
Preface

The aim of this book is to trace cancer metastasis from the primary sites to the regional lymph nodes and distant organs through the mechanism of local proliferation resulting in metastasis through the lymphovascular system. Rational therapy may be developed to curb the process of metastasis upon understanding these crucial steps of metastasis. Whether the cancer cells utilize the lymphatic or vascular channels or both to metastasize will be examined.

This book summarizes the *2nd International Symposium on Cancer Metastasis: Basis for Rational Therapy* being held in San Francisco from May 3–5, 2007 by bringing together the basic scientists and clinicians to ask the central question of the role of the lymphovascular system in the spread of cancer. Thus, this book is able to link the bench to the bedside and vice versa in understanding the mechanisms of cancer metastasis.

In human solid cancers, the nodal status is the most important prognostic indicator for patients' outcome. Recent developments in the sentinel lymph node (SLN) concept and technology have resulted in the application of such a procedure to define the first draining node or SLN as the primary gateway through which the cancer will spread.

Part I addresses several important developments in the biology and clinical aspects of cancer metastasis. Part II describes the relationship between tumor microenvironment and proliferation. Part III defines the process of lymphangiogenesis and angiogenesis with special reference to cancer metastasis. Part IV summarizes the development of multiple approaches in the imaging of cancer during its course of metastasis. Part V attempts to use the lymphatic system as a target to treat cancer. Part VI updates the latest cellular and molecular mechanisms of cancer metastasis. Part VII examines the role of molecular targeted therapy against growth factor receptors, signaling pathways and angiogenesis as therapeutic targets. Part VIII emphasizes the impact of tumor burden in the sentinel lymph nodes on the clinical outcome in several solid cancers. Part IX defines immune responses in the draining lymph nodes against cancer relating to immunotherapy against cancer. The role of cancer stem cells is being explored in Part X. With advent of molecular techniques, the genomic signatures of cancer may be developed and analyzed in Part XI. Parts XII and XIII summarize the therapeutic results of using new approaches in cancer treatment. Any promising leads from clinical trials in metastatic cancer may be used in the future as adjuvant therapies for occult metastatic deposits. Part XIV poses unanswered questions as future perspectives.

Perhaps, more uniquely, this book will bring the basic scientists, radiologists and clinicians together resulting in cross fertilization between these disciplines with intention to develop strategies to curb the process of metastasis.

San Francisco, CA

Stanley P. L. Leong, M.D.

2 Vascular Endothelial Growth Factor: Basic Biology and Clinical Applications

Napoleone Ferrara, MD

CONTENTS

INTRODUCTION
HISTORY OF VEGF
BIOLOGICAL EFFECTS OF VEGF-A
VEGF-A ISOFORMS
VEGF RECEPTORS
ROLE OF VEGF-A IN TUMOR ANGIOGENESIS
ROLE OF VEGF-A IN INTRAOCULAR NEOVASCULAR SYNDROMES
PERSPECTIVES
REFERENCES

ABSTRACT

Vascular endothelial growth factor (VEGF)-A is a well-characterized angiogenic factor involved in physiological and pathological growth of blood vessels. The homologous tyrosine kinases VEGFR-1 and VEGFR-2 are the main VEGF-A receptors. Much evidence indicates that VEGF-A is important in tumor angiogenesis. Humanized anti-VEGF-A monoclonal antibodies and two small-molecule inhibitors of VEGF receptor signaling have been approved by the US Food and Drug Administration (FDA) for cancer therapy. Furthermore, VEGF-A is implicated in intraocular neovascularization associated with active proliferative retinopathies and the neovascular form of age-related macular degeneration (AMD).

