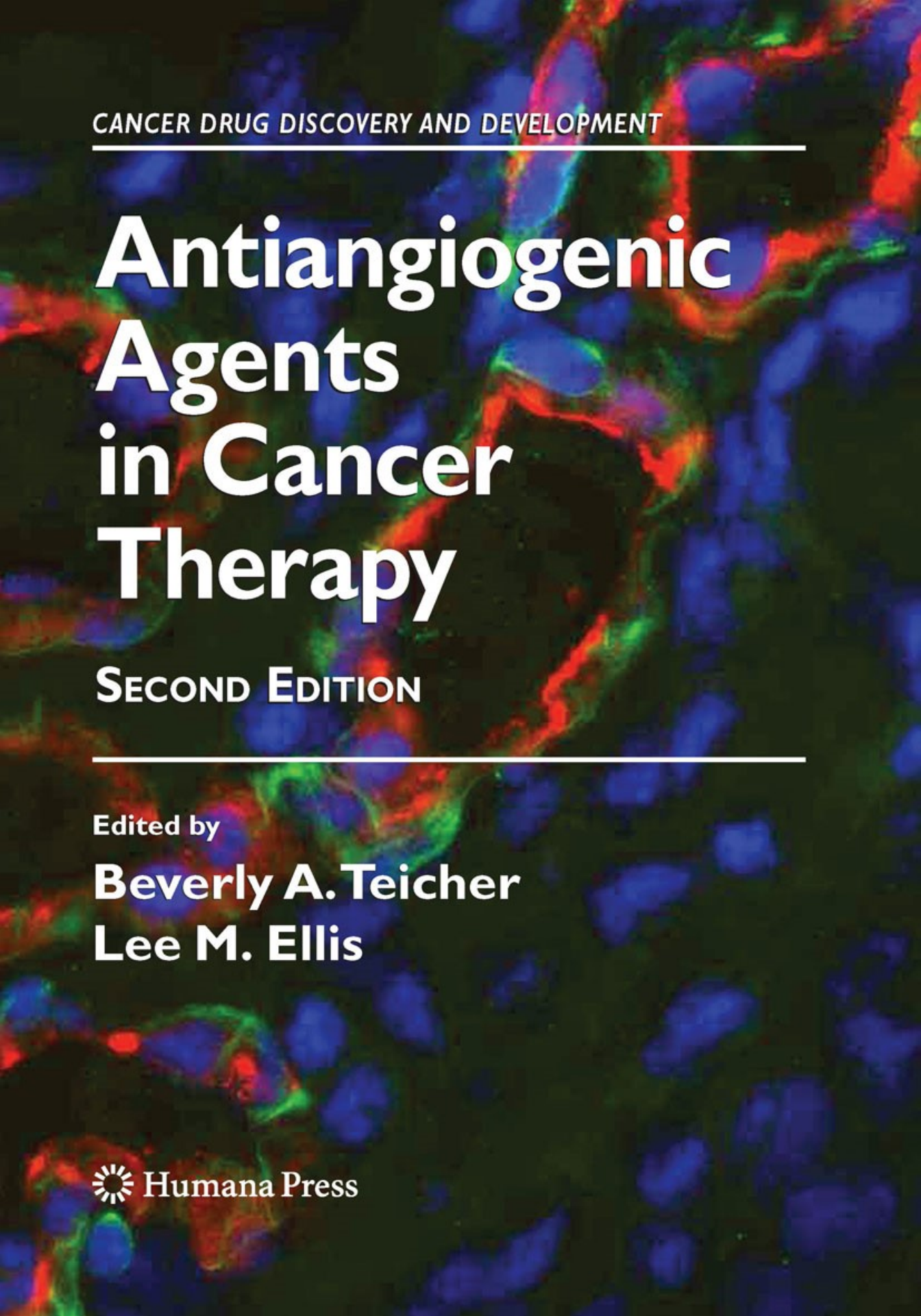
CANCER DRUG DISCOVERY AND DEVELOPMENT
Antiangiogenic Agents in Cancer Therapy
SECOND EDITION
Edited by
Beverly A. Teicher
Lee M. Ellis
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Antiangiogenic Agents in Cancer Therapy

Cancer Drug Discovery and Development

Beverly A. Teicher, Series Editor

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Antiangiogenic Agents in Cancer Therapy

Second Edition

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Preface

Angiogenesis as a therapeutic target for malignant disease has evolved from a pioneering idea outside of the mainstream of therapeutic development to an FDA-approved therapy widely used in patients with metastatic disease. The success in achieving such rapid progress in realizing the importance of angiogenesis in tumor growth its value as a therapeutic target, as well as reflects the impact of vocal pioneers in the field and the dedication, creativity, and insight of scientific investigators in oncology over the past 35 years.

