermal growth factor-like cysteine-rich motifs, a single transmembrane domain, and an intracellular region consisting of a tyrosine kinase domain split into two by a kinase insert region (Fig. 1).

Targeted gene disruption in mice has established distinct roles for Ties and their ligands in vascular development, but a unifying feature in the phenotypes of knock-out and transgenic studies of components of the Angiopoietin/Tie axis is vascular instability. Tie2-deficient embryos die between E9.5 and E12.5, the most striking defect being incomplete cardiac development characterized by a failure of the endocardium to adhere to the myocardial wall [175, 176]. $Tie1^{-/-}$ mice die later, between E13.5 and birth, and exhibit normal cardiac development indicating a role for this receptor at a later stage of embryogenesis [176]. Endothelial cells in the blood vessels of embryos lacking either

Ang1 [177] or Tie2 do not associate with support cells, and conditional loss of Tie2 *in vivo* increases endothelial cell death and haemorrhage [178]. Overexpression of Ang1 in skin increases formation of non-leaky blood vessels that are resistant to the permeability-increasing effects of VEGF [179, 180]. These findings strongly suggest a role for Ang1 and Tie2 in the stabilisation of developing blood vessels partly via direct effects on endothelial cells but also by promoting or maintaining interactions between endothelial and periendothelial pericytes and VSMC.

Tie receptor signalling

In common with many other receptor tyrosine kinases, Tie2 associates with the SH2 domain adapter protein GRB2 [181, 182]. Though a major function of GRB2 is to link tyrosine kinases to ras-dependent activation of ERKs, surprisingly, Ang1 is unable to activate ERKs 1/2 and is not mitogenic for endothelial cells [183, 184]. One of the most striking biological responses of endothelial cells to Ang1 is increased survival [185, 186]. Consonant with this, Ang1 stimulates several anti-apoptotic signalling pathways including PI3K-dependent Akt activation [187, 188], and upregulation of the IAP protein, survivin [189]. Tie2-mediated activation of the PI3K/Akt pathway is dependent on a multisubstrate docking site on Tie2 at Tyr1101 (Fig. 5) responsible for association of the p85 subunit of PI3K [187]. The importance of the survival function of Ang1 and Tie2 in vascular development *in vivo* is highlighted by the loss of endothelial cells that occurs in mice deficient in Tie2 [175, 178].

Several studies show that Ang1 stimulates endothelial cell migration [184–186] and that this function is important *in vivo* for vessel sprouting and vascular remodeling during development [175–177]. Ang1-induced endothelial cell sprouting in cultured porcine pulmonary arterial endothelial cells occurs via a PI3K-dependent increase in FAK tyrosine phosphorylation and induction of the proteolytic enzymes plasmin and MMP-2 [190]. Recruitment of the downstream-of-kinase-related (Dok-R) docking protein to Tie-2 also plays a key role in migratory responses to Ang-1 [191–193]. Dok-R binding to Tie2 and tyrosine phosphorylation results in recruitment of the SH2 domain adapter protein Nck and the p21-activating kinase, Pak1 [192]. Overexpression of either Dok-R or Pak increases Ang-1-induced migration. The PTB and plekstrin-homology domains of Dok-R mediate association with residue Y1106 of Tie-2 (Fig. 5) and mutation of this residue blocks both Ang1-stimulated Dok-R phosphorylation and restoration of the migratory potential of Tie2-deficient endothelial cells [193]. Tie2 also associates with the adapter protein GRB7 via Tyr 1100 and the tyrosine phosphatase SHP2 via Tyr 1111 [181, 182], both proteins being implicated in chemotactic signalling via FAK (Fig. 5). Tie2 also associates with the receptor-type phosphatases, HPTCA and vascular endothelial protein tyrosine phosphatase (VE-PTP) [43, 194]. As in the case of VEGFR2 associations with SHP-2, interactions with tyrosine phosphatases

