5 The Extracellular Matrix and VEGF Processing

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Summary

Tumor neovascularization requires the activation of a subset of endothelial cells from normal vascular beds, the digestion of the underlying basement membrane, and the directional migration of these cells toward an avascular site. The contribution of vascular endothelial growth factor (VEGF) to each one of these steps has received large experimental support, and it has been demonstrated that pharmacological and/or genetic inactivation of this growth factor can impact the angiogenic response and consequently suppress tumor growth. Thus, understanding the mechanisms that control VEGF levels has become an important focus of investigation. Today, we have a fairly comprehensive understanding of the mechanisms that regulate VEGF transcriptional rate and half-life. In contrast, little emphasis has been placed on the regulation of VEGF biology post-secretion. In this chapter, we focus our attention on the question of how VEGF becomes released from the extracellular environment and contributes to tumor neovascularization. We discuss this point in the larger context of matrix interaction with growth factors and their modulation by matrix metalloproteinases (MMPs).

Key Words: Matrix metalloproteinases; growth factors; angiogenesis; tumor microenvironment; endothelial cells; capillaries; neovascularization.

1. INTRODUCTION

It is generally accepted that the tissue/tumor microenvironment plays an active role in regulating the angiogenic response. Matrix molecules serve as substrate for migration of endothelial cells, as well as provide differentiation cues for the maturation and stabilization of new vascular beds *(1–3)*. Thus, the nature and physical features of the extracellular environment can provide differential permissive signals for angiogenesis progression and stabilization.

More pertinent to tumor angiogenesis, a cohort of extracellular matrix (ECM) fragments resulting from the cleavage-specific matrix proteins has been shown to be effective in the inhibition of vascular growth. These fragments, named "endogenous

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inhibitors of angiogenesis," were identified *in vivo* and were shown to modulate growth of neovessels in pathological conditions. Moreover, loss and gain of function studies have supported their biological relevance in the regulation of capillary density and in the modulation of pathological angiogenesis *(4–6)*. Thus, it has become critical to explore the dynamics of matrix degradation in the context of an angiogenic response to fully appreciate its impact on vessel growth.

Matrix remodeling is accomplished by a cohort of extracellular enzymes that include matrix metalloproteinases (MMPs), cathepsins, serine proteases, and aminopeptidases *(7)*. In the past, these molecules were considered to participate in the digestion and complete degradation of the matrix needed to repair and remodel tissues. Clearly, proteases are required for the digestion of ECM to allow cell migration. Specifically during angiogenesis, proteolysis is needed to remove the basement membrane of differentiated vessels and to detach pericytes enabling the migration of endothelial cells. More recently, however, the contribution of extracellular proteases in the fine modulation of multiple biological responses has gained a deeper appreciation.

During neovascularization, matrix proteases are required for the release of ECM-bound angiogenic growth factors. Angiogenic growth factors interact with multiple matrix proteins. It has been demonstrated both *in vivo* and *in vitro* that the extracellular milieu functions as a reservoir for growth factors that can be released by specific proteolysis. Thus, the activity of ECM enzymes can significantly enhance the angiogenic response through this property. In addition, matrix proteases are able to expose cryptic integrin-binding sites that would not be accessible in the absence of selective proteolysis. These fragments can facilitate migration and adhesion of endothelial cells during the neovascular response and can enhance or inhibit angiogenesis depending on their relative abundance and presentation to migrating endothelial cells. Finally, proteases are also required for the disruption of endothelial cell–cell adhesions, a process that is essential to the initiation of the neovascular response. For example, MMPs have been shown to cleave the ectodomain of VE-cadherin and thereby releasing endothelial cells from pre-existent blood vessels *(8)*.

Another pertinent aspect to this discussion is that growth factors themselves can be substrates for extracellular proteases. Indeed, extracellular proteolysis is a requirement for activation of some members of the vascular endothelial growth factor (VEGF) family, specifically VEGF-B and VEGF-C. Recently, we found that VEGF-A can also be processed extracellularly by a subset of MMPs and that this processing alters the type of vasculature induced by this growth factor *(9)*.

In this chapter, we will summarize our current understanding of growth factor interaction with matrix and the relevance of MMPs, as a prototype of extracellular proteases, in the modulation of growth factor function. Although much of the discussion will bring examples of several growth factors, our focus will be VEGF because of its prominent position in the angiogenic cascade.