This second edition of *Antiangiogenic Agents in Cancer Therapy* is intended to give a current perspective on the state of the art of angiogenensis and therapy directed at this process. Part I reflects the enormous progress in understanding the cell types, the growth factors, the environmental influences, and the genetic and physiologic abnormalities that mediate angiogenesis and its role in progression of malignant disease. Part II is a tribute to the intellect and creativity of those who developed working models of tumor angiogenesis. These scientists have developed in vivo systems and mechanical and computational tools to examine the structure and function of vessels in malignant tissues and their response to therapeutics in the preclinical setting. Part III is devoted to the role of angiogenesis inhibition in the therapy of malignant disease in humans. Clinical trial design for elucidating the activity of treatment agents and the vasculature and methods for imaging these effects are addressed. Selected malignant diseases are treated in each of several chapters with overviews of angiogenesis in those diseases and the impact of antiangiogenic agents in treatment and on therapeutic outcomes. In addition, clinical investigators provide a background on current directions of the use of these agents in clinical practice and ongoing trials. Antiangiogenesis remains a dynamic and evolving field in oncology. New therapeutic targets continue to emerge followed by the rapid development of new therapeutic agents to be investigated in clinical trials. Optimizing the therapeutic potential of antiangiogenic agents in combination with the other therapies in the armamentarium to fight cancer will be an ongoing challenge. *Antiangiogenic Agents in Cancer Therapy, Second Edition* represents a compendium of scientific findings and approaches to the study of angiogenesis in cancer that will be useful for many years.

Beverly A. Teicher, PhD
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I Basic Biology of Angiogenesis

1 Vascular Endothelial Growth Factor Family and Its Receptors

Daniel J. Hicklin, PhD

Summary

The vascular endothelial growth factors (VEGFs) are key regulators of blood and lymphatic vessel development during embryogenesis and in promoting new vascular growth during physiological and pathological processes in the adult. The VEGF family of ligands in mammals includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF). These ligands bind to and activate three receptor tyrosine kinases, designated VEGFR-1, VEGFR-2, and VEGFR-3. VEGF ligands bind to these receptors with overlapping ligand-receptor specificities, and activation may be further modulated through interaction with coreceptors such as the neuropilins (NRP-1 and NRP-2), integrins, or Vascular endothelial-cadherin (VE-cadherin). Ligand activation of VEGFRs triggers a network of distinct downstream-signaling pathways in a cell-type-specific manner that promotes vascular permeability, endothelial cell growth, migration, and survival. VEGF is an important survival factor for hematopoietic stem cells (HSCs) and stimulates the mobilization of endothelial progenitor cells (EPC) from the bone marrow to distant sites of neovascularization. A large body of experimental evidence has established VEGF as an essential molecule in promoting angiogenesis during tumor growth. These findings have led to the development of therapeutic agents that selectively target various VEGF ligands and their receptors. This chapter reviews the biology of VEGF and its receptors, emphasizing their important role for cancerous growth.

Key Words: Angiogenesis; cancer; growth factor; ligand; neuropilin; receptor; VEGF.

1. INTRODUCTION

Tumor growth and metastasis are dependent on the formation of new blood vessels from preexisting vasculature (angiogenesis) *(1,2)*. Angiogenesis supports tumor growth by providing a source of oxygen, nutrients, growth factors, proteolytic enzymes, and coagulation and fibrinolytic factors. Tumor angiogenesis is a complex process that is regulated by several proangiogenic and antiangiogenic molecules that maintain normal homeostasis and initiate the angiogenic process during pathological conditions *(3)*.

