CHAPTER 1

The Biology of Vascular Endothelial Cell Growth Factor Isoforms

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Abstract

The field of angiogenesis research was literally transformed overnight by the discovery of vascular endothelial growth factor (VEGF). Researchers quickly embraced VEGF in their different areas of vascular and angiogenesis research, and in the last two decades have discovered much about VEGF biology. It is now clear that VEGF is actually a collection of different isoforms. Through differential pre-mRNA splicing and protein processing, one VEGF gene gives rise to several different protein isoforms, which together orchestrate the complex processes of angiogenesis, vessel growth and adult vascular functions. The VEGF isoforms differ biochemically, and genetic experiments in mice have proven that the isoforms have different functions. Furthermore, certain VEGF isoforms associate with and likely play differential roles in various pathologic states. With better understanding of VEGF isoform biology, new insights into the complex mechanisms of VEGF-mediated vessel growth can be gained. In addition, findings about the specific VEGF isoform functions have important implications for VEGF-mediated therapeutic angiogenesis as well as anti-angiogenic therapy targeting VEGF.

Key Messages

- VEGF is a collection of different isoforms
- VEGF isoforms have different biochemical properties
- VEGF isoforms have overlapping and distinct functions during development
- VEGF is multifunctional for endothelial cells and also acts on other cell types
- Expression of a specific VEGF isoform is associated with pathological conditions

Introduction

Angiogenesis plays a critical role in the progression of many pathologies, including cancer. The search for angiogenic factors in the last decade has been largely driven by the hope that identification of such factors will lead to new treatments for these pathologies. Anti-angiogenic therapy is particularly promising for the treatment of cancer; the strategy of blocking tumor angiogenesis seems to offer the best approach yet for treating tumors resistant to conventional therapies.[1] The list of putative angiogenesis factors grows continuously, but VEGF, one of the first angiogenesis factors identified, is widely believed to be the most important regulator of both normal and pathological angiogenesis.

VEGF was first purified from tumor cells by Harold Dvorak and coworkers at the Beth Israel Hospital in Boston, USA. This factor was isolated based on its ability to enhance vascular

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permeability, and was therefore named "vascular permeability factor" or VPF.[2] Napoleon Ferrara at Genentech subsequently purified, and later cloned, a factor from medium conditioned by bovine pituitary folliculostellate cells that induced proliferation of vascular endothelial cells (EC). This substance was named VEGF.[3] When cDNAs from both the Dvorak and the Ferrara studies were sequenced, VEGF and VPF were found to be the same molecule.

It is now clear that VEGF elicits an array of EC responses in vitro, including stimulation of proliferation and migration, and induction of proinflammatory gene expression.[4] VEGF has been shown to guide blood cell and EC precursor migration in *Drosophila*,[5] *Xenopus*,[6] and likely in *Danio rerio*;[7] it is possible that in mammals the ancestral role of VEGF is to direct EC precursor, or angioblast, migration.[8] The findings in the *Drosophilia* and in the lower vertebrates suggest that at least the chemotaxic function of VEGF signaling is conserved during evolution, and the EC mitogenic and vascular permeability functions of VEGF may have evolved in more complex animals to modulate their vascular system.

Certain in vivo characteristics of VEGF further illustrate its role as the primary angiogenic factor. VEGF is a secreted angiogenic factor, and thus can act in a paracrine fashion.[4] Expression patterns of VEGF and its endothelial-selective receptors correlate both temporally and spatially with areas of vascular growth in developing embryos and during the female reproductive cycle.[4,9,10] Most importantly, embryos lacking either VEGF or its receptors fail to develop functional vessels.[11-14] Interestingly, heterozygous VEGF gene inactivation also results in severe disruption of vessel development and embryonic lethality, suggesting that correct dosage of VEGF is critical for normal vascular development. Indeed, further genetic experiments using a hypermorphic VEGF allele confirmed the tight dosage requirement of VEGF in cardiovascular development.[15] Lastly, antagonists of VEGF or its receptors can effectively block both normal and pathological angiogenesis in animals.[16-18]

In addition to its role in developmental angiogenesis, VEGF also modulates adult physiological angiogenesis and vessel function in numerous pathologies. In the adult, VEGF participates in regulation of the female reproductive cycle, wound healing, inflammation, vascular permeability, vascular tone, and hematopoiesis.[19] VEGF function also contributes to pathological angiogenesis in disorders such as cancer, rheumatoid arthritis, diabetic retinopathy and the neovascular form of macular degeneration.[4,20,21]

VEGF, also called VEGFA, belongs to the cystine-knot superfamily of growth factors that are characterized by the presence of eight conserved cysteine residues.[22,23] In addition to VEGFA, this superfamily of hormones and growth factors includes VEGFB, VEGFC, VEGFD, VEGFE encoded by the various *orf* viruses, and placental growth factor (PlGF1 and PlGF2 isoforms).[22] The VEGF family members are active as secreted, glycosylated homodimers.[23] They are closely related to and likely have a common ancestor with the platelet derived growth factor (PDGF) family of growth factors, which includes PDGFB, PDGFC, and PDGFD.[22] VEGF exerts its effects on EC through binding to the EC-selective high affinity receptors FLT1 (VEGFR1), KDR (VEGFR2) and neuropilins (NRP1 and NRP2).[4,24] VEGF is highly conserved across different vertebrate species, from mammals to fish, at both the polypeptide and genomic structure levels. VEGF protein from one species is therefore often functional toward EC from another species.

