Commentary

What Is the Role of Vascular Endothelial Growth Factor-Related Molecules in Tumor Angiogenesis?

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Growth of solid tumors depends on angiogenesis, the process by which new blood vessels develop from the endothelium of a pre-existing vasculature.¹ Tumors promote angiogenesis by secreting growth factors that stimulate endothelial migration, proliferation, proteolytic activity, and capillary morphogenesis.² Newly formed blood vessels supply the tumor with nutrients and oxygen, dispose of its metabolic waste products, and generate paracrine stimuli, which further promote tumor cell proliferation and invasiveness.^{1,3}

Among the known angiogenic factors, vascular endothelial growth factor (VEGF) has emerged as a central regulator of the angiogenic process in physiological and pathological conditions.⁴ This molecule was first named vascular permeability factor by Dvorak and co-workers because of its potent stimulatory effect on the permeability of the tumor microvasculature.⁵ Ferrara et al⁶ coined the term VEGF to describe a mitogenic factor that selectively stimulated endothelial cell proliferation and angiogenesis. The same molecule was independently discovered by others.^{7,8} Connolly et al⁹ reported that vascular permeability factor was mitogenic for endothelial cells and had the capacity to stimulate vascular proliferation. Sequence analysis of cDNAs demonstrated that vascular permeability factor and VEGF were the same molecule.⁴ Recently, the VEGF family of growth factors has expanded with the addition of four new molecules: placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D (Table 1).^{10–13} VEGF and VEGF-related molecules have homologous amino acid sequences including eight cysteine residues, which are also found in platelet-derived growth factors A and B, and bind to the same class of tyrosine kinase receptors.^{4,8} In this issue of the *American Journal of Pathology*, Salven et al¹⁴ report that VEGF-B and VEGF-C are expressed in a variety of human tumors. This commentary briefly reviews our knowledge of this field and critically evaluates the potential role of VEGF-related molecules in tumor angiogenesis and neoplastic progression.

VEGF

VEGF comprises four main isoforms produced by alternative splicing of mRNA: VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆.^{4,15} A fifth isoform, VEGF₁₄₅, has been found in placental cells and carcinoma cells from the female reproductive tract.¹⁶ VEGF molecules have a signal peptide sequence and are secreted as homodimers through conventional pathways.⁴ The mature form of VEGF is the VEGF₁₆₅ homodimer, which has a molecular weight of ~45 kd. VEGF₁₈₉ and VEGF₂₀₆ are the most basic isoforms, bind to heparin with greater affinity than VEGF₁₆₅, and are almost completely sequestered in the extracellular matrix. VEGF₁₂₁, which is slightly acidic because it lacks the basic amino acids responsible for heparin binding, is the most soluble isoform. In the extracellular compartment, plasmin cleaves a portion of the VEGF molecule, generating a 34-kd protein that consists of the first 110 NH₂-terminal amino acids.¹⁷ This may be a mechanism whereby bioactive VEGF is released by proteolysis from larger isoforms sequestered in the extracellular matrix.^{4,17}

VEGF binds with high affinity to two tyrosine kinase receptors: VEGF receptor (VEGFR)-1 (*Flt-1*) and VEGFR-2 (*KDR/Flk-1*).^{4,18,19} Binding of VEGF causes receptor dimerization followed by autophosphorylation of the receptor and signal transduction. There are significant differences between VEGFR-1 and VEGFR-2. For example, endothelial cells without endogenous VEGFRs migrate and proliferate in response to VEGF when transfected with VEGFR-2 but lack such responses if transfected with VEGFR-1.²⁰ VEGFR-1 is, however, capable of mediating PlGF-induced endothelial cell proliferation and plasminogen activator expression.⁶¹ Gene knockout experiments have confirmed a critical role in angiogenesis for both VEGFR-1 and VEGFR-2.^{21,22} Inactivation of the VEGFR-2 gene causes failure of vasculogenesis, the process of *de novo* formation of blood vessels from undiffer-

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Table 1. VEGF and VEGF-Related Molecules