2. INTERACTION OF GROWTH FACTORS WITH THE ECM

The ECM accounts for over 50% of the dry weight of the vasculature, and it is largely deposited toward the end of development *(10)*. As previously discussed, matrix proteins affect fundamental aspects of endothelial cell/vascular biology. These processes are complex and involve both external structural support and regulation of

multiple signaling pathways within the cell. ECM proteins provide a scaffold essential for maintaining the organization of vascular endothelial cells into blood vessels through adhesive interactions with integrins on the endothelial cell surface. In addition, the ECM modulates most if not all aspects of neovascularization such as endothelial cell proliferation, migration, morphogenesis, survival, control cytoskeleton and cell shape, and ultimately blood vessel stabilization through endothelial cell adhesion to the substrate.

The ECM is composed of complex arrangement of fibrous proteins and associated glycoproteins and proteoglycans *(11)*. The diversity of ECM components in the endothelial cell microenvironment provides an intricate level of complexity sufficient to exert significant and precise control over many aspects of neovascularization *(3)*. For example, basement membrane (a specialized form of ECM) of quiescent blood vessel contains different ECM components that when exposed out of context can both inhibit and promote angiogenesis. The basement membrane of normally quiescent endothelial cells is composed of type IV collagen, laminin, heparan-sulfate proteoglycans, perlecans, nidogen/entactin, SPARC/BM-40/osteopontin, type XV collagen, type XVIII collagen, and other molecules *(12–14)*. In a quiescent vessel, the basement membrane is highly cross-linked, and only certain domains of various constituents are exposed to (and can interact with) endothelial cells. In contrast, ECM undergoing remodeling (i.e., during tumor angiogenesis) exposes different constituents that can interact with endothelial cells, including collagen cryptic domains. Depending on the nature of the proteolysis (enzymes that participate in the process), the products of basement membrane digestion can be pro-migratory or anti-angiogenic *(1, 15)*.

As mentioned previously, the ECM is also a reservoir of cell-binding proteins and growth factors such as VEGF, basic fibroblast growth factor (bFGF), and transforming growth factor beta (TGF-β) *(16)*. Most angiogenic cytokines have affinity for heparin and heparan sulfate proteoglycan and directly bind to ECM scaffolds such as collagen type I and fibrin/fibronectin matrices *(16–18)*.

VEGF binds to heparin with high affinity, and both heparin and heparin sulfate can compete for binding of VEGF to the ECM. Heparinase I or II can induce the release of VEGF, providing further support for the notion that heparin-containing proteoglycans are binding sites for this growth factor *(19)*. In addition, VEGF has been shown to bind to fibrinogen, fibrin, and fibronectin. More importantly, these studies showed that bound VEGF retains functional activity *in vitro (20, 21)*. However, the nature of the interaction with a specific matrix molecule is likely to alter receptor usage and biological response.

The FGF family comprises 20 members of structurally homologous, functionally distinct small polypeptides with a central core of 140 amino acids. Among all, FGF1 (acidic FGF) and especially FGF2 (basic FGF) are most preferentially implicated in angiogenesis *(22, 23)*. FGF1 and FGF2 are about 18-kDa, single-chain, nonglycosylated proteins, share about 55% sequence homology with similar biological activities *(24)*. One characteristic shared by these two molecules is that they both strongly interact with heparin-like molecules and heparan sulfate proteoglycans of the ECM *(25, 26)*.

TGF-βs are cytokines with multiple key roles in modulating cell proliferation, differentiation, apoptosis, immune responses, tissue repair, and the ECM formation *(27)*. TGF-βs are produced as large latent complexes that are linked to one of four latent

TGF-β-binding proteins (LTBP) through disulfide bonds *(28)*. Although overall *in vivo* roles of LTBPs remain unidentified, they bind the latent TGF-β complex to ECM, likely through covalent linkage of LTBPs to ECM components such as fibrillin 1, decorin, biglycan, and beta glycan *(29,30)*. Therefore, in addition to the various intracellular, cell surface, and extracellular inhibitory proteins, TGF-β-mediated signaling is further controlled by the ECM-binding property of TGF-βs. It has been shown that binding of TGF-β1 to thrombospondin can activate the growth factor *(31)*. Thus, in this case, interaction with a matrix protein alone can be critical in the regulation of growth factor activity.