VEGF Isoform Structure

The VEGF gene is comprised of several exons separated by introns in all species characterized—from human to mouse, fish and frog.[22,25] As a result of differential pre-mRNA splicing, a single VEGF gene gives rise to many different VEGF isoforms.[23] Because the roles of the different VEGF isoforms in nonmammalian species have not been well characterized, and most studies using specific VEGF isoforms have utilized either mouse or human models, this chapter will focus on the human and murine VEGF isoforms.

The gene encoding VEGF is located on the short arm of chromosome 6 (6p21.1) in humans and on chromosome 17 (24.20 cM) in the mouse.[22] Both the human and the murine VEGF genes are comprised of eight exons, separated by seven introns (Fig. 1). Although in

theory any combination of the eight exons is possible, all differentially spliced variants of VEGF isoforms discovered to date contain the first 5 exons (1 to 5) plus different combinations of exons 6 to 8. As a result, all VEGF isoforms contain signal peptides (first 28 residues) and are thus secreted polypeptides, and they can all potentially form homodimers, because the dimerization domain is located in exons 2 to 5 (residues Cys51 and Cys61).[23] Because three residues tially spliced region of the VEGF pre-mRNA is limited to exons 6 to 8, the number of potential possible combinations of these exons is further increased by the fact that exons 6 and 7 have alternative internal splice donor and acceptor sites that further divide them into two different portions, referred to as 6a and 6b, 7a and 7b, respectively.[22,25]

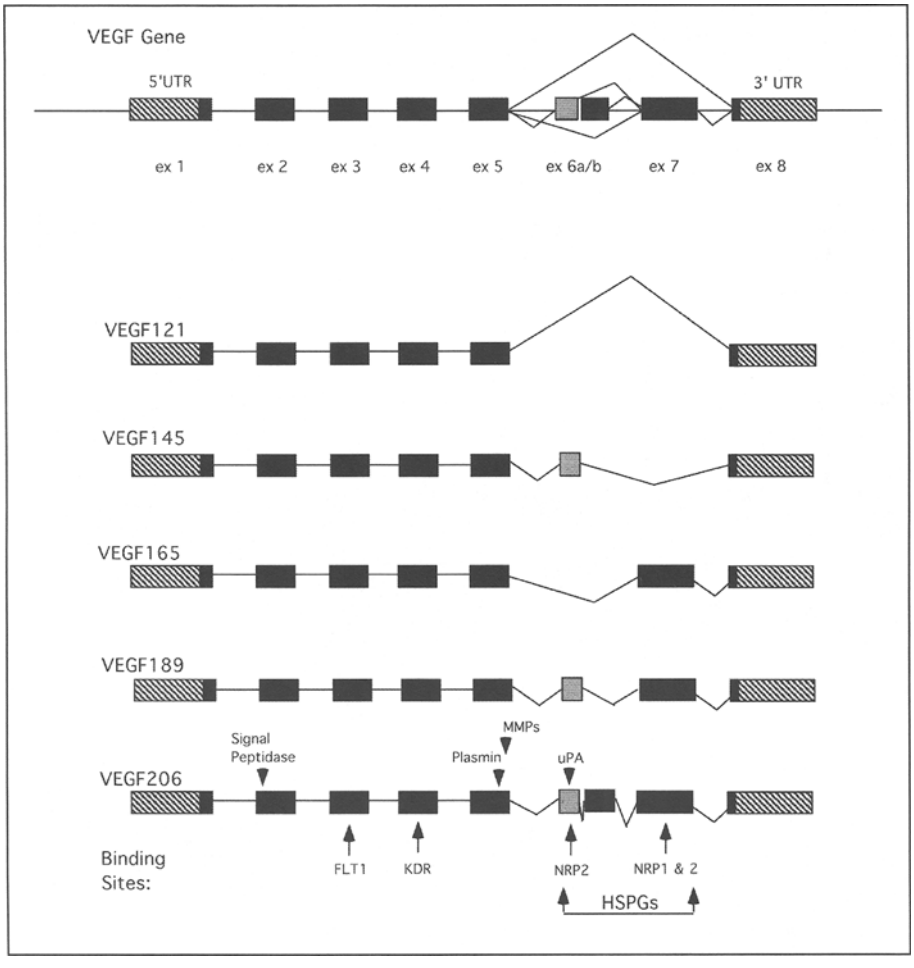

Figure 1. Genomic organization of the human VEGF gene and its alternative splice variants. By differential pre-mRNA splicing of exons 6 and 7, the human *vegf* gene gives rise to various isoforms (the most common 5 isoforms are shown). The different exons (ex) and domain sizes (a.a., amino acid residue) are shown below the gene. The 5'- and 3'-untranslated regions (UTR) are indicated by hatched boxes. Protease cleavage sites (arrowheads) and receptor binding sites (arrows) are indicated on the VEGF206 isoform.