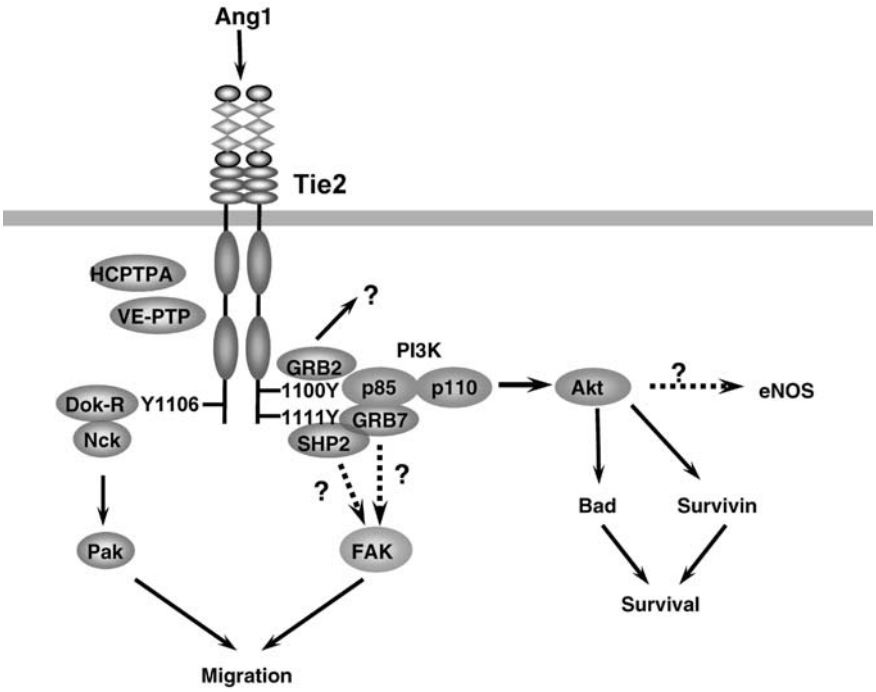

Figure 5. Tie signalling pathways. Angiopoietin (Ang)-1 is a major agonistic ligand for Tie2. Tyr 1100 in the carboxyl tail of Tie is a multifunctional docking site mediating association with the SH2 domains of the adapter proteins GRB2, GRB7, and the p85 subunit of PI3K. p85 binding mediates activation of the PI3K/Akt pathway which is responsible for survival functions of Ang1. Activation of eNOS via Akt is inferred, but not yet established. A function for GRB2 in downstream Tie2-mediated signalling has not yet been established, but Ang1 is not able to activate the ERK pathway. Tyr 1111 is also important for binding of the tyrosine phosphatase SHP2 and both SHP2 and GRB7 are implicated in activation of the FAK pathway that partly mediates effects of Ang1 on endothelial cell migration. The PTB and pleckstrin-homology domains of the downstream-of-kinase-related (Dok-R) docking protein mediate association with Tie2 residue Tyr 1106, resulting in recruitment of the SH2 domain adapter protein Nck and the p21-activating kinase, Pak1, a pathway that mediates Ang1-induced cell migration. Tie2 also associates with other SH2 domain proteins including the tyrosine phosphatases, HCPTPA and VE-PTP. (For colored picture see color plate 12)

have the potential to regulate Tie2 function through dephosphorylation at specific tyrosine residues forming binding sites.

The requirement of Tie1 for normal vascular development [176], and conservation of several signalling motifs in Tie 1 and Tie 2, suggests that Tie1 can transduce a signal, but the lack of a functional ligand has made investigation of Tie1 signalling problematic. The possibility cannot be precluded that, perhaps somewhat like VEGFR1, Tie1 may function primarily in a modulatory role and that the signalling function of its kinase domain is redundant. Tie1 does not appear to undergo tyrosine self-phosphorylation in endothelial cells under basal or stimulated conditions and activation of Tie2 is unable to cause

trans-phosphorylation of Tie1 [195]. Tie1 is itself subject to regulation through signalling pathways. PKC activation, inflammatory cytokines and VEGF can all induce MMP-mediated proteolytic cleavage of Tie1 *in vivo* to release a soluble extracellular domain [196–198]. Proteolysis is mediated by MMPs, and is followed by association between the intracellular domain of the truncated membrane-bound receptor and several tyrosine phosphorylated proteins including SHP-2 [198, 199]. The cleaved intracellular domain also heterodimerises with Tie2 [195], raising the possibility that Tie1 ectodomain cleavage can regulate Tie2 signalling.

Angiogenic regulation of gene expression

Individual studies have identified several VEGF-regulated genes, including interleukin-8, heparin binding-EGF-like growth factor (HB-EGF), COX-2 and Tissue factor [74, 200–202]. Upregulation of the transcription factor Ets1 has been implicated in VEGF-mediated cell migration [203], while Egr-1 and NFAT are implicated in the regulation of Tissue Factor and COX-2 expression by VEGF [74, 204, 205]. VEGF alone is unable to induce expression of functional Tissue Factor expression at the endothelial cell surface, but requires synergistic interaction with TNF-α to upregulate the protein [206, 207]. Whether NFAT or other transcriptional activators are essential for developmental or angiogenic effects of VEGF is as yet unclear.

Analysis of VEGF-regulated and angiogenesis-associated gene regulation using cDNA or oligonucleotide arrays identified several known VEGF-induced genes, including COX-2, HB-EGF, Tissue Factor and interleukin-8, as well as many genes not previously linked either to VEGF function or angiogenesis [208–212]. Interleukin-8, phospholipase A_2-γ, COX-2, the survival hormone stanniocalcin-1, and the blood proteinase regulator and VEGF-binding protein α2-macroglobulin, were also induced in mRNA profiling or cDNA microarray analysis of models of capillary morphogenesis [213–216].

Perspectives and concluding remarks

The preceding discussion has tried to highlight the multifunctionality and integration of VEGF signal transduction mechanisms. Cascades of kinases, biosynthetic enzymes and adapter proteins converge and branch at many points in functional VEGF signalling, emphasizing how linear pathways can integrate to form signal transduction networks (Fig. 3).

Though significant progress has been made towards mapping angiogenic signalling pathways, many challenges lie ahead. The biological and signalling roles of the VEGF receptors, VEGFR1 and neuropilin-1, and of Tie1 have not yet been clearly defined. It also remains to be resolved how intracellular signalling determines the distinct biological responses evoked by different angio-

genic factors such as VEGF and Ang1. At least part of the answer is likely to be that while individual signalling responses are common to different ligand receptor systems, the total complement of signalling cascades triggered by different factors is unique. A striking difference between VEGF and Ang1, for example, is the inability of Ang1 and its receptor Tie2 to activate the ERK pathway and its corresponding inability to promote cell proliferation [171]. Ang1 is able to promote Akt-mediated endothelial cell survival, but VEGF stimulates other signal transduction pathways, including PLC-γ activation, that so far have not been identified downstream of Tie2. These differences may help to explain the role of Ang1/Tie2 as a vascular stability factor, whereas VEGF alone produces unstable and leaky vessels. Similarly, though FGF-2 and VEGF signalling shows a significant degree of overlap particularly in the activation of the ERK and PLC-γ pathways, FGF-2 appears to stimulate a more restricted signalling programme than VEGF, and this may help to explain differences in the angiogenic roles of these factors and in the characteristics of the vessels they produce (compare Figs 3–5). While distinct signalling repertoires are likely to play a major role in producing specific biological effects, further fine-tuning may be determined by differences in the amplitude and duration of particular signalling events, synergistic or regulatory interactions between them, and by spatio-temporal regulation of expression of angiogenic factors and their receptors. Analysis of differences in the gene expression profiles of different angiogenic factors may also provide an important key to understanding the molecular basis of functional specificity in angiogenesis. Little is known about such differences, but at least one comparison between VEGF and FGF-2 revealed very restricted overlap in gene expression [211]. Genomic analysis of high-density arrays complemented by studies of the factor-specific proteomes of endothelial cells and vessels will clearly help to answer this question.