Ligand	Isoforms	Chromosome*	Receptor	Vascular target
VEGF	VEGF ₁₂₁ VEGF ₁₆₅ VEGF ₁₈₉ VEGF ₂₀₆ VEGF ₁₄₅	6p	VEGFR-1 VEGFR-2	Hematic endothelium
PlGF	PlGF-1 PlGF-2 PlGF-3	14q	VEGFR-1	Hematic endothelium
VEGF-B	VEGF-B ₁₆₇ VEGF-B ₁₈₆	11q	VEGFR-1	Hematic endothelium
VEGF-C	VEGF-C	4q	VEGFR-2 VEGFR-3	Lymphatic endothelium Hematic endothelium
VEGF-D	VEGF-D	Xp	VEGFR-2 VEGFR-3	Lymphatic endothelium Hematic endothelium

*Chromosome localization refers to human genes.

entiated mesenchyme.²¹ VEGFR-2 knockout embryos are unable to form blood islands and to generate hemopoietic precursors. Targeted mutation of the VEGFR-1 gene does not affect the differentiation of endothelial cells but causes a disorganized assembly of the developing vasculature.²² Because of vascular abnormalities, both VEGFR-1 and VEGFR-2 knockout embryos die *in utero* between days 8.5 and 9.5. Embryos having a single (VEGF+/-) or both VEGF (VEGF -/-) alleles defective suffer from a failure of vasculogenesis, leading to intra-uterine death by day 10 to 12.²³ The demonstration of a vasculogenic defect also in heterozygous embryos (VEGF+/-) suggests that there is a critical threshold of VEGF levels below which new blood vessels are unable to form.²³

VEGF stimulates the migration and proliferation of arterial, venous, and microvascular endothelial cells as well as angiogenesis *in vivo* and *in vitro*.^{4,6,7,9} VEGF promotes the balanced degradation of the extracellular matrix around the sprouting endothelium by inducing the expression of urokinase-type plasminogen activator, tissue-type plasminogen activator, plasminogen activator inhibitor-1,²⁴ and interstitial collagenase.²⁵ By enhancing the permeability of venules to circulating proteins including fibrinogen, VEGF is believed to facilitate the perivascular deposition of fibrin, which further potentiates angiogenesis.²⁶ Through its capacity to induce nitric oxide, VEGF may also mediate the vasodilation and increased blood flow that precede angiogenesis.²⁷

PlGF

The PlGF gene encodes three alternatively spliced isoforms with different secretion patterns, heparin binding affinities, and dimerization properties: PlGF-1, PlGF-2, and PlGF-3.^{10,28-30} PlGF binds with high affinity to VEGFR-1 but not to VEGFR-2.³¹ The expression of PlGF is restricted to the placenta and is not observed in the majority of normal adult tissues.³⁰ Because it forms heterodimers with VEGF, which are less potent than VEGF homodimers, PlGF may reduce the bioavailability of active VEGF molecules.³² PlGF, however, can also potentiate the activity of suboptimal concentrations of VEGF.³¹

Recently, Ziche et al³³ reported that recombinant PlGF-1 purified from overexpressing eukaryotic cells was as effective as VEGF and basic fibroblast growth factor in stimulating angiogenesis in the rabbit cornea assay. They also noticed that the mitogenic effect of PlGF-1 was dependent on the endothelial cell type. On this basis, they suggested that the preferential target of PlGF-1 is the endothelium of postcapillary venules, whereas VEGF stimulates with equal potency both macrovascular and microvascular endothelium.

VEGF-B

VEGF-B is present in a variety of normal organs and is particularly abundant in heart and skeletal muscle. There are two alternatively spliced isoforms of VEGF-B: VEGF-B₁₆₇ and VEGF-B₁₈₆.^{11,34,35} VEGF-B₁₆₇ is a highly basic heparin-binding protein which, similarly to VEGF₁₈₉ and VEGF₂₀₆, remains associated with the cell or the extracellular matrix and is not released into the culture medium unless cells are treated with heparin. VEGF-B₁₈₆ homodimers, on the other hand, are readily secreted.³⁴ VEGF-B can form disulfide-linked heterodimers with VEGF and influence the bioavailability of this molecule, as described for PlGF.^{11,34} VEGF-B stimulates endothelial cell proliferation, but it does not bind to VEGFR-2. Recent studies indicate that the function of VEGF-B is mediated by VEGFR-1 (Olefsson, Kerpelainen, Mandriota, Pepper, Aase, Kumar, Ganji, Jelisch, Shibuya, Alitalo, and Eriksson, unpublished manuscript).