Despite this variety of potential differential splicing combinations, it appears that there are only three major VEGF isoforms produced in all vertebrates, and these differ by the presence or absence of peptides encoded by exons 6 (24 amino acids) and 7 (44 amino acids). The major human VEGF isoforms are VEGF121, VEGF165 and VEGF189, with the numbers indicating the number of amino acids in the mature polypeptides (Fig. 1). The major murine VEGF isoforms are VEGF120, VEGF164 and VEGF188,[26] each containing one less amino acid than the human orthologue. In vivo, VEGF121(120), VEGF165(164) and VEGF189(188) isoforms are produced by certain cells in a tissue-specific pattern with the clear exception of most vascular endothelial cells,[27] and many of the less-abundant VEGF isoforms are associated with specialized cell types or tumor cells (see below).

Biochemistry of the Major VEGF Isoforms

The different VEGF isoforms have distinct biochemical properties (Fig. 2). As VEGF120(121) does not bind heparan sulfate, it is readily diffusible. VEGF164(165) has moderate affinity for heparan sulfate; it is partially sequestered on the cell surface and in the extracellular matrix (ECM),[28] likely due to heparan sulfate proteoglycans (HSPGs) binding. The HSPGs-binding activity of VEGF164(165) is conferred by the 15 basic residues within the peptide encoded by exon 7, which is defined as the heparin-binding domain of VEGF.[23] VEGF188(189) has high affinity for heparan sulfate due to the presence of the additional basic residues and a strongly heparin-binding domain encoded by exon 6. As a result, VEGF188(189) is mostly associated with the cell-surface and ECM.[28] (In this chapter, the nomenclature for the human and murine VEGF isoforms will be used interchangeably).

The VEGF isoforms localized or sequestered at the cell surface or ECM constitute a reservoir of angiogenic growth factors that can be mobilized by various enzymes. For example, heparinase and matrix metalloproteinases (MMPs) can release matrix-bound VEGF isoforms from HSPGs,[23,28] whereas plasmin can cleave the heparin-binding domain of the matrix-bound

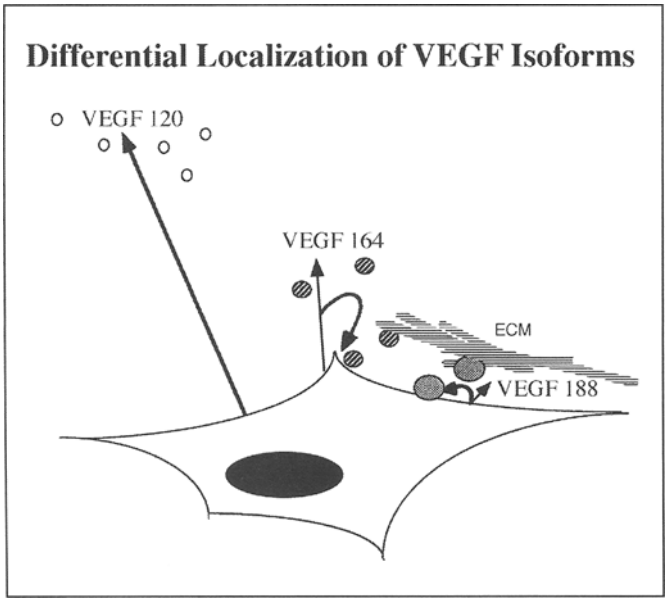

Figure 2. Distinct biochemistry of the different VEGF isoforms. Schematic representation of the differential extracellular localization of the three murine VEGF isoforms based on their different affinities for heparin sulfate.