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One of the major pathways involved in the process of tumor angiogenesis and lymphangiogenesis is the vascular endothelial growth factor (VEGF) family of ligands and receptors *(4, 5)*. Overexpression of VEGF has been associated with tumor progression and poor prognosis in several human malignancies including carcinomas of the breast, colon, kidney, liver, lung, pancreas and prostate, and stomach (reviewed in refs 6, 7). During cancerous growth, activation of the VEGF/VEGFR axis triggers multiple signaling networks that result in increased vascular permeability, endothelial cell mitogenesis, migration, survival, and mobilization of various progenitor cell populations from the bone marrow to sites of tumor growth and metastasis *(5, 6, 8)*. A large body of experimental evidence has subsequently shown that interfering with VEGF or VEGFR function can potently inhibit tumor growth and angiogenesis *(6,9,10)*. Owing to its central role in tumor angiogenesis, the VEGF/VEGFR pathway continues to be a major focus of cancer research and in the development of new therapies for this disease.

2. VEGF FAMILY OF LIGANDS AND RECEPTORS

In mammals, the VEGF gene family of angiogenic and lymphangiogenic growth factors consists of five glycoproteins referred to as VEGF-A, VEGF-B, VEGF-C, VEGF-D, and placenta growth factor (PlGF) *(5,11,12)*. A homolog of VEGF, referred to as VEGF-E, has been identified in the genome of the parapoxvirus Orf virus and shown to have VEGF-like activities *(13)*. Recently, another VEGF homolog, referred to as VEGF-F, was identified from snake venom *(14)*. The VEGF ligands bind to and activate three structurally similar type III receptor tyrosine kinases, designated VEGFR-1, VEGFR-2, and VEGFR-3 (Fig. 1). The assortment of VEGF ligands has distinctive-binding specificities for each of these receptors, which contribute to their diversity of function. VEGF-A binds to both VEGFR-1 and VEGFR-2 *(15)*. VEGF-B and PlGF bind exclusively to VEGFR-1 *(16,17)*. Heterodimers of VEGF-A and PlGF have been identified, which can bind to and activate VEGFR-2 *(18,19)*. The VEGFR-3 is a specific receptor for VEGF-C and VEGF-D *(20,21)*. VEGF-C and VEGF-D can be proteolytically processed that allow binding to VEGFR-2 as well. VEGF-E binds specifically to VEGFR-2, whereas VEGF-F can bind both VEGFR-1 and VEGFR-2. The neuropilins NRP-1 and NRP-2 *(22)* can also act as coreceptors for certain VEGF–VEGFR complexes and along with other molecules such as integrins *(23)* and Vascular endothelial-cadherin (VE-cadherin) *(24)*, can modulate VEGF–VEGFR activation and signaling.

Gene targeting studies have shown that VEGFs and VEGFRs are essential during vasculogenesis during development *(25–28)*. In the adult, VEGFs play a role in physiological processes such as wound healing, endochondral bone formation, and follicular growth and development of the corpus luteum during menstrual cycling. VEGF ligands and their receptors also have important roles in pathological conditions such as age-related macular degeneration (AMD), various inflammatory diseases, polycystic ovary syndrome, endometriosis, rheumatoid arthritis, and psoriasis. For a review of the VEGF biology in normal and pathological angiogenesis, see ref. *(29)*.