Another key challenge is the identification of signalling important for endothelial cell differentiation and the developmental demarcation of arteries and veins. The transmembrane ligand ephrin B2 is specifically expressed in developing arteries while its tyrosine kinase receptor EphB4 is more restricted to veins [217, 218]. Targeted disruption of either ephrin B2 or ephB4 results in a similar phenotype characterized by a range of vascular abnormalities [218, 219]. Loss of signalling through the Notch receptor causes defects in arteriovenous differentiation in Zebra fish [220], while VEGF functions upstream of Notch and downstream of the secreted polypeptide, sonic hedgehog, in determining arterial endothelial differentiation [221]. These findings suggest that arteriovenous differentiation involves a complex interaction between several distinct signalling systems.

Much also remains to be learned about how early signalling events in angiogenesis are hard-wired to cellular functions and long-term biological responses. Gene (and protein) expression will also play a key role in this respect. The step-change in knowledge generated by current genomic and future proteomic analysis is likely to transform our understanding of angiogenic signalling, by

providing crucial insights into the mechanisms underlying both the distinct biological roles of key factors, and how these mechanisms are linked and coordinated to form the system of integrated signalling networks necessary for blood vessel formation.

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Index

Color plates

Color plate 1

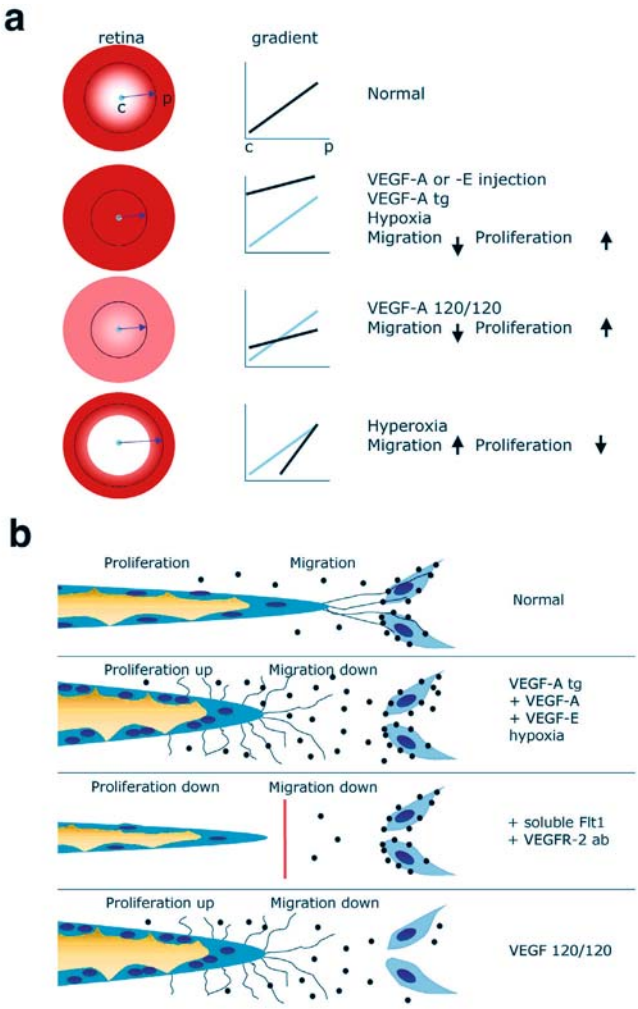


Figure 1 from page 8

(a) Graphical representation of retinas with the graded distribution of VEGF-A in red. C represents the retinal center (optic nerve and vessel entrance) from which vessels sprout towards the periphery (p). The blue circle and its radius (arrow) represent the distance of vessel spreading at approximately postnatal day 5 in the mouse retina in the various experimental situations indicated to the right (referred to as migration). The graphs in the middle illustrate how the VEGF gradients change across the retinal radius (central to peripheral) in the experimental situations indicated. Proliferation implies the increase in cell number in the sprout stalks. (b) Schematic illustration of sprout morphology in the various experimental conditions. Black dots represent VEGF-A molecules and their distribution. The VEGF-secreting cells are shown ahead of the sprout and the distribution and orientation of the filopodia at the tip and in the stalk is shown.

Color plate 2

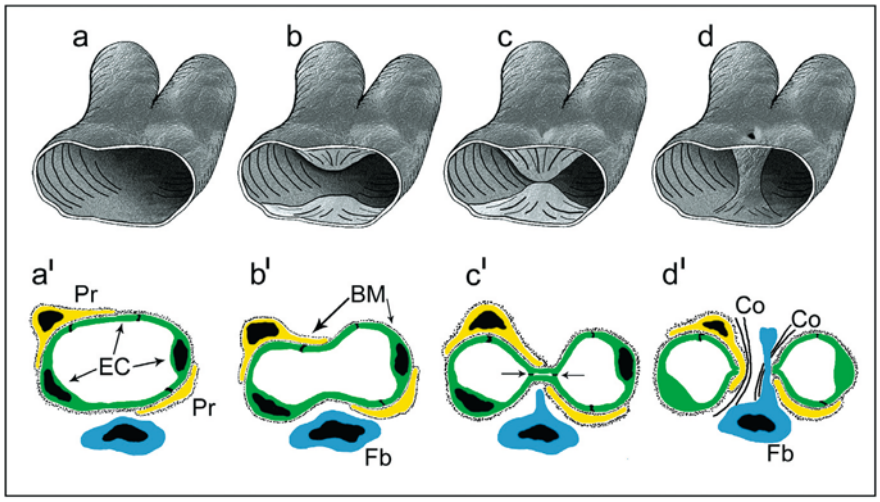
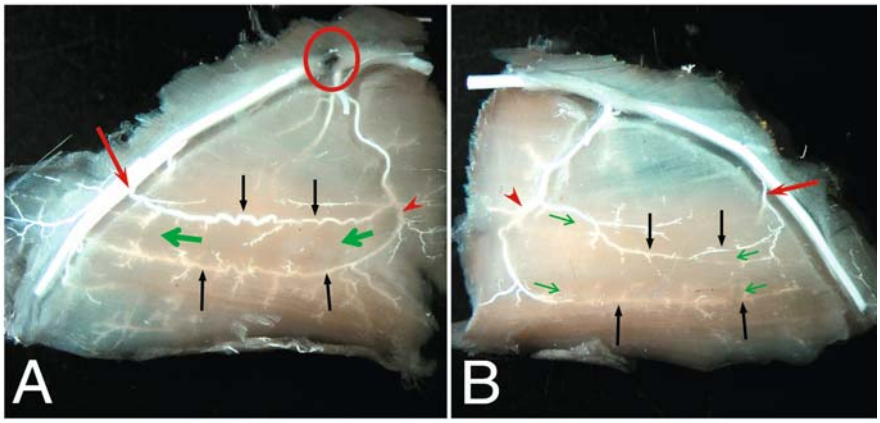