VEGF isoforms, producing a truncated, but biologically active, VEGF110 (VEGF109 in the mouse).[28] This enzyme-mediated VEGF release represents a fast and effective way to mobilize VEGF and increase its effective concentration in different local environments. Therefore one of the functions for the different VEGF isoforms is to modulate the availability of VEGF by being sequestering on the cell surface or in the ECM. Although the details of this protease-mediated VEGF release pathway have not been elucidated in vivo, MMP9 plays a major role in VEGF-mediated tumor angiogenesis in the RipTag pancreatic tumor model[29] and during bone development,[30] suggesting that protease-mediated VEGF release does indeed play a prominent role in the VEGF pathway. In addition to releasing matrix-sequestered VEGF, it has been reported that a nMMP with anti-angiogenic properties, ADAMTS1, directly binds to VEGF and dampens downstream VEGF signaling.[31] More recently, it has been reported that many different MMPs can cleave the matrix-bound VEGF isoforms intramolecularly, creating two fragments: a soluble and biologically active N-terminal fragment that can bind to the VEGF receptors, and a shorter matrix-binding C-terminal fragment that has no apparent biological activity.[32] Interestingly, the authors also reported distinct angiogenic outcomes produced by the matrix-bound VEGF and the MMP-processed soluble N-terminal VEGF fragment, providing further proof that matrix association can modulate the function of the different VEGF isoforms. These findings clearly suggest that interactions of various MMPs and possibly other proteases with VEGF occur in vivo, and that these interactions can directly modulate both the availability and the activity of VEGF.

Not only do the VEGF isoforms display differences in localization and availability, they also have different affinities for their high affinity receptors, FLT1 and KDR, expressed on the EC surface. Binding of FLT1 by both VEGF121 and VEGF165 is inhibited by heparin. Binding of KDR by VEGF165 is enhanced by low concentrations of heparin, but is inhibited by high concentrations of heparin.[23] Binding of KDR by VEGF121 is not affected by heparin. Besides differential binding affinities for FLT1 and KDR, the neuropilin family of cell-surface receptors exhibits differential specificity for VEGF isoforms. Because NRP1 binds to the peptide encoded by exon 7, VEGF165 and probably VEGF189 bind to NRP1, but VEGF121 does not.[24] Although binding by VEGF165 may not induce NRP1 signal transduction directly, due to the lack of a conventional signaling cytoplasmic domain, it was reported that cell surface NRP1 can increase the binding affinity of VEGF165 for KDR,[24,33] but not for VEGF121. This suggests that NRP1 functions as a coreceptor in EC cells. Interestingly, it has been reported that VEGF165, but not VEGF121, can promote survival of breast carcinoma cells in vitro in a neuropilin-dependent manner, suggesting that NRP1 may function as a signaling VEGF165-specific receptor in nonendothelial cells.[34] Indeed, a chimeric receptor containing the transmembrane and intracellular domain of NRP1 and the extracellular domain of epidermal growth factor (EGF) was reported to mediate human umbilical vein EC (HUVEC) migration upon EGF stimulation.[35] These results suggest that the NRP1 receptor is capable of transducing signals in certain cell types and in certain contexts. Considering the important role of the NRP1 receptor reported for vascular development[23,36] and for guidance of EC tip cells and vessels,[37] more studies into the exact signaling role of the NRP1 will provide further insights into the biology of VEGF isoforms and their roles in modulating angiogenesis. Another neuropilin family member, NRP2, has also been shown to bind VEGF165 but not VEGF121.[38] Interestingly, NRP2 was reported to function as a receptor for the less-abundant VEGF145 isoform,[38] suggesting that both exon 6 and exon 7 of VEGF can facilitate the binding to NRP2 by VEGF145 and VEGF165, respectively. However, the functional role of this selective interaction between VEGF isoforms and NRP2 in angiogenesis remains to be determined.

It is likely that the difference in biochemical properties described above translates into distinct biological activities for the various VEGF isoforms. For example, it has been reported that the VEGF isoforms can have different mitogenic activity for EC in vitro and in vivo.[23] However, others have found that VEGF120 and VEGF164 do not differ in their ability to support EC proliferation, but that vessel networks differ in tissues developing in the absence of specific

isoforms and in tumors overexpressing the various VEGF isoforms in vivo.[32,39-43] The differences in localization, availability, receptor-binding affinity and bioactivity likely contribute to distinct roles for the individual VEGF isoforms during vascular development, and imply that the presence of the different VEGF isoforms is critical for normal vascular development. Therefore, knowledge of the differential functions of the different isoforms will be crucial for designing an effective VEGF-mediated angiogenesis therapy to promote normal vessels growth in patients suffering from ischemic vascular diseases.[44]

VEGF Isoforms in Vascular Development

VEGF mediates angiogenesis both during development and in the adult. While the roles of specific VEGF isoforms in normal adult angiogenesis remain largely unexplored, contributions of VEGF isoforms to developmental angiogenesis have been identified using transgenic mouse models.