As mentioned previously, many growth factors can also become anchored to heparan sulfate proteoglycans (i.e., syndecans, perlecan, and versican) either on the surface of endothelial cells or within the surrounding ECM by binding to the heparan sulfate. Syndecans are a family of transmembrane core proteins carrying with attachment sites for three to five heparan sulfate or chondroitin sulfate chains *(32)*. By virtue of the presence of heparan sulfate, syndecan interacts with a large number of heparin-binding growth factors such as FGF, VEGF, TGF-β, and platelet-derived growth factor (PDGF). Subsequently, the heparan sulfate chains of syndecans facilitate interactions between heparin-binding growth factors with various ECM molecules, including fibronectin *(32)*. Overexpression of syndecan-1 or shedding of its ectodomain inhibits FGF-2-induced cell proliferation *(33)*.

The consequence of the ECM binding to the growth factors is broad. For PDGFB, its binding to the ECM is critical to the recruitment of tumor pericytes and their integration into the vascular wall *(34)*. ECM can also mask growth factors, for example binding of decorin, biglycan and beta glycan to TGF-β competes for receptor activation and thereby diminish TGF-β signaling *(35)*. Growth factor immobilization may also provide important guidance cues for directional growth and morphogenesis. In fact, only the heparin-binding VEGF isoforms (VEGF164 and VEGF188) generate extracellular gradients that are required for directional migration of endothelial cells *(18,36)*.

Therefore, a concrete understanding of binding portfolio of each growth factor is critical to gain insights into complex biological processes, such as angiogenesis. Along the same lines, the interface between this growth factor-rich matrix and its body of degrading enzymes offers multiple opportunities for therapeutic intervention.

3. MMPS: DEGRADATION AND PROCESSING OF THE EXTRACELLULAR ENVIRONMENT

Pericellular proteases play an important role in angiogenesis and vasculogenesis. They comprise MMPs, serine proteases, cysteine cathepsins, and membrane-bound aminopeptidases *(37)*. We will center our comments to MMPs.

MMPs are a family of over 20 zinc-containing endopeptidases that can degrade/process various components of the ECM *(1)*. Although each member has its own substrate specificity, redundancies exist, and MMPs in concert are capable of degrading a wide spectrum of matrix proteins, and therefore they are considered to be the major proteases involved in the remodeling of the endothelial basement membrane and interstitial matrix *(37)*. Quiescent endothelial cells produce little or no MMPs, whereas the activated (i.e., angiogenic, in wound healing, and in inflammation) endothelial cells strongly up-regulate the expression of several MMPs *in vitro*.

Recently, it has become clear that MMPs' role in angiogenesis is more complex than simply degrading the ECM to facilitate invasiveness of endothelial cells. For example, MMPs have been shown to generate both pro- and anti-angiogenic molecules by proteolytic cleavage of ECM components. MMPs cleave type IV collagen to expose cryptic αvβ3-binding sites. Following cleavage, type IV collagen loses its binding to integrin α1β1 but binds to αvβ3 integrin with higher affinity and this promotes angiogenesis *(15)*. This cleavage is associated with increased MMP-2 expression and activation. Exposure of these cryptic sites occurs within the endothelial basement membranes in angiogenic and tumor blood vessels but not in quiescent vessels. Relevant to anti-angiogenic activities of MMPs, the C-terminal non-collagenous I (NC1) domain of several collagen chains, generated by proteolytic cleavage, shows anti-angiogenic activity *(16)*. These include endostatin (the NC1 domain of type XVIII collagen α1 chain), tumstatin (type IV collagen α3 chain NC1), and arrestin (type IV collagen α1 chain NC1). Endostatin can be released by MMP-3, MMP-9, MMP-12, MMP-13, and MMP-20, as well as by several cathepsins *(38)*. Endostatin binds to cell surface proteoglycans, to VEGFR-2, and to integrin α5β1 to inhibit VEGF- and bFGF-induced endothelial cell migration and to induce apoptosis *(39)*. In addition, endostation blocks the activation and activity of MMP-2, MMP-9, MMP-13, and MT1-MMP *(40, 41)*. Tumstatin, which can be released by MMP-9, inhibits endothelial cell proliferation and promotes apoptosis. Decreased levels of tumstatin in MMP-9 null mice were shown to be responsible for increased tumor growth *(39, 42)*. With respect to other ECM molecules proteolyzed by MMPs, fibronectin is concentrated at the pericyte–endothelial cell interstitium, and its degradation by MMPs gives rise to biologically active fragments *(43)*. Among these, 145-kDa fibronectin fragment inhibits endothelial cell proliferation and stimulates pericytes and VSMC proliferation, suggesting a role for this fragment in vessel maturation *(44)*.