Figure 1 from page 19
Three-dimensional schematic illustrating the steps in the generation of new vascular segments by intussusceptive growth. The process begins with the protrusion of portions of the walls from opposite sides into the vessel lumen (a, b). After contact has been established and fortified (c), the endothelial bilayer becomes perforated centrally and a transluminal pillar is formed (d).
a'–d': Two-dimensional representation of the events depicted in a–d. Endothelial cells (EC) situated on opposite sides of a capillary protrude into its lumen until they contact each other (a'–c'). Once established, this contact is fortified by the formation of interendothelial junctions and then reorganized in such a manner that the endothelial bilayer is perforated centrally. The endothelial cells then retract, and the newly formed pillar increases in girth after being invaded by fibroblasts (Fb) and pericytes (Pr), which lay down collagen fibrils (Co in d').

Color plate 3



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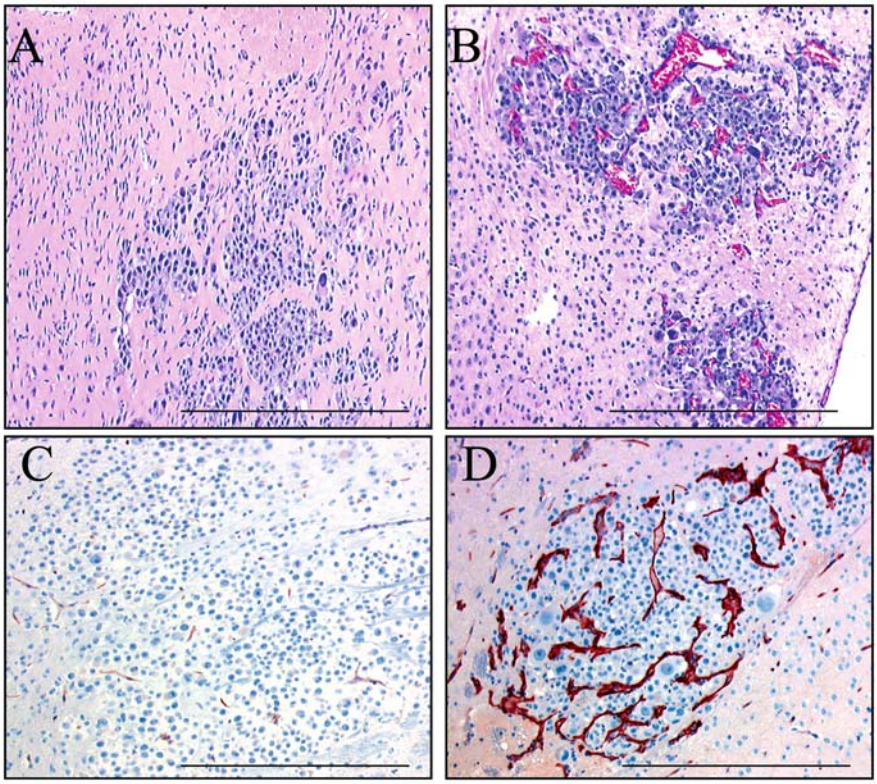
Microphotographs of murine adductor muscles containing collateral arteries filled with bismuth/gelatin contrast agent. In a physiologically normal limb (B), collateral arteries (black arrows) can be identified as small arteries connecting the distal femoral artery (red arrow) to the arteria profunda (red arrowhead). Small green arrows show the direction of the blood flow. When the femoral artery is occluded (A, red circle), the blood flowing through the collateral arteries changes direction and intensity. Two weeks after surgery, the collateral arteries (black arrows) have significantly grown in size and have acquired a corkscrew appearance.

Color plate 4



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Representative example of an infiltratively growing tumor in mouse brain. Mel57 human melanoma cells were injected in the internal carotid artery of mice as described [16]. After 3 weeks animals were sacrificed, the brains removed, fixed in buffered formalin and slices subjected to H&E staining (A) or CD34 endothelial staining (C). Blood vessels in these tumors are all pre-existent, as illustrated by the presence of an intact blood brain barrier and lack of upregulation of CD34 and other endothelial markers (C, and not shown). B/D: Results from a similar experiment, but now with Mel57 cells that were stably transfected with VEGF165. Note the highly dilated vessels in the VEGF-expressing tumors. B) H&E staining, D) CD34 endothelial staining. Bars represents 500 μm. Original magnification: 250 x.

Color plate 5

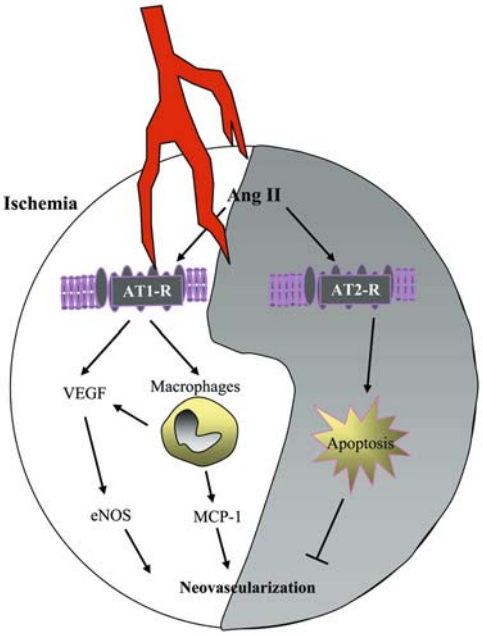


Figure 1 from page 84
Ang II and post-ischemic neovascularization. AT1 receptor positively regulates the angiogenic process in the setting of ischemia. Conversely, AT2 receptor activation hampers new vessel growth, suggesting that Ang II may tightly regulate the revascularization reaction.