Experiments that directly measured the VEGF isoform mRNA levels in the mouse revealed that the relative levels of the three major VEGF isoforms vary among different adult organs. Differential mRNA levels of the three major VEGF isoforms during murine embryonic development also suggest that expression of the different isoforms is developmentally regulated. Whereas the three major VEGF isoforms are expressed in all embryonic organs examined, the relative levels of each isoform varied from organ to organ, and the isoform composition changed over developmental time in the same organ.[26] For example, adult lung, heart and liver express relatively high levels of VEGF188 mRNA (52% of the total VEGF message in the lung and 36% in the heart and liver), whereas brain, eye, spleen and kidney express relatively low levels of VEGF188 mRNA (6%, 5%, 11% and 17% of the total VEGF mRNA, respectively). During development, in the embryonic day 13.5 lung, VEGF188 makes up only about 10% of total VEGF mRNA, but at embryonic day 17.5, just before birth, about 50% of the total VEGF mRNA produced in the lung is the VEGF188 isoform. The levels of VEGF188 remain high at about 50% of total VEGF mRNA in the adult lung. These findings are consistent with the concept that the VEGF isoforms serve different functions during vascular development and angiogenesis in the adult.[26]

To directly assess the different functions of the three major VEGF isoforms during development and in the adult, and in an attempt to avoid the early embryonic lethality from the total gene inactivation,[13,14] VEGF isoform-specific gene targeting approaches were used. Using exon-specific deletion and cDNA replacement (knock-in) strategies, VEGF alleles were created that permitted expression of only one of the three VEGF isoforms: VEGF120, VEGF164 or VEGF188.[39,40] Mice were then generated in which the normal VEGF gene was replaced with one or two isoform-specific alleles. The isoform-specific mouse models yielded two immediate and significant findings. First, heterozygous mice in which one of the two VEGF alleles could produce only a single VEGF isoform were viable and developed normally (Table 1). Given that single-allele VEGF inactivation, as well as the slight increase in VEGF levels resulting from a hypermorphic mutation, both lead to embryonic lethality,[15] these observations suggest that the isoform-specific allele likely produced similar levels of VEGF transcript compared to the wild-type allele. Indeed, RNase protection assays confirmed that the total levels of VEGF transcript in the heterozygous and the homozygous VEGF isoform-specific mice were comparable to that of wild-type mice.[39] A second important finding was that although not all VEGF isoform-specific homozygous embryos were viable (Table 1), their phenotypes were much less severe than those of VEGF-null mice. This result is consistent with the concept that the different VEGF isoforms all support EC growth, but have subtle differences in function, such that one isoform can only partially replace the function of the others during embryonic development and in the adult (Table I).

Analysis of the $VEGF^{120/120}$ and $VEGF^{188/188}$ homozygous mice, in which both VEGF alleles produce only a single VEGF isoform (VEGF120 and VEGF188, respectively), has yielded further insight into the roles of the VEGF isoforms in vascular development. Homozygous $VEGF^{120/120}$ mice are not viable and exhibit very distinct vascular defects. Most of the $VEGF^{120/120}$ embryos die in utero, or soon after birth. All $VEGF^{120/120}$ embryos exhibit decreased angiogenesis,

Table 1. The phenotypes of VEGF isoform-specific mice

Homozygous	***vegf120/120*** Perinatal (90%) or early postnatal (10%) lethality. Extensive vascular hemorrhages in most organs. Decreased vascular branching and impaired postnatal angiogenesis[26,39,43] (Fig. 3).	***vegf164/164*** Viable, normal appearance[40] (P. D'Amore and Y. S. Ng, unpublished data). No obvious vascular phenotypes in the embryo (C. Ruhrberg and D.T. Shima, unpublished data). Yolk sac vesselsare patterned normally (Fig. 3).	***vegf188/188*** Embryonic lethality from embryonic day 10 (90%); 10% reach adulthood, though with decreased body size/weight and subtle vascular defects in the retina[40] (P. D'Amore and Y. S. Ng, unpublished data). Excessive branching and small caliber of vessels in several embryonic organs.[43] Hyper-fused vessel networks in the yolk sac (Fig. 3).
Heterozygous	***vegf120/+*** Viable, normal appearance, but decreased vascular branching in several embryonic organs[26,39,43] (Fig. 3).	***vegf164/+*** Viable, normal appearance, normal vessel patterning in all organs examined[40] (P. D'Amore and Y. S. Ng, unpublished data; C. Ruhrberg and D. T. Shima, unpublished data).	***vegf188/+*** Viable, normal appearance[40] (P. D'Amore and Y. S. Ng, unpublished data). Vascular phenotype not examined.
Compound heterozygous	***vegf164/120*** Viable, normal (P. D'Amore and Y. S. Ng, unpublished data). Viability suggests that the combination of VEGF164 and VEGF120 isoforms is sufficient for normal vascular development.	***vegf188/120*** Viable, normal (P. D'Amore and Y. S. Ng, unpublished data). Normal vascular development in several organs.[43,46] These observations suggest that the combination of VEGF188 and VEGF120 isoforms is sufficient for normal vascular development.	***vegf188/164*** Viable, normal (P. D'Amore and Y. S. Ng, unpublished data). The vascular phenotype has not been examined. Viability suggests that the combination of VEGF188 and VEGF164 isoforms is sufficient for normal vascular development.