In addition to degrading ECM components and activating other enzymes, MMPs can enhance the availability/bioactivity of growth factors and cytokines. Degradation of ECM releases ECM/basement membrane-sequestered angiogenic factors such as VEGF, bFGF, and TGF-β. In tumor angiogenesis, MMP-2 and MMP-9 have been shown to be critical for the "angiogenic switch" when tumors first become vascularized by the selective release of VEGF *(45)*. Similarly, overexpression of MMP-9 in human breast cancer MCF-7 cells resulted in increased tumor angiogenesis, tumor growth, and VEGF/VEGFR-2 complex formation suggesting that MMP-9 regulates the release of VEGF from the ECM *(46)*.

MMPs can target many non-ECM proteins, including growth factors, growth factor receptors, cell-associated molecules, and cytokines. MMPs release active growth factors by cleavage of growth factor precursor or growth factor-binding proteins. For example, MMP-3 and MMP-7 have been shown to cleave the membrane-bound precursor of heparin-binding EGF (HB-EGF), releasing active HB-EGF, whereas tumor necrosis factor-α (TNF-α) is released from the cell surface by MMP-1, MMP-3, and MMP-7 *(47)*. MMP-1, MMP-3, MMP-7, or MMP-13 releases active VEGF165 from connective tissue growth factor (CTGF)/VEGF complex by direct cleavage of CTGF *(48)*. MMP-2 and MMP-9 proteolytically activate latent TGF-β1 and TGF-β2 *(47, 49)*. Also recently, it was shown that bone morphogenetic protein 1 (BMP1)-like MMP cleaved latent TGF-β1-binding protein LTBPs at two specific sites, thus liberating a large latent complex of TGF-β from ECM and resulting in subsequent activation of TGF-β1 *in vitro (50)*.

Relevant to angiogenesis, MMP-9 cleaves the pro-inflammatory, pro-angiogenic cytokine IL-8 increasing its activity by tenfold *(51)*. On the other hand, MMP-2 cleaves the FGF receptor 1 (FGFR1) releasing the soluble ectodomain of FGFR1 that can still bind FGFs, but lacks signaling capacity *(52)*. Also, MMPs cleave the ectodomain of VE-cadherin, a major cell–cell adhesion molecule in endothelial cells *(8)*. MT1-MMP processes the αvβ3 integrin into two disulfide-linked fragments that retain RGD-ligand binding, and this processing enhances integrin signaling through focal adhesion kinase, contributing to enhanced adhesion and cell migration *in vitro*nectin. MMP-2, MMP-7, MMP-9, and MMP-12 have the capacity to hydrolyze plasminogen to form the potent angiogenesis inhibitor, angiostatin *(53–55)*.

4. VEGF PROCESSING: A NEW ROLE FOR MMPS IN ANGIOGENESIS

VEGF signaling is essential for specification, morphogenesis, differentiation, and homeostasis of vessels during development and in the adult *(56–60)*. Furthermore, this signaling pathway is an integral component of pathological angiogenesis during tumor expansion *(61)*. In fact, decreased levels of VEGF result in suppression of vascular expansion and concomitant reduction of tumor growth and metastasis *(62,63)*. Therefore, it is not surprising that VEGF levels are under exquisite transcriptional and translational control, and slight alterations in expression levels can have devastating effects during development. In fact, unlike most mammalian genes, inactivation of only one allele results in embryonic lethality at mid-gestation due to severe cardiovascular defects *(64,65)*. Interestingly, a twofold increase of VEGF can also lead to lethality *(66)*. Thus, both decrease and increase in VEGF levels translate into significant pathological effects to the vasculature and to the organism as a whole. Therefore, regulation of VEGF synthesis, secretion, and availability carries important implications in the modulation of the angiogenic response.