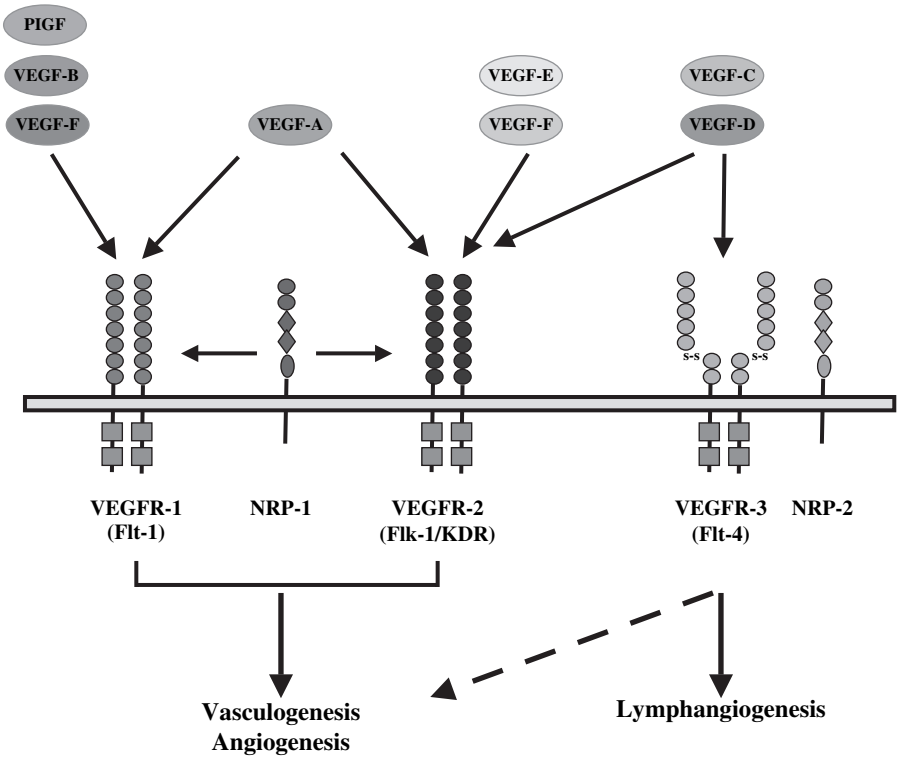

Fig. 1. Binding specificity of VEGF ligands and their receptors. The VEGF family consists of seven ligands: VEGF-A, -B, -C, -D, -E, and PlGF. VEGF ligands have specific binding affinities to VEGFR-1, -2 and -3 as shown. NRP-1 and -2 are co-receptors for specific isoforms of VEGF family members as shown and increase binding affinity of these ligands to their respective receptors. (Please see color insert.)

2.1. VEGF-A

The VEGF-A gene is located on chromosome-6 and is encoded by eight exons *(30)*. The VEGF-A gene undergoes alternative splicing to yield mature isoforms of 121, 145, 165, 183, 189, and 206 amino acids *(11, 12, 31)*. The $VEGF_{121}$ isoform is a secreted diffusible ligand. $VEGF_{165}$ is the predominant isoform and exists in both a soluble and an extracellular matrix (ECM)-bound form *(32, 33)*. $VEGF_{165}$ (and $VEGF_{189}$ and $VEGF_{206}$) can be released from the ECM as a diffusible form by plasmin cleavage generating a bioactive fragment. Alternatively, VEGF can be released from the ECM by matrix metalloproteinase 9 (MMP9) to liberate soluble ligand and initiate angiogenesis *(34)*. VEGF-A is essential for vasculogenesis during development. Homozygous or heterozygous deletion of the VEGF gene in mice is embryonically lethal resulting in defects in vasculogenesis and cardiovascular abnormalities *(25, 35)*. The expression patterns of VEGF-A isoforms are tissue specific, implying that these isoforms have defined functions during vasculogenesis and angiogenesis *(36)*.

VEGF-A (primarily $VEGF_{165}$) is commonly overexpressed in a several human solid tumors and hematologic malignancies *(4, 6, 7, 9, 32, 37)*. VEGF-A expression is upregulated in tumor cells, surrounding stromal cells including endothelial cells, smooth muscle cells, and fibroblasts, and also expressed by various infiltrating bone

marrow-derived cell populations. Selective gene targeting studies in mice have shown that VEGF-A is essential for efficient tumor angiogenesis *(38)*. The important role of VEGF-A in tumor angiogenesis has been further established in studies showing that various anti-VEGF inhibitors can potently inhibit angiogenesis and tumor growth in preclinical models *(6,9)*. One of the first studies used a neutralizing murine anti-VEGF monoclonal antibody that inhibited angiogenesis and growth of human tumor xenografts *(39)*. A number of subsequent studies using neutralizing antibodies to VEGF, soluble VEGF receptors/receptor hybrids, or VEGF antisense approaches have shown similar results *(6,40–42)*.