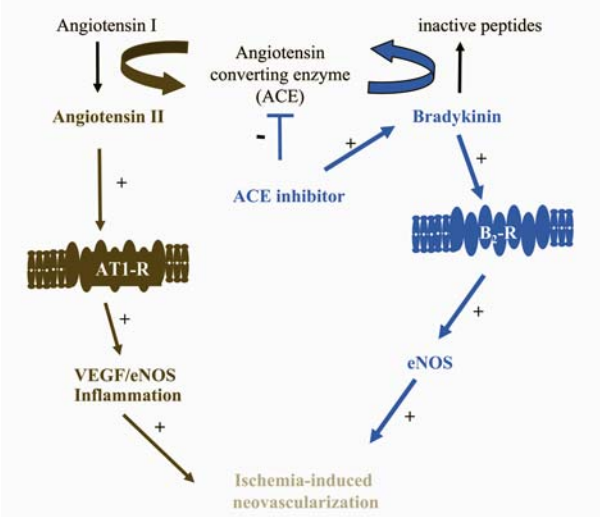
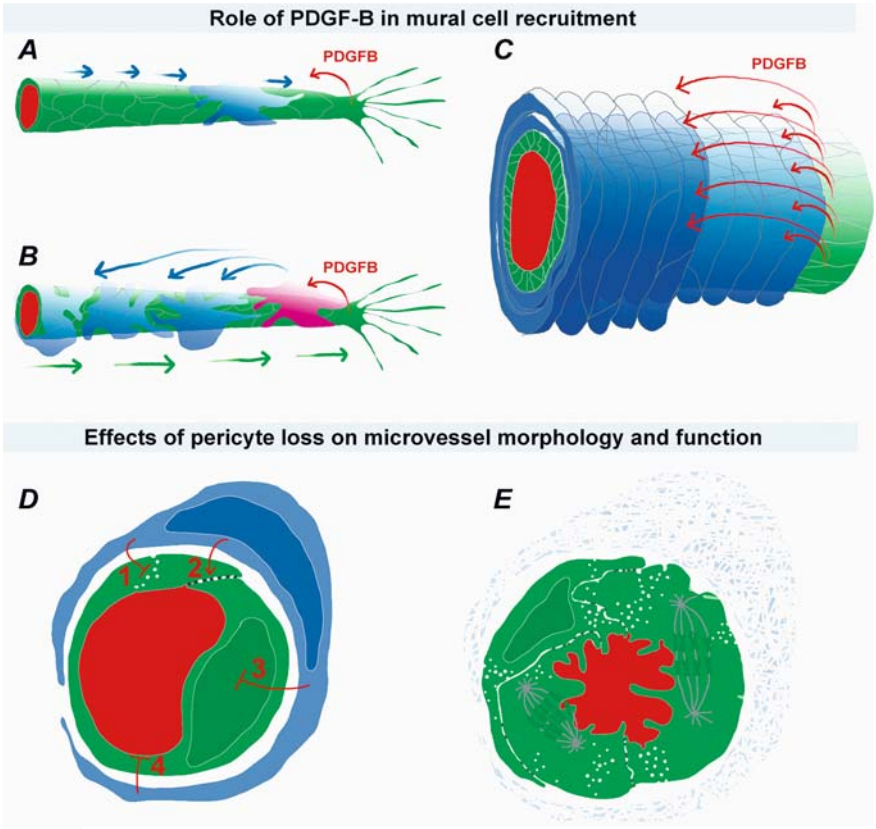


Figure 2 from page 88
The ACE inhibitor paradox. Ang II has been shown to stimulate the angiogenic process through AT1 stimulation and activation of VEGF and inflammation-related pathways. ACE inhibitor inhibits Ang II formation but also blocks the breakdown of bradykinin. Activation of the bradykinin signaling may likely account for increased neovascularization in response to ACE inhibition. AT1, Angiotensin II receptor type I; B2R, Bradykinin receptor type 2.

Color plate 6



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Role of PDGFB in mural cell recruitment and effects of pericyte loss on vessel morphology and function.

A) Schematic model of PDGF-B expression and function in pericyte recruitment in conjunction with angiogenic sprouting. PDGF-B expressed by the endothelial tip cell promotes pericyte co-migration. B) Alternative angiogenic scenario: the leading pericyte (pink) "hitch-hikes" with the migratory tip cell and responds to tip-cell derived PDGF-B by proliferation, leaving daughter cells behind to invest the elongating sprout. C) Role of PDGF-B in arterialization. PDGF-B produced by the endothelium stimulates mural cell proliferation thereby increasing their density and triggering organization in concentric layers typical of arterial vascular smooth muscle cells. D) Hypothetical role of pericytes in CNS microvessels deduced from mouse mutants of the PDGF-B/PDGFRß signaling pathway. Pericytes inhibit vesicle transport and transcytosis (1), stimulate maturation of endothelial junctions (2), inhibit endothelial proliferation (3) and inhibit endothelial luminal membrane formation (4). E) Absence of pericytes leads to increased vesicle transport and transcytosis, the formation of abnormal inter-endothelial junctions, increased endothelial cell proliferation and increased luminal membrane surface with numerous membrane folds.