Key Words: vascular endothelial growth factor; endothelium; angiogenesis; tyrosine kinases; tumor growth

1. INTRODUCTION

Angiogenesis is known to be fundamental to a variety of physiological processes including embryonic and postnatal development, reproductive functions, and wound healing (1). Furthermore, neovascularization plays an important pathogenic role in tumorigenesis and in the vision

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loss associated with ischemic retinal disorders and the wet form of age-related macular degeneration (AMD). Research performed in recent decades has established that angiogenesis is a complex and coordinated process, which requires a series of signaling steps in endothelial and mural cells elicited by numerous families of ligands (reviewed in [2]). Moreover, a variety of endogenous inhibitors of angiogenesis have been identified, including endostatin, tumstatin, and vasostatin (3). However, despite such complexity and potential redundancy, vascular endothelial growth factor (VEGF)-A appears to be necessary for growth of blood vessels in a variety of normal and pathological circumstances (4). *VEGF-A* is the prototype member of a gene family that includes also placenta growth factor (PlGF), VEGF-B, VEGF-C, VEGF-D, and the orf-virus-encoded VEGF-E (reviewed in [5,6]).

Definitive clinical studies, resulting in approval by the US Food and Drug administration (FDA) of several drugs, have established that VEGF-A is an important therapeutic target for cancer and wet AMD (reviewed in [7]). This chapter summarizes the basic biology of VEGF-A and provides an update on the clinical progress in targeting VEGF.

2. HISTORY OF VEGF

Independent lines of research contributed to the discovery of VEGF (4). In 1983, Senger et al. (8) reported the identification in the supernatant of a guinea pig tumor cell line of a permeability-enhancing protein, which was named “vascular permeability factor” (VPF). However, these efforts did not yield the full purification of the VPF protein. The lack of amino acid sequence information precluded cDNA cloning and elucidation of the identity of VPF. Therefore, very limited progress in understanding the role of VPF took place over the subsequent several years.

In 1989, we reported the isolation of an endothelial cell mitogen from the supernatant of bovine pituitary cells, which we named “vascular endothelial growth factor” (VEGF) (9). The NH₂-terminal amino acid sequence of VEGF did not match any known protein in available databases (9). Subsequently, Connolly’s group at Monsanto Co. reported the isolation and sequencing of VPF (10). By the end of 1989, we isolated cDNA clones encoding bovine VEGF₁₆₄ and three human VEGF isoforms: VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉ (11). The Monsanto group described a human VPF clone, which encoded a protein identical to VEGF₁₈₉ (12). These studies indicate that, unexpectedly, a single molecule was responsible for both mitogenic and permeability-enhancing activities.

3. BIOLOGICAL EFFECTS OF VEGF-A

VEGF-A stimulates the growth of vascular endothelial cells derived from arteries, veins, and lymphatics (11,13). VEGF-A induces angiogenesis in a variety of *in vivo* models (13). Administration of VEGF also induces rapid and transient increases in microvascular permeability in several experimental model systems (reviewed in [14]).

Inactivation of a single VEGF-A allele results in embryonic lethality between day 11 and day 12, indicating that during early development there is a critical VEGF-A gene-dosage requirement (15). VEGF-A plays an important role also in early postnatal life. Administration of VEGF inhibitors, including monoclonal antibodies and soluble receptors, results in growth arrest and lethality in mice when the treatment is initiated at day 1 or day 8 postnatally (16,17). VEGF is important for endochondral bone formation and growth plate angiogenesis and morphogenesis. VEGF-A blockade reversibly inhibits skeletal growth (18). Another key function of VEGF-A is the regulation of the cyclical angiogenesis that occurs in the female reproductive tract (19). VEGF-A is also a survival factor for endothelial cells, both *in vitro* and

in vivo (20–23). VEGF induces expression of the anti-apoptotic proteins Bcl-2, A1 (21), and survivin (24) in endothelial cells. In vivo, VEGF's pro-survival effects are developmentally regulated. VEGF inhibition results in apoptotic changes and regression of the vasculature of neonatal, but not adult mice (16). Endothelial cells are the primary targets of VEGF-A, but several studies have reported mitogenic and non-mitogenic effects of VEGF-A on non-endothelial cell types including neurons (reviewed in [25]). It is now well established that VEGF-A promotes monocyte chemotaxis (26,27).

4. VEGF-A ISOFORMS

Alternative exon splicing results in the generation of four main VEGF-A isoforms, which have respectively 121, 165, 189, and 206 amino acids after the signal sequence is cleaved (VEGF₁₂₁, VEGF₁₆₅, VEGF₁₈₉, VEGF₂₀₆) (28,29). Less frequent splice variants have also been reported, including VEGF₁₄₅, VEGF₁₈₃, VEGF₁₆₂, and VEGF_{165b} (reviewed in [13]).