2.2. VEGF-B and PlGF

The VEGF-B gene is located on chromosome-11 and contains eight exons *(43)*. Two isoforms of VEGF-B have been identified, referred to as VEGF-B_{167} and VEGF-B_{186}. VEGF-B_{167} binds heparin sulfate and is sequestered in the ECM, whereas VEGF-B_{186} does not bind heparin and is found as a soluble, diffusible molecule. VEGF-B binds specifically to VEGFR-1 and the coreceptor NRP-1. The role of VEGF-B during development and in postnatal angiogenesis is not completely understood. VEGF-B-deficient mice are healthy and fertile but develop hearts with reduced size *(44,45)*. VEGF-B-deficient mice also display vascular dysfunction after coronary occlusion and impaired recovery from experimentally induced myocardial or cerebral ischemia. Recent experimental evidence advocating a role for VEGFR-1 in pathological angiogenesis, including cancer (described later), raises the possibility that VEGF-B may be important in certain diseases requiring angiogenesis. However, there is currently no evidence to support this role for VEGF-B.

The PlGF gene has been mapped to chromosome-14 and is encoded by seven exons *(30)*. Four isoforms of PlGF have been identified—PlGF-1, PlGF-2, PlGF-3, and PlGF-4. PlGF-1 and PlGF-3 are non-heparin binding, whereas PlGF-2 and PlGF-4 contain heparin-binding regions *(46)*. All PlGF isoforms bind exclusively to VEGFR-1. PlGF expression was first identified in the placenta, but it is also known to be expressed in the heart and lungs *(47)*. The precise role of PlGF in angiogenesis is unclear at present. PlGF also appears to play a prominent role in the process of arteriogenesis *(48)*. Studies have shown that PlGF can indirectly promote endothelial cell survival and angiogenesis through upregulation of VEGF-A *(49)*. PlGF null mice are viable, but its loss results in impaired angiogenesis and tumor growth, collateral growth during ischemia, inflammation, and wound healing suggesting a role for PlGF in pathological states in the adult *(51)*. Overexpression of PlGF in various tissues, or by tumor cells, results in stimulation of angiogenesis that can be blocked by VEGFR-1 inhibition *(51)*.

2.3. VEGF-C and VEGF-D

The VEGF-C and VEGF-D genes are located on chromosomes 4 and X, respectively *(43,52)*. The VEGF-C and VEGF-D gene products are produced as precursor molecules that are proteolytically processed at the cell surface *(53)*. The VEGF homologs, VEGF-C and VEGF-D, play key roles during embryonic and postnatal lymphangiogenesis *(54)*. Homozygous deletion of the VEGF-C gene in mice is embryonically lethal, and heterozygous deletion results in postnatal defects associated with

defective lymphatic development *(55)*. Interestingly, VEGF-D null mice lack profound lymphatic vessel defects *(56)*, suggesting that this ligand does not play an essential role during development or that a compensatory mechanism for lymphatic development exists. Transgene expression of VEGF-C or VEGF-D induces lymphangiogenesis in mouse models *(57, 58)*.

VEGF-C and VEGF-D are proposed to play a role in tumor growth by inducing the formation of lymphatic vessels, which in turn is hypothesized to promote lymph node metastasis *(59–61)*. VEGF-C and VEGF-D do not appear to influence the growth of primary tumors although their role in primary tumor growth and angiogenesis require further study. Several correlative studies have shown an association between tumor expression of VEGF-C or VEGF-D and lymph node metastasis in human malignancies *(62)*.As VEGF-C and VEGF-D can signal through VEGFR-2, these ligands may also play a role in new blood vessel growth during tumor growth. Specific blockade of VEGF-C-induced tumor lymphangiogenesis and metastasiswas achieved in preclinical models using soluble VEGFR-3 inhibitors *(63–65)*. In addition, inhibition of tumor cell VEGF-C expression by a VEGF-C RNAi approach suppressed lymphangiogenesis and metastasis in a murine breast cancer model *(66)*. A blocking antibody to VEGF-D inhibited tumor lymphangiogenesis and lymphatic metastasis of VEGF-D-dependent mouse tumors *(60)*.

2.4. VEGF-E and VEGF-F

VEGF-E is a viral protein encoded by the parapoxvirus Orf virus that infects sheep and goats *(13)*. The VEGF-E gene product shares approximately 22% sequence identity to VEGF-A and does not contain a heparin-binding domain. VEGF-E preferentially binds to VEGFR-2 and NRP-1 and potently stimulates endothelial cell proliferation and vascular permeability. Another VEGF-like molecule, referred to as VEGF-F, was recently identified in the venom of the viper snake *(14)*. VEGF-F consists of two VEGF-like proteins designated vammin and VR-1. These two proteins share 50% sequence homology to VEGF-A and, like VEGF-E, bind selectively to VEGFR-2. However, distinct from VEGF-E, the VEGF-F molecule contains a heparin-binding region.