Mice expressing different combinations of VEGF isoforms were produced by crossing heterozygous mice carrying one wild-type and one isoform-specific *vegf* allele.

decreased vascular branching and vascular hemorrhage,[39] suggesting that the vessels in these mice are leaky and lack the normal vascular morphology found in wild-type mice. Close examination of the vascular beds in organs from the $VEGF^{120/120}$ mice of different developmental stages revealed extensive vascular remodeling defects and decreased vascular branching and density (Fig. 3).[26,43] These results suggest that the freely diffusible VEGF120 isoform alone can support the initial stages of vascular development, but that VEGF120 cannot replace the functions of the heparin-binding VEGF isoforms such as VEGF164 in the fine patterning of the vasculature. There is also a strong correlation between the severity of vascular phenotypes observed in the $VEGF^{120/120}$ embryos and the organ expression patterns of the various isoforms; that is, the organs that express relatively high levels of the heparin-binding VEGF isoforms (VEGF164 and VEGF188) exhibited the most severe, abnormal vascular phenotypes. For example, high levels of

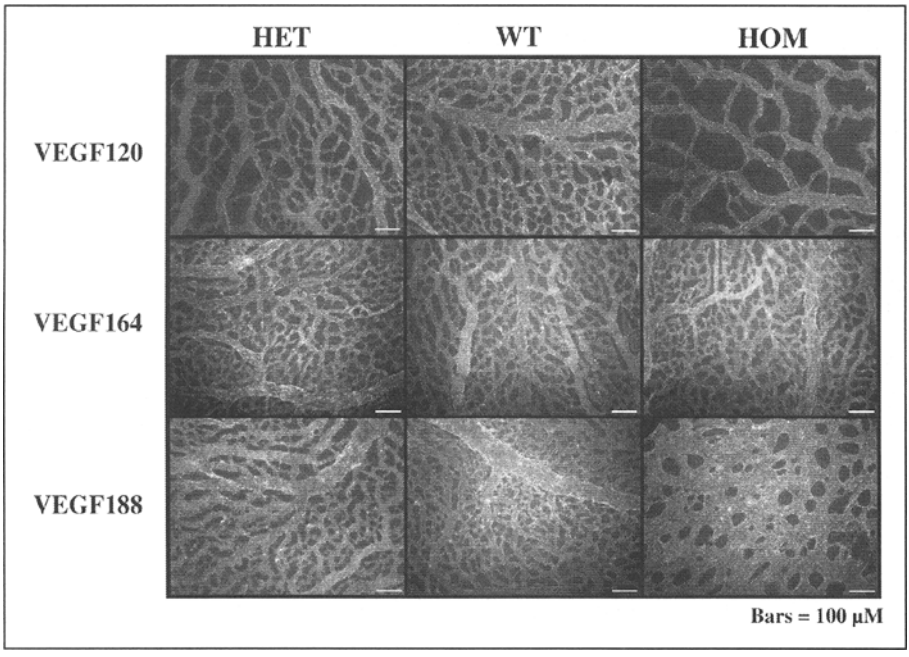

Figure 3. Vascular patterning of the VEGF isoform-specific mice. Embryonic day 10.5 yolk sacs showing the different vascular patterns formed in the VEGF120, VEGF164, and VEGF188 isoform-specific mice. In the heterozygous (HET) and homozygous (HOM) VEGF120 yolk sacs, the lack of vessel sprouting results in sparser vascular networks as compared to wildtype (WT), whereas the HOM VEGF188 yolk sac displayed enlarged and sheet-like vessels likely resulting from fusion of vessels due to excessive vascular sprouting and/or branching. Vessels in the VEGF164 yolk sacs are indistinguishable from the WT.

VEGF188 expression are temporally and spatially associated with the maturation of the lung, and analysis of the lungs from $VEGF^{120/120}$ mice revealed extensive microvascular defects and retarded alveolarization. Thus, although the VEGF120 isoform alone appears to support initial vessel growth in the lung, remodeling or maturation/maintenance of the final vascular bed, including further angiogenesis, is defective in the absence of the heparin-binding VEGF isoforms.[26]

Because the $VEGF^{120/120}$ mice produce only freely soluble VEGF, defects in these mice provide information about potential roles of heparin-binding isoforms such as VEGF164. Analysis of organs in $VEGF^{120/120}$ mice that are vascularized by angiogenic sprouting, such as the hindbrain suggested that matrix-bound VEGF164 is required to establish a normal concentration gradient of VEGF. This concentration gradient, formed when VEGF164 binds to the cell surface and ECM, can effectively direct the chemotactic activity of the EC by modulating the behavior of filopodia on endothelial vessel tips during vessel sprouting[43] (see Chapter 6 by H. Gerhardt). As the VEGF120 isoform is freely diffusible, these gradients cannot form properly in $VEGF^{120/120}$ mice. These observations establish the importance of localized VEGF in establishing a concentration gradient, and help to explain the unique role of the heparin-binding function of VEGF isoforms in vascular development.