Color plate 7

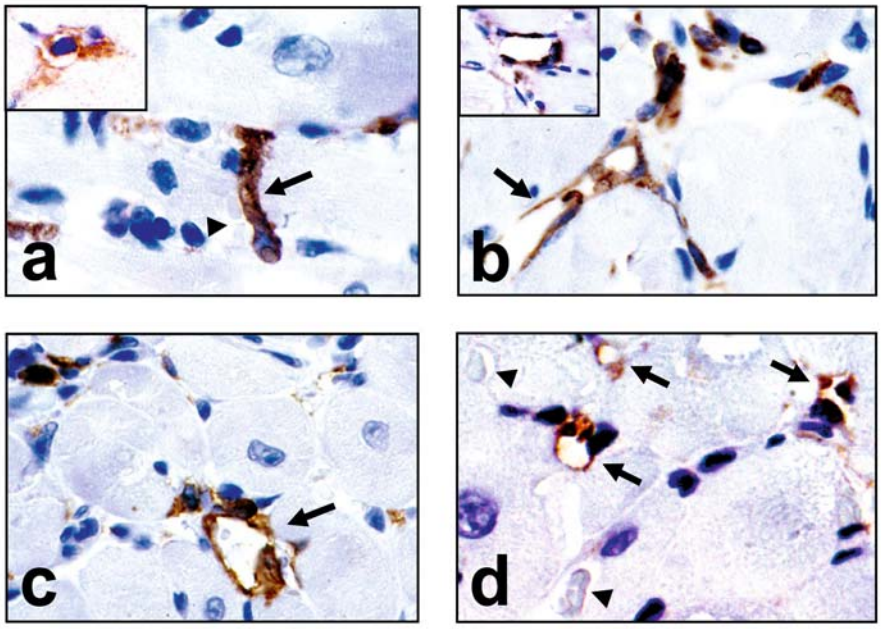

Figure 1 from page 135
Potential contribution of mononuclear leukocytes to formation of microvascular conduits in the hearts of transgenic mice expressing MCP-1 under a cardiac-specific promoter. (a) Structure positive for CD18 (pan leukocyte antigen, arrow) running in parallel with a capillary, identified by erythrocytes (arrowhead). Note the counterstained (blue) nucleus of a cell apparently penetrating the extracellular matrix. Insert: MC/MPh positive for MMP12 and presenting a surrounding proteolysis rim. (b) Space in the myocardium, lined by CD18 positive cells (arrow). Insert: a lumen edged by Mac-3 positive macrophages. (c) Erythrocytes present within a CD18 positive microvascular structure (arrow). (d) Structures populated by CD34 positive cells (arrows). Note that bona fide capillaries (identified by erythrocyte content, arrowheads) are CD34 negative. All samples were analyzed by DAB immunocytochemistry (brown) with hematoxylin counterstaining (blue). Negative controls (omission of the primary antibody) were all negative (not shown). Original magnifications: x120. Reproduced with permission from [102].

Color plate 8

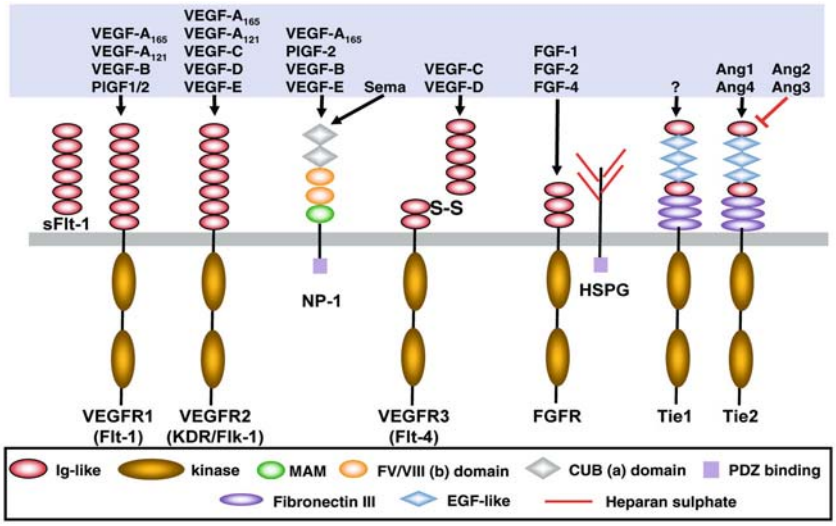

Figure 1 from page 270

Angiogenic factors and receptors. VEGF (VEGF-A) binds to two related tyrosine kinase receptors, VEGFR1 (Flt-1) and VEGFR2 (KDR/Flk-1), each possessing an extracellular domain containing seven Ig-like loops, a single hydrophobic membrane-spanning domain, and a large cytoplasmic domain comprising a single catalytic domain containing all the conserved motifs found in other RTKs which is interrupted by a non-catalytic region, called the kinase insert. The extracellular domain of VEGFR1 is also independently expressed as a soluble protein (sFlt-1). VEGFR3 (Flt-4), a receptor for VEGF-C and VEGF-D, undergoes proteolytic processing to yield disulphide-linked 120 and 75 kDa polypeptides. Neuropilin-1 (NP-1) is a non-tyrosine kinase receptor for $VEGF_{165}$, PlGF-2, VEGF-B and VEGF-E, but does not bind to $VEGF_{121}$. NP-1 has a short cytoplasmic domain containing a carboxyl-terminal PDZ domain-binding motif, a single transmembrane domain and a large extracellular region comprising a membrane proximal C or MAM (meprin, A5, γ) domain, tandem b1 and b2 domains related to the C1 and C2 domains of coagulation factors VIII and V, and tandem CUB domains (a1 and a2), homologous to regions of complement components C1r and C1s. NP-1 is also a receptor for semaphorins (sema). FGFs binds to a family of related RTKs, FGFRs1-4, consisting of an extracellular domain containing three Ig-like regions, a single hydrophobic membrane-spanning domain, and a split cytoplasmic tyrosine kinase domain. FGFs require heparan sulphate proteoglycans (HSPGs) for receptor dimerisation and activation. One HSPG co-receptor for FGF-2, syndecan-4, contains a carboxyl-terminal PDZ domain-binding motif. Angiopoietins bind to Tie2 receptors comprising an extracellular domain with three fibronectin type III homology domains, and one complete and one partial Ig-like domain separated by three epidermal growth factor-like cysteine-rich motifs, a single transmembrane domain, and an intracellular bipartite tyrosine kinase domain. No ligand for Tie1 has been identified.