VEGF-A (also known as simply as VEGF) exists as five different isoforms termed according to the number of amino acids, they are 121, 145, 165, 189, and 206 in humans (in mouse each isoform lacks one amino acid). These forms are generated by alternative splicing of a single pre-mRNA and differ in their ability to bind to heparan sulfate and to ECM molecules. The gene encoding VEGF-A comprises 14 kb and contains eight exons. Exons 2–5 code for the receptor-binding sites, whereas exons 6a, 6b, and 7 code for residues that bind to matrix proteins. These last three exons can be selectively spliced generating the different protein isoforms. The last exon (exon 8) is present in all isoforms. VEGF121 lacks exons 6a, 6b, and 7 and is the only highly soluble form. All other variants bind to ECM proteins restricting access of the growth factor to receptors on target cells. The affinity for matrix proteins is thought to be proportional to the length of the carboxy-terminal end (Fig. 1).

How can matrix-bound VEGF become free from its interactions with the ECM? A favored hypothesis has been that MMPs mediate its release by degradation of ECM proteins. Although this possibility is likely correct, we have been interested in testing an alternative (yet not exclusive) possibility, namely that VEGF-A could be cleaved intramolecularly to specifically mediate release. We showed that VEGF could indeed be cleaved by several MMPs, releasing a dimeric fragment of 32 kDa able to phosphorylate VEGFR2 and induce angiogenesis *in vivo (9)* (Fig. 2). The location of the cleavage site (113 amino acid) indicates that all VEGF forms (including VEGF120) are susceptible to this event.

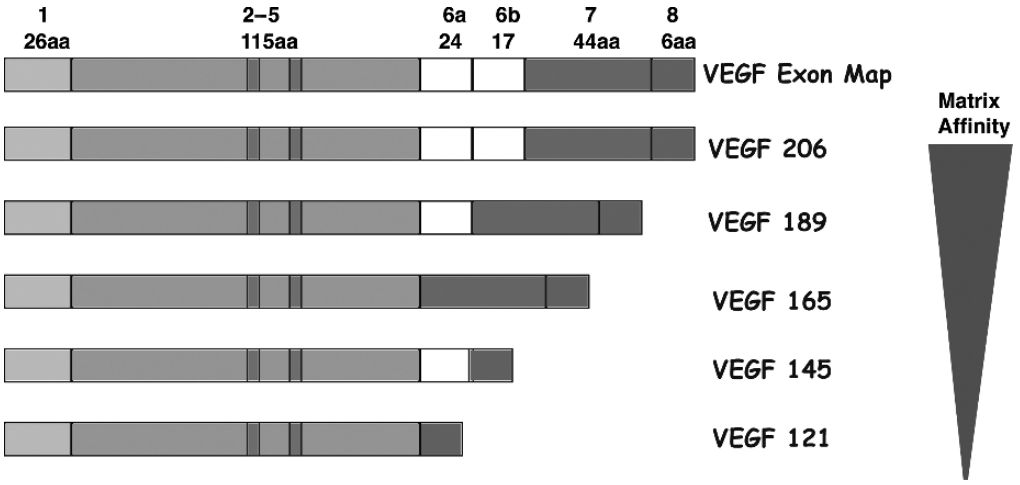

Fig. 1. Structure of vascular endothelial growth factor (VEGF) isoforms and matrix affinity. VEGF is coded by eight exons. Exons 2–5 code for the receptor-binding region, whereas exons 6a, 6b, and 7 code for amino acids involved in binding to the extracellular matrix (ECM). These can be alternatively spliced to give rise to multiple isoforms.