Like VEGF₁₆₅, native VEGF is a heparin-binding homodimeric glycoprotein of 45 kDa (9, 30). In contrast, VEGF₁₂₁ lacks heparin-binding properties (31). VEGF₁₈₉ and VEGF₂₀₆ bind to heparin with affinity comparable to that of bFGF (31). Whereas VEGF₁₂₁ is a freely diffusible protein, VEGF₁₈₉ and VEGF₂₀₆ are almost completely bound to heparin-like moieties in the cell surface or in the extracellular matrix. VEGF₁₆₅ has intermediate properties in terms of heparin-affinity and bioavailability (32). The long isoforms may be released in a diffusible form by proteolytic cleavage. Early studies showed that plasmin is able to cleave VEGF₁₆₅ at the COOH terminus, generating VEGF₁₁₀, a bioactive fragment consisting of the first 110 NH₂-terminal amino acids (31,33). Interestingly, recent studies have shown that various matrix metalloproteinases (MMPs)—especially MMP-3—may also cleave VEGF₁₆₅ to generate diffusible, nonheparin-binding fragments (34). Proteolytic processing of VEGF₁₆₅ by MMP-3 occurs in steps, with sequential cleavage at residues 135, 120, and finally at residue 113 (34). Thus, the final product of MMP-3 processing, VEGF₁₁₃, is expected to be biologically and biochemically very similar to the plasmin-generated VEGF fragment.

5. VEGF RECEPTORS

VEGF-A binds two highly related receptor tyrosine kinases (RTK), VEGFR-1 (35), and VEGFR-2 (36). VEGFR-1 was the first RTK to be identified as a VEGF receptor more than a decade ago (37), but the precise function of this molecule is still debated in the field (13). The functions and signaling properties of VEGFR-1 appear to be varying with the developmental stage and the cell type, e.g., endothelial versus non-endothelial cells. VEGFR-1 binds not only VEGF-A but also PlGF and VEGF-B and fails to mediate a strong mitogenic signal in endothelial cells (38,39). Non-mitogenic functions mediated by VEGFR-1 in the vascular endothelium include the release of growth factors (40) and the induction of MMP-9 (41). Furthermore, VEGFR-1 mediates hematopoiesis (42) and monocyte chemotaxis (27) in response to VEGF-A or PlGF.

VEGFR-2 also binds VEGF-A with high affinity (36,43). VEGF-C and VEGF-D may also bind and activate VEGFR-2, following their proteolytic cleavage (6). The key role of VEGFR-2 in developmental angiogenesis and hematopoiesis is underscored by lack of vasculogenesis and failure to develop blood islands and organized blood vessels in Flk-1 null mice (44). There is now agreement that VEGFR-2 is the major mediator of the angiogenic and permeability-enhancing effects of VEGF-A. VEGFR-2 undergoes dimerization and strong

ligand-dependent tyrosine phosphorylation in intact cells and results in a mitogenic, chemotactic, and pro-survival signal. Several tyrosine residues have been shown to be phosphorylated (for review see [45]).

In 1998, Soker et al. (46) identified a receptor for isoforms of VEGF-A containing the exon 7-encoded heparin-binding domain. Surprisingly, this receptor proved identical to Neuropilin-1 (NRP1), a molecule that had been previously shown to be implicated in axon guidance as a receptor for members of collapsin/semaphorin family (46). NRP1 appears to present VEGF₁₆₅ to VEGFR-2 in a configuration that enhances the effectiveness of VEGFR-2-mediated signal transduction (46).

6. ROLE OF VEGF-A IN TUMOR ANGIOGENESIS

Many tumor cell lines secrete VEGF-A in vitro (reviewed in [13]). In situ hybridization studies have demonstrated that the VEGF mRNA is expressed in many human tumors (14). A variety of transforming events also lead to induction of VEGF gene expression. Oncogenic mutations or amplification of ras lead to VEGF up-regulation (47). Renal cell carcinomas have a particularly high level of VEGF-A expression, consistent with the notion that inactivating mutation in the von Hippel–Lindau (VHL) tumor suppressor gene, resulting in high transcription of the hypoxia-inducible factor (HIF)-target genes under normoxic conditions, occur in ~50% of such tumors (48).