Color plate 9

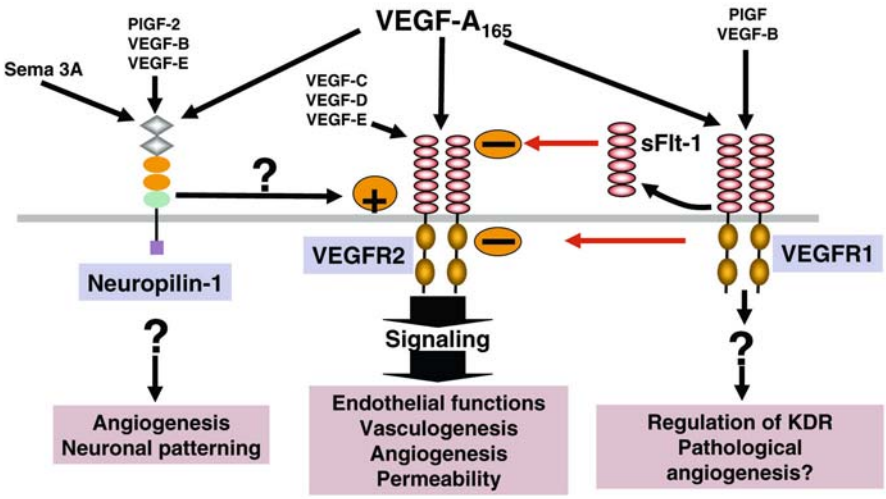

Figure 2 from page 272
Biological roles of VEGF receptors. VEGFR2 is the major receptor responsible for VEGF signalling and biological functions in endothelial cells, and plays a key role in mediating vasculogenesis, endothelial cell differentiation, and angiogenesis in development, and disease-associated neovascularisation. VEGFR1 is thought to act as a decoy receptor during embryogenesis, by regulating functions of VEGF mediated via VEGFR2, either by direct inhibition of VEGFR2, or by inhibition of VEGF by sFlt-1. VEGFR1 may play a role in pathophysiological angiogenesis in the adult. NP-1 is essential for embryogenic angiogenesis, but the precise role of this receptor in the function of VEGF is unclear. NP-1 has no defined signalling role as yet.

Color plate 10

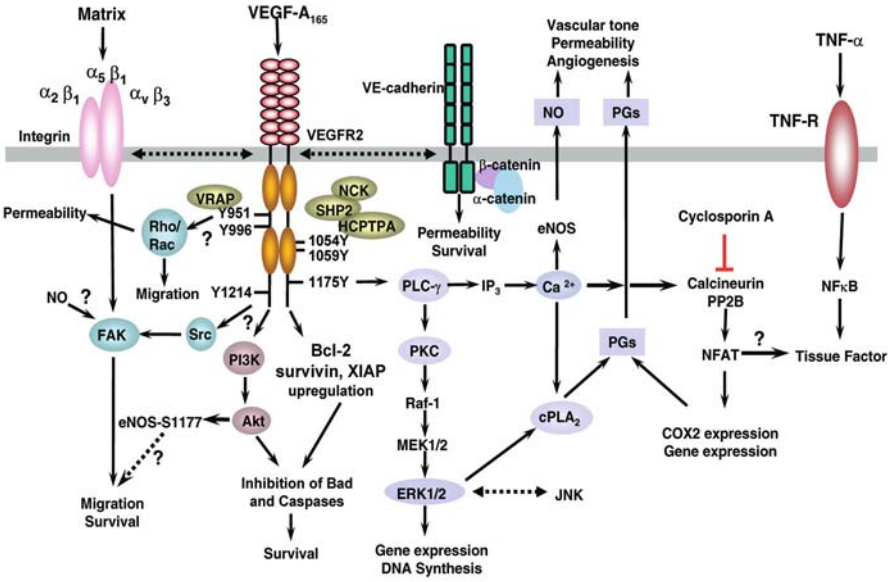

Figure 3 from page 273

Functional VEGF signalling in endothelial cells. Most biologically relevant VEGF signalling is mediated via VEGFR2. Activation of VEGFR2 occurs through ligand-induced dimerisation and receptor autophosphorylation at multiple tyrosine residues in the intracellular domain. VRAP and PLC-γ associate with Y951 and Y1175, respectively, but the role of other sites is yet to be defined, though VEGFR2 associates with several other SH2 domain proteins including Nck and the tyrosine phosphateses SHP2 and HCPTPA. VEGF-dependent endothelial cell survival is mediated in part via PI3K-mediated activation of the anti-apoptotic kinase Akt, though the mechanism of PI3K activation is unclear. Akt phosphorylates and inhibits the pro-apoptotic protein Bad, leading to inhibition of caspase activity. Upregulation of the anti-apoptotic proteins Bcl2, survivin and XIAP may also mediate inhibition of terminal effector caspases. Akt also causes Ca^{2+}-independent eNOS activation through phosphorylation at ser 1177, and this pathway may also be essential for migration. Increased tyrosine phosphorylation of FAK mediated in part through Src contributes to VEGF-dependent survival and migration signalling, but the mechanism responsible for VEGFR2-mediated activation of this pathway is unclear. Direct interactions (indicated by bidirectional dotted arrow) and convergent FAK signalling between VEGFR2 and integrin receptors for collagen, fibronectin and vitronectin, may also play a role in survival functions of VEGF. VEGFR2 residue Tyr 951 mediates activation of Rho and Rac via an unknown mechanism and this pathway is also implicated in migration signalling. Rac activation may mediate VEGF-induced fenestration contributing to the permeability-increasing effect of VEGF. Interaction between VEGFR2 and VE-cadherin is important for survival signaling and may also regulate permeability. A major mitogenic signalling mechanism for VEGF is the PLC-γ pathway resulting in

hydrolysis of phosphatidylinositol 4,5-bisphosphate, generation of inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol and subsequent mobilization of intracellular Ca^{2+} and PKC activation. PKC mediates activation of ERKs 1/2 via Raf-1 and MEK, and this pathway is a major mediator of both mitogenesis and cPLA2 activation leading to generation of COX-derived prostanoids (PGs), including PGI_2 and PGE_2. Ca^{2+} signalling is also important for eNOS activation and NO generation, and activates the serine/threonine phosphatase, calcineurin (PP2B), a target for the immunosuppressant, cyclosporin A, leading to activation of the transcription factor, nuclear factor of activated T-cells (NFAT) and induction of COX-2 and tissue factor gene expression. VEGF also interacts synergistically with TNFα to promote tissue factor expression. Question marks and dotted lines indicate areas where the mechanism involved in unclear, or where a link is inferred but not yet established.