VEGF-C

VEGF-C is produced as a propeptide that is proteolytically cleaved to a 21-kd molecule.^{12,35,36} Unlike some of the VEGF, VEGF-B, and PlGF isoforms, VEGF-C does not bind to heparin. VEGF-C increases vascular permeability and stimulates the migration and proliferation of endothelial cells, but at a significantly higher concentration than VEGF.¹² VEGF-C binds to and induces autophosphorylation of the tyrosine kinase receptor *Flt-4*, which has been renamed VEGFR-3.¹² VEGF-C also activates

VEGFR-2 but not VEGFR-1.¹² Proteolytic processing of VEGF-C generates several VEGF-C forms with increased activity toward VEGFR-3, but only the fully processed VEGF-C can bind to VEGFR-2.³⁶ VEGF-C has greater affinity for VEGFR-3 than VEGFR-2. The other members of the VEGF family, with the exception of VEGF-D (see below), are unable to activate VEGFR-3. VEGF-C is expressed during embryonal development in regions where lymphatics sprout from venous vessels.¹² VEGF-C is present also in adult tissues, where it is postulated to play a role in the maintenance of lymphatic endothelial differentiation.¹² VEGFR-3 is highly expressed in angioblasts, veins, and lymphatics during embryonal vasculogenesis but becomes largely restricted to the lymphatic endothelium in adult tissues.^{12,37} VEGFR-3 is also expressed in the high venular endothelium of lymph nodes.³⁷ Because of their patterns of expression, VEGF-C and VEGFR-3 have been implicated in lymphangiogenesis, the process of formation of new lymphatics. Exogenous VEGF-C selectively stimulates lymphatic proliferation in the chorioallantoic membrane, whereas VEGF promotes angiogenesis from blood vessels.³⁸ Consistent with these observations, targeted overexpression of VEGF-C in the skin of transgenic mice causes lymphatic hyperplasia.³⁹ The targeted effect of VEGF-C is probably linked to the formation of VEGFR-2/VEGFR-3 heterodimers in lymphatic endothelial cells.³⁹

VEGF-D

VEGF-D, the newest member of the VEGF family, is 48% identical to VEGF-C.¹³ Both VEGF-C and VEGF-D have long NH₂- and C-terminal extensions, which set these growth factors apart as a subfamily of VEGF-related proteins. VEGF-D is induced by *c-fos*⁴⁰ and is strongly expressed in the fetal lung during development. In the adult it is primarily found in skeletal muscle, heart, lung, and intestine.¹³ VEGF-D is a ligand for both VEGFR-2 and VEGFR-3, but does not bind to the VEGFR-1.⁴¹ In addition, VEGF-D stimulates the proliferation of endothelial cells.⁴¹

Regulation of VEGF and VEGF-Related Molecules

Members of the VEGF family are regulated by different mechanisms. For example, serum, growth factors, and inflammatory cytokines increase VEGF and VEGF-C mRNAs but have no effect on VEGF-B.^{42,62} Amplification of the *ras* oncogene causes up-regulation of VEGF⁴ but leaves unaltered the expression of VEGF-B and VEGF-C.⁴² Hypoxia strongly induces VEGF mRNA expression but has no significant effect on VEGF-B, VEGF-C, or PlGF.^{4,42} A mutant form of p53 potentiates the expression of VEGF mRNA but has no effect on VEGF-B or VEGF-C mRNA.^{42,43} Renal cell carcinoma cells lacking the von Hippel-Lindau (VHL) tumor suppressor gene or expressing an inactive form of this gene exhibit abnormal regulation of VEGF production, which is reversed by introduc-

tion of the wild-type VHL gene.⁴⁴ Human chorionic gonadotropin induces expression of VEGF by granulosa-luteal cells, decreases production of VEGF-C, and has no effect on VEGF-B.⁴⁵ Among the VEGF-related molecules mRNAs, the VEGF-B mRNA is the most stable and is not regulated by any of the factors examined so far.