In 1993, monoclonal antibodies targeting VEGF-A were reported to inhibit the growth of several tumor cell lines in nude mice (49). Inhibition of tumor growth has been achieved also with other anti-VEGF-A treatments, including small-molecule inhibitors of VEGFR-2 signaling (reviewed in [50]), anti-VEGFR-2 antibodies (51), and soluble VEGF receptors (52,53).

Although tumor cells frequently represent the major source of VEGF-A, tumor-associated stroma is also an important site of VEGF production (52). Recent studies have shown that tumor-derived PDGF-A may be especially important for the recruitment of an angiogenic stroma that produces VEGF-A and potentially other angiogenic factors (54,55).

Combining anti-VEGF treatment with chemotherapy (56) or radiation therapy (57) results in a greater anti-tumor effect than either of these therapies alone. The mechanism of such potentiation is under debate. One hypothesis by Jain (58) is that antiangiogenic agents normalize the tumor vasculature. Alternatively, chemotherapy-induced damage to tumor endothelial cells may be amplified by blockade of a key pro-survival factor like VEGF (56).

6.1. Clinical Trials in Cancer Patients with VEGF Inhibitors

Several VEGF inhibitors have been developed as anti-cancer agents. These include a humanized anti-VEGF-A monoclonal antibody (bevacizumab; AvastinTM) (59,60), an anti-VEGFR-2 antibody (51), various small molecules inhibiting VEGFR-2 signal transduction (50), and a VEGF receptor chimeric protein (53). For recent reviews, see (61–63).

The clinical trial that resulted in FDA approval of bevacizumab (February 2004) was a randomized, double-blind, phase III study in which bevacizumab was administered in combination with bolus-IFL (irinotecan, 5FU, leucovorin) chemotherapy as first-line therapy for previously untreated metastatic colorectal cancer (64). Median survival and progression-free survival were increased by the addition of bevacizumab survival (64). Although bevacizumab was generally well tolerated, some serious and unusual toxicities were observed. Hypertension requiring medical intervention with standard anti-hypertensive therapy developed in 11% of bevacizumab-treated patients. In addition, gastrointestinal perforation was noted in ~2% of

patients. In a combined analysis of five randomized trials involving bevacizumab, including the pivotal trial in colorectal cancer, the incidence of arterial thromboembolic complications, including stroke, myocardial infarction, transient ischemic attacks, and unstable angina was approximately double the incidence seen with chemotherapy alone.

The clinical benefit of bevacizumab is being evaluated in a broad variety of tumor types and lines of therapy. Several combination studies with biologicals are also ongoing, which include inhibitors of tyrosine kinase (bay 43-9006), the proteasome (bortezomib), and mTor (CCI-779). Bevacizumab combined with weekly paclitaxel in women with previously untreated metastatic breast cancer provided a substantial improvement in the primary endpoint of progression-free survival (11.0 versus 6.1 months, $p < 0.001$) relative to paclitaxel alone (reviewed in [77]). Combining bevacizumab with paclitaxel and carboplatin in patients with previously untreated, nonsquamous, NSCLC provided a significant improvement in the primary endpoint of overall survival (12.5 versus 10.2 months, $p = 0.007$) (65). An earlier phase II study of bevacizumab in NSCLC had identified pulmonary bleeding as a significant adverse event in this tumor type (66). Squamous cell histology was identified as a major risk factor for bleeding, and these patients were excluded from the phase III study. This markedly reduced the rate of serious bleeding associated with bevacizumab use (65). Also, combining bevacizumab with 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) in patients with previously treated metastatic colorectal cancers provided a significant improvement in the primary endpoint of survival (67).