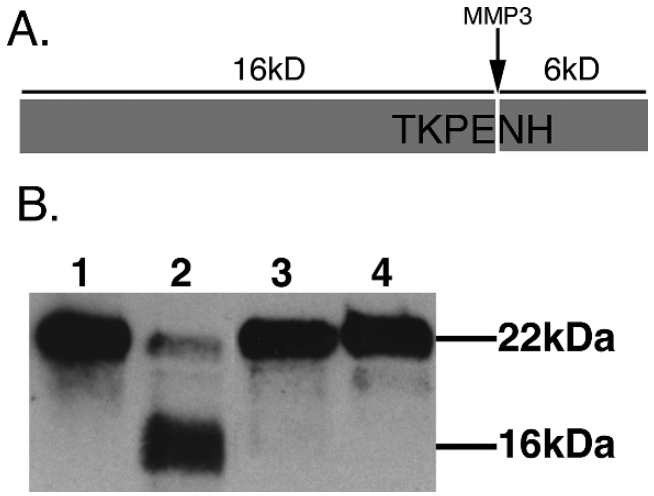

Fig. 2. Vascular endothelial growth factor (VEGF) cleavage by matrix metalloproteinase-3 (MMP-3). (**A**) Schematic representation of VEGF monomer and the site for MMP cleavage. (**B**) Biotinylated VEGF165 was incubated with MMP-3 in the presence of specific inhibitors indicated below. The digestion products were resolved in tricine gels and were detected by avidin-HRP. Lanes 1, VEGF; 2, VEGF + MMP-3; 3, VEGF + MMP-3 + EDTA; 4, VEGF + MMP3 + BB94. EDTA blocks MMPs function and BB94 is a pan-MMP inhibitor. 22-kDa, glycosylated mVEGF165 monomer and 16-kDa, cleaved fragment.

In that same study, we determined the significance of VEGF processing to tumor angiogenesis and compared the effects of MMP-cleaved VEGF and an MMP-resistant VEGF to wild-type VEGF. Our findings demonstrated that bound (MMP resistant) and soluble VEGF (MMP cleaved) are able to activate VEGFR2 equivalently *in vitro*, yet each form elicits distinct modes of vascular expansion *in vivo*. Signaling initiated by matrix-bound VEGF resulted in the formation of a highly branched vasculature, in contrast, signaling initiated by soluble (MMP cleaved) VEGF leads to vascular hyperplasia and hypertrophy with poor sprouting response (Fig. 3) *(9)*.

To gain further insight onto the cellular effects mediated by soluble and bound VEGF, we evaluated their effects *in vitro*. Purified growth factors at identical concentrations were included in fibrinogen/fibronectin gels before polymerization, and the behavior of endothelial cells bound to sepharose beads was examined. Our data showed that MMP-resistant VEGF induced capillary morphogenesis, whereas cleaved VEGF (VEGF113) induced the proliferation of endothelial cells in sheets *(9)*. These results indicate that local and discrete VEGF164 cleavage is likely to occur as endothelial cells migrate and grow as sheets, whereas in the absence of such digestion, VEGF mediates the organization of cord-like structures. Although further mechanistic exploration of these findings is in progress, it is likely that specific recruitment of distinct downstream signaling targets is a key molecular initiator of these morphogenetic events.

VEGF is known to signal through two receptor tyrosine kinases: VEGFR1 (flt-1 or FLT-1) and VEGFR2 (flk-1 or KDR) *(67)*. In addition, a non-tyrosine kinase receptor, neuropilin-1, is known to bind to VEGF and modulate the responses to VEGFR2 *(68)*. The two tyrosine kinase receptors share 44% homology and consist of seven extracellular immunoglobulin-like domains, a single transmembrane region, a split tyrosine kinase domain that is interrupted by a 70 amino acid kinase insert, and a C-terminal tail.

VEGFR1 exists in both transmembrane and soluble forms, and although it displays high affinity, it negatively regulates vasculogenesis and angiogenesis during early development *(69)*. Genetic inactivation of VEGFR1 results in increased hemangioblast commitment toward the endothelial fate, an outcome that leads to profuse vascular disorganization due to endothelial cell overgrowth *(69)*. These data, as well as additional information from several laboratories, indicate that VEGFR1 functions as a decoy