Expression of VEGF and VEGF-Related Molecules in Tumors

VEGF has been found in lung, thyroid, breast, gastric, colonic, kidney, bladder, ovary, and cervical carcinomas, as well as in angiosarcomas, germ cell tumors, and neoplasms of the central nervous system (reviewed by Ferrara and Davis-Smyth⁴). PlGF was down-regulated in thyroid carcinomas and overexpressed in renal cell carcinomas.^{46,47} Studies with cultured cells showed that VEGF-B was expressed by a variety of tumor cells, whereas VEGF-C was detected in a limited number of prostatic, lung, and renal carcinoma cell lines.⁴² The work by Salven et al¹⁴ demonstrates different patterns of expression of VEGF, VEGF-B, and VEGF-C in breast carcinomas, squamous cell carcinomas, lymphomas, melanomas, sarcomas, adenomas, and papillomas.¹⁴ VEGF-B was found by Northern blot analysis in approximately 90% of the tumors, whereas VEGF-C was detected in half of the cases. Interestingly, 10 tumors expressed VEGF-C and/or VEGF-B but not VEGF, and four carcinomas failed to produce any type of VEGF or VEGF-related molecule. In a similar study, using *in situ* hybridization and reverse transcriptase polymerase chain reaction, Sowter et al⁴⁸ found that mRNAs encoding for VEGF and VEGF-B were up-regulated in ovarian carcinomas relative to the normal ovarian surface epithelium. Conversely, neither VEGF-C nor PlGF was expressed at a substantial level.

Potential Role of VEGF-Related Molecules in Tumor Angiogenesis

Although more studies are needed to better define the significance of these observations, a picture is emerging showing heterogeneity of VEGF and VEGF-related molecules expression in human neoplasms. This heterogeneity is not surprising, because of the distinct regulatory mechanisms that control the expression of these growth factors. Based on the VEGF/VEGF-related molecule profiles reported by Salven et al,¹⁴ tumors can be divided into different groups depending on which factors predominate. Tumors that do not produce significant amounts of VEGF may use VEGF-related molecules to stimulate angiogenesis. Although they are less potent than VEGF, VEGF-related molecules have the capacity to directly activate VEGFRs and potentiate the activity of VEGF when this factor is produced at low levels.³¹ In tumors that do not express VEGF, VEGF-B, or VEGF-C, angiogenesis may be promoted by PlGF, VEGF-D, or other VEGF-related molecules that have yet to be de-

scribed. It is also reasonable to invoke VEGF-independent mechanisms involving other angiogenic factors.

Potential Role of VEGF-Related Molecules in Lymphangiogenesis and Cancer Spread

The discovery of VEGF-C and VEGF-D, which are both capable of activating the lymphatic endothelial receptor VEGFR-3, has reopened the question of lymphatic proliferation in tumors. Achen et al⁴¹ have proposed that expression of VEGF-C and VEGF-D at a particular site in developing embryos can attract the growth of both hematic and lymphatic endothelial cells, whereas expression of VEGF, a molecule that activates VEGFR-2 but not VEGFR-3, would only attract the hematic endothelium. A similar hypothesis can be postulated for tumors, but previous studies have failed to demonstrate lymphangiogenesis in neoplasms.⁴⁹ The reported absence or paucity of lymphatics in tumors is intriguing, because lymphatics can proliferate in experimental conditions.^{50–52} In addition, lymphatic endothelial cells have been isolated and shown to form capillary tubes when stimulated with endothelial growth factors.⁵³ Evaluation of lymphatics in tumors is made difficult by the attenuated morphology of lymphatic capillaries and the lack of markers for lymphatic endothelial cells. The discovery of VEGFR-3 and the development of VEGFR-3-specific probes should facilitate the identification of lymphatics in tissue sections. Using this approach, it would be of interest to evaluate whether tumors that produce VEGF-C and/or VEGF-D have a greater number of lymphatics and a more aggressive behavior than tumors negative for these factors. Should these studies confirm the lack of lymphangiogenesis in human tumors, it will be important to investigate the mechanisms responsible for this phenomenon. A possible explanation could be that growth of lymphatics is blocked by the high interstitial pressure of tumors. In addition, lymphatic endothelial cells may be sensitive to inhibition by tumor cell-derived angiostatic factors such as endostatin.⁵⁴