Besides bevacizumab, several other types of VEGF inhibitors are being developed (reviewed in [8,68,63]). Among these, a variety of small-molecule RTK inhibitors targeting the VEGF receptors are at different stages of clinical development. The most advanced are SU11248 and Bay 43-9006. SU11248 inhibits tyrosine phosphorylation of VEGFRs, platelet-derived growth factor receptors (PDGFRs), c-kit, and Flt-3 (69) and has been reported to have efficacy in imatinib-resistant gastrointestinal stromal tumor (GIST) (70). AG-013736, which has a similar spectrum of kinase inhibition as SU11248, has also shown therapeutic promise in metastatic renal cell carcinoma in a Phase II monotherapy study (68). SU11248 is FDA-approved for the treatment of Gleevec-resistant GIST and metastatic renal cell carcinoma (71). Phase III data indicate that Bay 43-9006 monotherapy results in a significant increase in progression-free survival in patients with advanced renal cell carcinoma (72). In 2006, Bay 43-9006 and SU-11248 were approved by the FDA for the treatment of metastatic renal cell carcinoma.

7. ROLE OF VEGF-A IN INTRAOCULAR NEOVASCULAR SYNDROMES

The expression of VEGF-A mRNA is spatially and temporally correlated with neovascularization in several animal models of retinal ischemia (20,73). This is consistent with the fact that VEGF-A gene expression is up-regulated by hypoxia, via HIF-dependent transcriptional activation (74). In 1994, it was reported that the levels of VEGF-A are elevated in the aqueous and vitreous humor of human eyes with proliferative retinopathy secondary to diabetes and other conditions (75,76). Subsequently, animal studies using various VEGF inhibitors, including soluble VEGF receptor chimeric proteins (77), anti-VEGF-A monoclonal antibodies (78), and small-molecule VEGF RTK inhibitors (79), have directly demonstrated the role of VEGF as a mediator of ischemia-induced intraocular neovascularization.

AMD is the most common cause of severe, irreversible vision loss in the elderly (80). AMD is classified as nonexudative (dry) or exudative (wet or neovascular) disease. Although the exudative form accounts for ~10–20% of cases, it is responsible for 80–90% of the visual loss associated with AMD (81). Pharmacologic therapies for neovascular AMD have been approved by the FDA. One is verteporfin (Visudyne[®]) photodynamic therapy (PDT) (82) for only

predominantly classic lesions, in which 50% or more of the lesion consists of classic choroidal neovascularization (CNV). The other is Pegaptanib sodium (Macugen[®]) (83) approved in December 2004 for all angiographic subtypes of neovascular AMD. Although both treatments can slow the progression of vision loss, only a small percentage of treated patients experience any improvement in visual acuity. Most recently (June 2006), ranibizumab was approved by the FDA for the treatment of all subtypes of neovascular AMD (84).

7.1. Clinical Studies of Anti-VEGF Therapy for Neovascular AMD: Pegaptanib and Ranibizumab

Pegaptanib sodium injection (Macugen[®]) and ranibizumab (Lucentis[®]) are the first ocular anti-VEGF treatments evaluated in large, randomized, controlled clinical trials for the treatment of neovascular AMD. Both are administered locally by intravitreal injection into the back of the eye. Pegaptanib sodium is a pegylated oligonucleotide aptamer that binds to and inactivates VEGF₁₆₅ (85). In a combined analysis of the VISION trials—two identical, large, controlled, double-masked, randomized, multicenter clinical trials involving patients with all CNV lesion types—pegaptanib sodium prevented moderate vision loss (the primary endpoint, which was defined as loss <15 letters of vision) in 70% of subjects compared with 55% for the control group at one year ($p < 0.001$) (83). However, on average, patients in the pegaptanib sodium group lost ~8 letters at one year, compared with a loss of ~15 letters in the sham injection group ($p < 0.002$). The proportion of subjects who experienced a moderate gain in vision (defined as a change of ≥ 15 letters at one year from baseline) was 6% in the pegaptanib sodium group versus 2% in the sham injection group ($p = 0.04$). Key adverse events observed in the pegaptanib sodium groups were uncommon and included endophthalmitis in 1.3%, traumatic lens injury in 0.7%, and retinal detachment in 0.6% of patients.

Ranibizumab (Lucentis[®]) is a recombinant, humanized Fab that binds to and potently neutralizes the biological activities of all known human VEGF-A isoforms, as well as the proteolytic cleavage product VEGF₁₁₀ (84,86). Ranibizumab is currently being evaluated in two large, phase III, multicenter, randomized, double-masked, controlled pivotal trials in different neovascular AMD patient populations.