2.5. The VEGF Receptors

VEGF ligands mediate their biological effects through selective binding and activation of three different type III receptor tyrosine kinases—VEGFR-1, VEGFR-2, and VEGFR-3. VEGFR-1 (also referred to as fms-like tyrosine kinase 1, Flt-1) *(67)* and VEGFR-2 (also referred to as kinase-insert-domain-containing receptor, KDR *(68)*, and the murine homologue, fetal liver kinase-1, Flk-1) *(69)* were originally identified on endothelial cells. VEGFR-1 and VEGFR-2 are also expressed on various hematopoietic cell lineages in the adult. These two receptors share 44% homology and possess a characteristic structure consisting of seven extracellular immunoglobulin (Ig)-like domains, a single transmembrane domain, and a consensus tyrosine kinase domain interrupted by a kinase insert domain. VEGFR-3 (also referred to as fms-like tyrosine kinase 4, Flt4) *(70)* was cloned form human leukemia cells and has been found to be primarily associated with lymphangiogenesis *(71, 72)*. VEGFR-3 is distinct from VEGFR-1 and VEGFR-2 in that it is proteolytically processed at the fifth Ig domain yielding two subunits that are held together by a disulfide bond. Activation of the

VEGFRs triggers a network of distinct downstream-signaling pathways involved in proliferation, migration, and survival. For recent reviews on VEGFR signaling, see refs *73, 74*.

2.5.1. VEGFR-1

VEGFR-1 is a receptor for all VEGF-A isoforms and a specific receptor for VEGF-B and PlGF. VEGFR-1 is expressed on endothelial, hematopoietic, and smooth muscle cells. VEGFR-1 is critical for developmental vasculogenesis. VEGFR-1 null mice die in utero between 8.5 and 9.5 because of excessive hemangioblast proliferation and poor organization of vascular structures *(26)*. Despite its important role in development, the precise function of VEGFR-1 in the process of angiogenesis, as well as other processes such as hematopoiesis, is still under investigation. VEGFR-1 was initially thought to be a negative regulator of VEGF activity either by acting as a decoy receptor for VEGF or by downregulating VEGFR-2-mediated signaling *(75, 76)*. VEGF-mediated stimulation of VEGFR-1 autophosphorylation and signaling in endothelial cells is weak when compared to signaling through VEGFR-2 *(77)*. A repressor motif has been identified in the juxtamembrane region of VEGFR-1 that impairs PI-3-kinase signaling and endothelial cell migration in response to VEGF stimulation *(78, 79)*. However, other studies have indicated that VEGFR-1 has a positive, functional role in certain cell types—participating in monocyte migration *(80, 81)*, recruitment of endothelial cell progenitors *(82)*, increasing the adhesive properties of natural killer cells *(83)*, and inducing growth factors from liver sinusoidal endothelial cells *(84)*.

Activation of VEGFR-1 by PlGF results in transphosphorylation of VEGFR-2 in endothelial cells coexpressing these receptors *(85)*. Furthermore, VEGF/PlGF heterodimers were capable of activating intramolecular VEGFR cross-talk through formation of VEGFR-1/VEGFR-2 heterodimers. Other studies have shown that during pathological conditions, such as tumorigenesis, VEGFR-1 is a potent, positive regulator of angiogenesis *(50, 51, 86)*. Hence, current evidence now suggests that the function of VEGFR-1 differs with stages of development, various states of physiological and pathological conditions, and the cell type in which it is expressed.