VEGF-C and VEGF-D may promote tumor progression by mechanisms other than lymphangiogenesis. For example, lymphatic endothelial cells may respond to VEGF-C and VEGF-D by secreting chemotactic factors for cancer cells, thereby promoting lymphatic invasion and lymph node metastases. VEGF-C and VEGF-D may regulate the contractility of lymphatics by stimulating nitric oxide production, as reported for VEGF in arteries.²⁷ This in turn may affect the extent of lymphatic permeation and the rate of metastasis, because lymph is propelled to lymph nodes by periodic contractions of lymphatic vessels.⁵⁵ In addition, cancer cells that produce VEGF-C or VEGF-D and metastasize to lymph nodes may have a growth advantage because of their capacity to stimulate both hematic and lymphatic endothelia.³⁷ A similar consideration applies to lymphoma cells the angiogenic activity of which may be mediated, at least in part, by VEGF-C and/or VEGF-D.^{14,56}

VEGF-Related Molecules in Tumors: Therapeutic Implications

The heterogeneity of VEGF and VEGF-related molecules expression in tumors has important implications for the design of anti-angiogenic therapy in cancer patients. Experimental animal models have demonstrated that tumor growth can be inhibited with anti-VEGF antibodies (reviewed by Ferrara and Davis-Smyth⁴). This effect is due to the anti-angiogenic activity of the antibodies, which starve tumor cells by cutting off their blood supply. It is also possible that a restricted number of tumors capable of expressing VEGFRs may be directly inhibited by anti-VEGF therapy.^{57,58} A clinical trial is currently evaluating the safety and efficacy in cancer patients of a humanized anti-VEGF antibody.⁵⁹ Although it is too early to make predictions, the report by Salven et al¹⁴ suggests that there may be a subset of tumors that do not use VEGF to stimulate angiogenesis and are therefore resistant to anti-VEGF treatment. On this basis, tumor angiogenesis may be more effectively blocked using a cocktail of antibodies directed against VEGF and VEGF-related molecules. An additional approach would be to target VEGFRs with either blocking antibodies or tyrosine kinase inhibitors. It is, however, still unclear how interactions among VEGF and VEGF-related molecules affect tumor angiogenesis. Future studies with experimental models of angiogenesis should elucidate the potential antagonism, synergy, or additive effects of different combinations of VEGF and VEGF-related molecules. It will also be of interest to evaluate whether there is a relation between the expression of VEGF-related molecules, the density of microvessels in histological section, and the aggressiveness of the tumor, as reported for VEGF.⁶⁰ Finally, studies should be carried out to evaluate the potential role in the lymphatic spread of cancer of lymphangiogenic factors such as VEGF-C and VEGF-D.

Summary and Conclusion

The work by Salven et al¹⁴ demonstrates that there is redundancy and heterogeneity of VEGF and VEGF-related molecules expression in human tumors. Although the biological significance of these observations remains to be elucidated, this report suggests that tumors from different patients use distinct repertoires of VEGF and VEGF-related molecules to stimulate angiogenesis. In addition, some tumors produce lymphangiogenic molecules such as VEGF-C, which may contribute to lymph node metastasis. These findings confirm the complexity of tumor angiogenic regulation and raise challenging new questions for both basic researchers interested in the molecular mechanisms of tumor progression and clinical investigators involved in the design of growth factor-based anti-angiogenic therapy for cancer patients.

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