The MARINA trial randomized subjects with minimally classic (<50% of the lesion consisting of classic CNV) or occult without classic CNV to monthly sham injections or monthly intravitreal injections of one of two doses of ranibizumab (87). In the primary analysis at one year, the study met its primary endpoint, with a significantly greater proportion of ranibizumab subjects avoiding moderate vision loss than sham-injected subjects. Moreover, on average, ranibizumab-treated subjects gained vision at one year compared with baseline while sham-injection subjects lost vision. A significantly larger percentage of subjects treated with ranibizumab gained ≥ 15 letters at one year than did the sham-injection group. Key serious ocular adverse events occurring in ranibizumab-treated subjects included uveitis and endophthalmitis and were uncommon.

The ANCHOR trial randomized subjects with predominantly classic CNV to verteporfin PDT with monthly sham ocular injections or to monthly intravitreal injections of one of two doses of ranibizumab with a sham PDT procedure. In the primary analysis at one year, the study met its primary endpoint, with a significantly greater proportion of ranibizumab subjects avoiding moderate vision loss compared with subjects treated with verteporfin PDT (88). In addition, on average, ranibizumab-treated subjects gained vision at one year compared with baseline while verteporfin PDT subjects lost vision, and a significantly larger percentage of subjects treated with ranibizumab gained ≥ 15 letters at one year than did the verteporfin PDT group.

8. PERSPECTIVES

Research conducted for almost two decades has established that VEGF-A is important for regulation of the normal angiogenesis processes. Moreover, VEGF inhibition has been shown to suppress pathological angiogenesis in a variety of cancer models, leading to the clinical development of a variety of VEGF inhibitors. Definitive clinical studies have proved that VEGF inhibition, by means of bevacizumab in combination with chemotherapy, provides a significant clinical benefit, including increased survival, in patients with previously untreated metastatic colorectal cancer (64). Furthermore, SU11248 and Bay 43-9006 have been recently approved by the FDA for metastatic renal cell carcinoma, and their mechanism of tumor suppression consists, at least partly, of inhibition of VEGF signaling (61–63).

A particularly active area of research concerns the elucidation of the mechanisms of refractoriness or resistance to anti-VEGF therapy. Tumor cell-intrinsic or treatment-induced expression of angiogenic factors may be implicated (89,90). Very recent studies have provided evidence that, at least in some murine models, refractoriness to anti-VEGF therapy is related to the ability of the tumor to recruit CD11b + Gr1 + myeloid cells, which in turn promote angiogenesis (91). It remains to be established whether these findings also apply to human tumors.

Most clinical studies with VEGF inhibitors have been conducted in patients with advanced malignancies. Preclinical studies suggested that such agents may be particularly effective when tumor burden is low (92). Thus, the clinical benefit of these therapies might be greater if the treatment were initiated at earlier stages of malignancy. Adjuvant clinical trials with bevacizumab in breast, colorectal, and nonsmall-cell lung cancer patients are presently ongoing and the results should be known within the next few years.

Reliable markers are needed to monitor the activity of antiangiogenic drugs. Circulating endothelial cells and their progenitor subset are a potential candidate, as is MRI dynamic measurement of vascular permeability/flow in response to angiogenesis inhibitors, but neither has been clinically validated (62). Emphasizing the difficulty of identifying predictive markers, a recent study found that VEGF-A and thrombospondin expression or microvessel density in tumor sections do not correlate with clinical response to bevacizumab in patients with metastatic colorectal cancer and patients showed a survival benefit from the treatment, irrespective of these parameters (93).

VEGF inhibitors have demonstrated a marked clinical benefit also in wet AMD. Blockade of all VEGF-A isoforms and bioactive fragments with Ranibizumab not only slowed down vision loss, but unexpectedly appears to have the potential to enable many AMD patients to obtain a meaningful and sustained gain of vision. Further research is needed to determine whether the vision gain conferred by ranibizumab extends beyond 24 months and whether additional intraocular neovascular syndromes may benefit from this treatment.

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