2.5.2. VEGFR-2

VEGFR-2 is considered the principle mediator of VEGF-A-stimulated function in vasculogenesis and angiogenesis. VEGFR-1 is expressed on endothelial cells, hematopoietic cells, and neurons. Hetero- and homozygous VEGFR-2 knockout mice die in utero of defects in blood island formation and vascular development demonstrating the critical dependence of this receptor during the process of vasculogenesis *(27)*. VEGFR-2 is also the principle VEGF-A-signaling receptor for microvascular permeability, endothelial cell proliferation, invasion, migration, and survival during angiogenic processes *(32, 79, 87)*. VEGFR-2-mediated proliferation of endothelial cells involves activation of a phospholipase-C–gamma-Raf–MAP kinase-signaling pathway *(88)*, whereas survival and migration are believed to involve phosphatidylinositol 3-kinase (PI3K) and focal adhesion kinase (FAK), respectively *(89)*. Specific activation of VEGFR-2 with VEGF-E has demonstrated potent endothelial cell activity *in vitro* and *in vivo* strongly supporting the notion that activation of VEGFR-2 alone can efficiently stimulate angiogenesis. As described above,

coexpression and activation of VEGFR-1 can negatively or positively influence the activation and signaling of VEGFR-2.

Studies with neutralizing anti-VEGFR-2 antibodies, or VEGFR-2-selective tyrosine kinase inhibitors, have shown that these approaches are capable of potently inhibiting tumor angiogenesis and primary and metastatic tumor growth in a variety of preclinical models *(90–97)*. A neutralizing anti-Flk-1 mAb (DC101) suppressed the growth and metastasis of human tumor xenografts in mice, and this antitumor effect was associated with decreased microvessel density, tumor cell apoptosis, decreased tumor cell proliferation, and tumor necrosis *(90,93,94)*. Similar effects have been shown with small molecule VEGFR-2-selective tyrosine kinase inhibitors *(91,92)*. Anti-VEGFR-2 treatment in various tumor models has been combined with cytotoxic, metronomic, or radiation therapy, resulting in improved antitumor effects *(96–98)*.

2.5.3. VEGFR-3

VEGFR-3 is a receptor tyrosine kinase originally cloned from a human leukemia cell line and human placenta *(71,72,99)*. VEGFR-3 preferentially binds VEGF-C and VEGF-D. VEGFR-3 expression in the adult is limited to lymphatic endothelial cells. Homozygous deletion of the VEGFR-3 gene in mice leads to embryonic death at day 10–12.5, with an underdeveloped yolk sac, poor perineural vasculature, and pericardial fluid accumulation *(28)*. Hereditary functional mutations of the VEGFR-3 tyrosine kinase domain have been identified in human kindreds with lymphedema. In adult tissues, VEGFR-3 expression has been correlated with transient lymphangiogenesis in wound healing *(100)*. Thus, VEGFR-3 has critical and diverse functions, assisting in cardiovascular development and remodeling of primary vascular networks during embryogenesis and facilitating postnatal lymphangiogenesis. Moreover, some evidence supports a continuing role of VEGFR-3 in the vasculature and suggests that it modulates VEGFR-2 signaling to maintain vascular integrity *(101)*.

VEGFR-3 activation and upregulation of its ligands have been observed in several human cancers with elevated levels of VEGF-C or VEGF-D associated with lymph node metastasis in patients *(61,101–104)*. Of interest, it appears that in addition to lymphatics, some tumor-associated blood vessels may also express VEGFR-3 *(102)*. Overexpression of VEGF-C or VEGF-D and activation of VEGFR-3 in preclinical models of human breast tumor xenografts, or genetic models of pancreatic islet cell carcinoma, were shown to enhance tumor-associated lymphangiogenesis and dissemination of tumor cells to regional lymph nodes *(60,105)*.

A number of recent studies have evaluated VEGFR-3-specific inhibitors in preclinical tumor models. VEGFR-3 blockade using a neutralizing monoclonal antibody reduced the incidence of lymph node and organ metastasis in a VEGF-C-overexpressing breast tumor model *(106)*. In another study, treatment with VEGFR-3 antibody in a mouse tumor model reduced lymphatic hyperplasia, inhibited transit of tumor cells to draining lymph nodes, and consequently suppressed lymph node metastasis *(107)*. However, growth of tumor cells already seeded in lymph nodes was unaffected by VEGFR-3 therapy in this model.

2.5.4. Neuropilins, Integrins, and VE-cadherin

A number of molecules, most notably neuropilins, integrins, and VE-cadherin, have been identified as coreceptors and/or modulators of VEGF-binding specificity