$VEGF^{188/188}$ mice, too, display abnormal vascular development. About half of the homozygous $VEGF^{188/188}$ embryos die around embryonic day 10.5, exhibiting developmental delay and extensive vascular defects (D'Amore and Ng unpublished data), and the remaining half develop to term with subtle vascular defects in most organs examined.[40] Because the VEGF188 isoform is highly localized to ECM and cell surface HSPGs, proteolytic cleavage is required to

release the biologically active form of soluble N-terminal VEGF fragment from the cell surface and ECM in tissue culture experiments.[23,28,32] The fact that some $VEGF^{188/188}$ mice are viable suggests that the VEGF188 isoform is processed into soluble receptor-binding N-terminal fragment and in ECM-binding C-terminal fragments by proteases in vivo. The $VEGF^{188/188}$ mouse model supports the idea that post-translational proteolytic processing of VEGF isoforms in vivo could potentially create additional isoform proteins beyond those formed by differential pre-mRNA splicing. However, the functional significance of post-translational modification/processing of VEGF isoforms remains unclear, and more studies will be required to elucidate the biology of this pathway in vascular development and neoangiogenesis.

VEGF Isoforms in Diseases

A theory is emerging that particular VEGF isoforms might associate with certain disease states;[45] in this case, specific VEGF isoforms might be considered pathological. Although the mechanism of the disease/pathological VEGF isoform association is unclear, understanding of isoform specificities for diseases might permit development of better and more specific anti-VEGF therapies to treat these conditions.

Amongst the better-defined VEGF-mediated pathologies are the proliferative intraocular neovascular syndromes in diseases such as diabetic retinopathy, retinopathy of prematurity (ROP), and the wet form of macular degeneration. In all of these disease states, VEGF not only causes uncontrolled neovascular growth that damages the retina, but also promotes vascular leakage and vitreous hemorrhages that eventually lead to blindness.[21] Recently, VEGF164 has been identified as the major pathological VEGF isoform in the eye.[21,46] In experimental murine models of diabetes and ROP, VEGF164 was more potent than VEGF120 in inducing both endothelial intercellular adhesion molecule 1 (ICAM-1) expression and chemotaxis of leukocytes, which together lead to increased inflammation of the retinal vessels. Furthermore, VEGF164 was more potent than VEGF120 in inducing vascular leakage and blood-retinal barrier breakdown. Interestingly, the vascular inflammation and leakage caused by VEGF in these models were significantly reduced following administration of a pan-VEGF isoform antagonist or a VEGF164-specific, RNA-based aptamer antagonist.[21] These observations suggest that VEGF164 is the major disease-causing isoform in models of neovascular eye disease, and highlight the importance of understanding the different contributions of specific VEGF isoforms in vascular pathologies. Therefore, anti-angiogenic therapy targeted to individual VEGF isoforms might increase the specificity and potentially the efficacy of the therapy.

Another disease that is dependent on angiogenesis is cancer, as solid tumors incorporate new vessels to support and promote tumor growth and metastatic spread.[1] Although not all tumor angiogenesis is VEGF dependent, most tumor types studied to date display upregulation of VEGF mRNA and protein.[4] It is therefore likely that VEGF is at least partly responsible for tumor angiogenesis in most cancer types. Interestingly, tumor vessels are mostly unstable, leaky and immature; these characteristics result from the high levels of VEGF in the tumor.[47] VEGF164 and VEGF120 appear to be the most widely expressed VEGF isoforms in tumors. One early study in which murine brain tumors over-expressed one of the three major VEGF isoforms, showed that both VEGF120 and VEGF164 promoted rapid growth of vessels that were highly unstable and leaky. In contrast, the VEGF188 over-expressing brain tumor supported vessels that were slower growing, nonhemorrhagic vessels that were relatively normal in appearance.[41] These results demonstrated that all three VEGF isoforms can stimulate angiogenesis, but that the characteristics of the resulting vessels depend on the specific isoform(s) expressed.