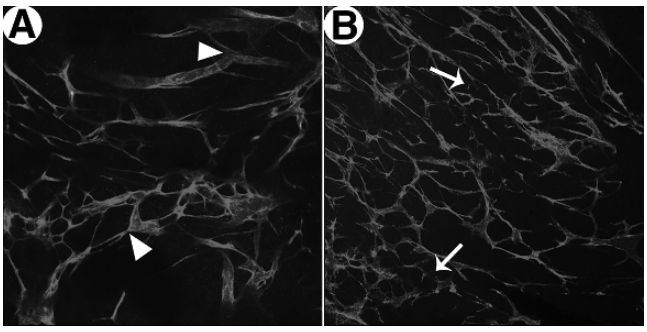

Fig. 3. Distinct tumor vessel phenotype by different vascular endothelial growth factor (VEGF) forms. Platelet/endothelial cell adhesion molecule (PECAM) staining of tumor sections expressing cleaved form of VEGF (**A**) and MMP-resistant form of VEGF (**B**). Arrowheads point to dilated and fused vessels. Arrows point to excess vessel branching. Note the difference in vessel density between **A** and **B**. (Please see color insert.)

receptor and regulator of VEGFR2 function by controlling the levels of VEGF at the cell surface *(70)*. Furthermore, VEGFR1 has been shown to display pro-angiogenic properties during inflammatory responses. Its contribution appears to be relevant in rheumatoid arthritis although it is unclear whether this activation is mediated by VEGF-A, VEGF-B, placental growth factor (PLGF), or a combination thereof *(71)*.

Our current understanding indicates that the sum of pro-angiogenic responses initiated by VEGF originates primarily through activation of VEGFR2 *(67)*. VEGFR2 has been shown to induce proliferation, migration, survival, and permeability *(67)*. However, much remains to be learned about how this single receptor regulates downstream signals to specify each of these possible responses. Six autophosphorylation sites have been identified on the intracellular domain of VEGFR2: 951, 996,1054, and 1059 are located in the kinase domain, and 1175 and 1214 are located within the C-terminal tail *(67)*. Using a combination of genetic deletion and cell biological approaches, tyrosine 1175 has been identified as a VEGFA-dependent autophosphorylation site essential to developmental angiogenesis *(72)*. Other individual, and some combinations of, tyrosine sites have been thought to play different roles in signal transduction pathways that affect neovascularization of tumors and permeability events.

In terms of specific intracellular signal transduction pathways, it is well accepted that VEGFR2 activates PLC gamma and phosphatidylinositol 3-kinase (PI3K) through binding to phosphorylated $VEGFR2_{Tyr1175}$ *(73)*. Activation of PI3K pathway through Tyr1175 leads to signaling through AKT/PKB and regulation of endothelial cell survival *(74, 75)* and in binding to the adaptor protein Sck/ShcB *(76)*. In addition, Shb also binds to phosphorylated Tyr1175 although the biological significance of this binding is yet to be determined *(77)*.

Tyrosine 951 also has important downstream-signaling events. Once phosphorylated, this residue binds to TSAd [T-cell-specific adaptor; also known as VEGFR-associated protein (VRAP)] *(78)*. The phosphorylated Tyr-951-TSAd complex regulates cell migration and has been implicated in neovascularization of tumors *(78,79)*. In addition, VEGF-A induces the formation of a complex between TSAd and Src, which indicates that TSAd might regulate Src activation and vascular permeability downstream of VEGFR2 *(78, 80)*.

Targeted mutation of Tyr1212 (corresponding to human Tyr1214) to phenylalanine (Tyr1212Phe) results in mice that are viable and fertile *(72)*. However, phosphorylation of Tyr1214 has been implicated in VEGF-induced actin remodeling through the sequential activation of cdc42 and p38MAPK *(81)*. Inhibition of the p38MAPK augments VEGF-induced angiogenesis in the chorioallantoic membrane (CAM) *(82, 83)* without an accompanying increase in vascular permeability *(82)*. In addition, p38MAPK induces the phosphorylation of the heat-shock protein-27, a molecular chaperone that positively regulates VEGF-induced actin reorganization and migration *(84,85)*. In light of these findings, it is unclear how to reconcile the outcome of the mutant mouse (Tyr1212Phe) *(72)*. Either additional sites are involved or compensatory mechanisms might overcome the mutation. Perhaps, a detailed evaluation of the p38MAPK activation in the mutant mice could shed light on this paradox.

An integration between the selective activation of tyrosine residues and their specific downstream targets together and the soluble versus bound VEGF-induced activation of VEGFR2 will likely shed light on the molecular orchestration that leads to the morphogenesis of differential vascular beds.

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