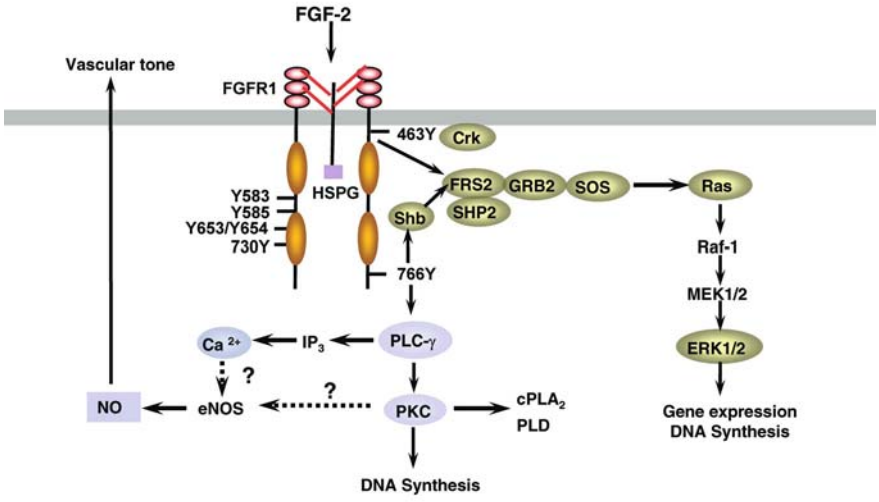

Figure 4 from page 283
FGF-2/FGFR1 endothelial signalling. Dimerisation and activation of FGFR1 by FGF-2 requires HSPGs. FGFR1 is phosphorylated at multiple sites, most of which do not have a defined function. The SH2 domain adapter protein Crk associates with the juxtamembrane tyrosine 463. Tyr 766 mediates binding to PLC-γ. Binding of the adapter protein, FRS2, to the juxtamembrane region of FGFR1 via a phosphotyrosine-binding (PTB) domain in FRS2, mediates recruitment of GRB2 and ras-dependent activation of ERKs. Binding of the SH2 domain adapter protein, Shb, to Tyr 766 regulates FRS2 tyrosine phosphorylation and ERK activation. FGF-2 induces NO generation, but the mechanism involved is unclear, though PLC-γ-mediated PKC activation and increased intracellular Ca^{2+} are implicated.

Color plate 12

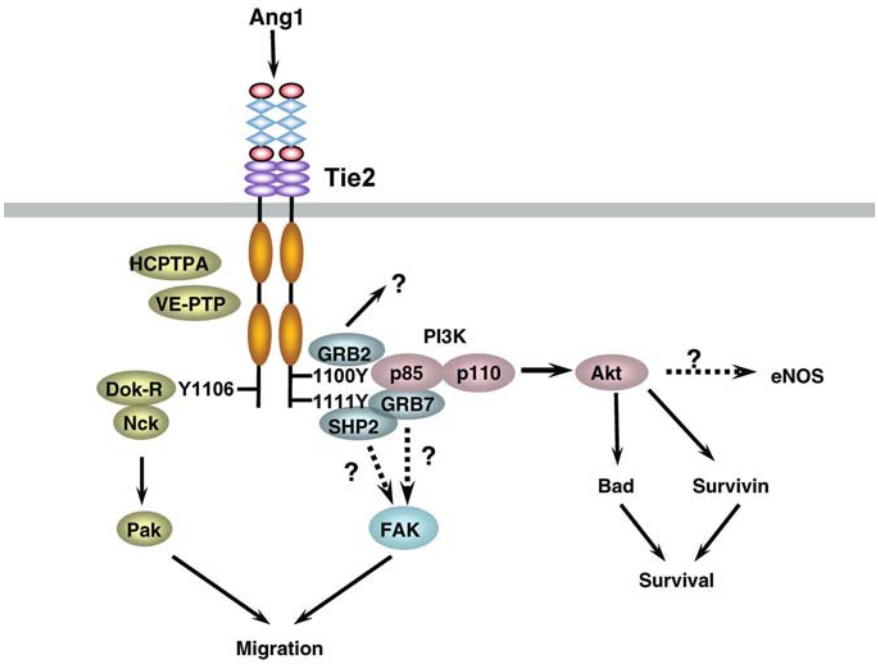

Figure 5 from page 286

Tie signalling pathways. Angiopoietin (Ang)-1 is a major agonistic ligand for Tie2. Tyr 1100 in the carboxyl tail of Tie is a multifunctional docking site mediating association with the SH2 domains of the adapter proteins GRB2, GRB7, and the p85 subunit of PI3K. p85 binding mediates activation of the PI3K/Akt pathway which is responsible for survival functions of Ang1. Activation of eNOS via Akt is inferred, but not yet established. A function for GRB2 in downstream Tie2-mediated signalling has not yet been established, but Ang1 is not able to activate the ERK pathway. Tyr 1111 is also important for binding of the tyrosine phosphatase SHP2 and both SHP2 and GRB7 are implicated in activation of the FAK pathway that partly mediates effects of Ang1 on endothelial cell migration. The PTB and pleckstrin-homology domains of the downstream-of-kinase-related (Dok-R) docking protein mediate association with Tie2 residue Tyr 1106, resulting in recruitment of the SH2 domain adapter protein Nck and the p21-activating kinase Pak1, a pathway that mediates Ang1-induced cell migration. Tie2 also associates with other SH2 domain proteins including the tyrosine phosphatases, HCPTPA and VE-PTP.