Because this tumor study was complicated by the fact that VEGF was also endogenously expressed by the tumor cells, a more refined study was conducted in which tumor cells derived from VEGF-deficient fibroblasts were used to create VEGF isoform-specific tumor cells.[42] In the mouse, these VEGF isoform-specific cells all supported vessel growth in the fibrosarcomas, but the new tumor vessels displayed different vascular characteristics depending on the expressed isoform. The VEGF164-expressing cells induced tumor growth and vessel density similar

to those observed for wild-type tumor cells. The VEGF120-specific cells only partially rescued tumor growth, and vascular density in these tumors was reduced. Although the VEGF188-specific cells formed smaller tumors than wild type cells, the vessels in these tumors were present at a higher density and displayed a more highly branched phenotype than observed for the wild type tumor. Furthermore, by mixing the VEGF120- and VEGF188-specific cells, both the vascular density and tumor growth were comparable to those in the wild type tumor. Taken together, these results suggest that different VEGF isoforms play distinct but cooperative roles during tumor angiogenesis. Although VEGF164 and VEGF120 were more potent than VEGF188 in inducing both tumor growth and angiogenesis, the isoform-specific fibrosarcomas also demonstrated that the VEGF isoforms could act cooperatively to make normal-appearing and functional vessels during tumor vascularization.[42]

Specific VEGF isoform expression has also been associated with the progression of a particular tumor type in humans,[48] and may be an indicator for the prognosis of the disease. For example, high levels of VEGF189 expression were associated with nonsmall-cell lung cancer (NSCLC) and were an indicator of poor prognosis in patients, compared to NSCLC expressing low levels of VEGF189. High levels of VEGF165 expression were associated with poor prognosis in osteosarcoma.[48] Although it is not clear whether the predominance of a particular VEGF isoform in a tumor is the cause or a consequence of the disease, such associations might be used as disease markers with potential diagnostic or therapeutic value.[45]

Novel VEGF Isoforms

With the improved sensitivity of RT-PCR detection techniques and better design of PCR primers based on the VEGF genomic DNA sequence, many novel and low-expression VEGF isoforms have been discovered in diverse tissue types. Relatively low levels of VEGF206 (exons 1-5, 6a, 6b, 7 and 8) and VEGF145 (exons 1-5, 6a, 8) transcripts have been detected in normal human placental tissues, and VEGF145 was the major isoform expressed in several carcinoma cell lines derived from the female reproductive system.[23] VEGF183 (exons 1-5, truncated 6a, 7 and 8) is similar to VEGF189 but with a shorter exon 6a, and has been reported to have wide tissue distribution.[23] A new heparin-binding VEGF162 (exon 1-5, 6a, 6b, 8) has recently been discovered in human ovarian carcinoma cells, and has been reported to have angiogenic activity.[49] VEGF165b (exon 1-5, 7, distal splice site of exon 8) is very similar to VEGF165 in protein sequence and size, but inhibits VEGF165 by competing for KDR binding,[50] and thus may function as an endogenous, competitive inhibitor of all VEGF isoforms for receptor binding.

Pre-mRNA splicing is very important in normal development, as it creates protein diversity in complex organisms. This mechanism is also a natural target for various disease processes, including carcinogenesis. Since mice expressing only VEGF164 are viable,[40] the novel isoforms may be unnecessary for the normal development and survival of adults (Table 1). These novel isoforms may, however, play important roles in disease states. Because upregulation of novel VEGF isoforms has been observed predominately with abnormal tissues, it will be important to determine if these isoforms play a role in disease progression. In vivo animal models will be needed to correlate and confirm expression of novel VEGF isoforms, to exclude potential tissue culture artifacts. Further studies of these isoforms, with their different combinations of C-terminal exons, might help to better elucidate the functions of VEGF in vivo.

Conclusions

The central role of VEGF in both normal and pathological angiogenesis has been established, and the complex biology of VEGF, including roles of the different isoforms and receptors, is starting to be elucidated. From the vascular biology point of view, however, much remains unclear regarding the differential functions of the VEGF isoforms. For example, do specific pairings of isoforms with VEGF receptors contribute to the different functions of the VEGF isoforms? Do the isoforms have differential effects on vascular permeability, which is a major problem associated with many vascular pathologies? VEGF was once considered an

endothelial-specific growth factor that mediating angiogenesis and permeability, but it is now clear that VEGF has additional functions beyond the vasculature that affect cell types including, for example, neural cells, bone-forming cells and immune cells (see Chapters 8 by J. Haigh, J.M. Krum and C. Ruhrberg; Chapter 7 by C. Maes and G. Carmeliet; and Chapter 3 by J.J. Haigh). The roles of the VEGF isoforms in mediating inflammation are poorly understood. Furthermore, the differential effects of the VEGF isoforms on the nervous system, likely via the NRP1/2 receptors, in development and in diseases remain largely unexplored. With the increasing interest in the use of anti-VEGF therapy to treat various diseases that are associated with abnormal angiogenesis, inflammation, and vessel hyperpermeability, more attention must be paid to understanding the exact roles of VEGF isoforms in normal physiology and vascular pathologies. A better understanding of the isoform functions in building and maintaining normal vessels during development will also allow the design of better pro-angiogenic therapies; specific VEGF isoforms might be particularly beneficial for patients suffering from ischemic diseases.

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