REVIEW ARTICLE

VEGF-A: a critical regulator of blood vessel growth

Napoleone Ferrara

Genentech, Inc., San Francisco, USA

Correspondence: N. Ferrara, Genentech, Inc. 1 DNA Way, So. San Francisco, CA 94080, USA
<nf@gene.com>

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ABSTRACT. Angiogenesis is required for a variety of normal and pathological, proliferative processes. Numerous regulators of angiogenesis have been identified and characterized over the last decades. Among these, vascular endothelial growth factor (VEGF)-A appears especially important in normal development and in disease processes. Several VEGF inhibitors have been approved by the FDA for the treatment of tumors or the neovascular form of age-related macular degeneration. This article examines the molecular and biological characteristics of VEGF and also discusses preclinical and clinical studies with VEGF inhibitors and the lessons learned from these studies.

Keywords: VEGF, VPF, angiogenesis, tumor

Angiogenesis is a complex process that results in the establishment of microvascular networks required for pre/postnatal development and for tissue repair in the adult [1-4]. The cardiovascular system is the first organ system to develop and reach a functional state in an embryo [5]. Importantly, without the onset of angiogenesis, most tumors cannot grow beyond 1 to 2 mm due to diffusion limitations and thus may remain dormant [6]. Tumor cells appear to utilize developmental programs resulting in the upregulation of proangiogenic factors and, possibly, downregulation of inhibitory ones [7].

The observation that tumor growth can be accompanied by increased vascularity was reported more than a century ago (for review, see [7]). In 1939, Ide *et al.* postulated the existence of a tumor-derived "blood vessel growth stimulating factor" [8]. In 1945, Algire *et al.* progressed these concepts, proposing that "the rapid growth of tumor transplants is dependent upon the development of a rich vascular supply" [9]. These investigators hypothesized that the acquisition by the tumor cells of the ability to promote vascular proliferation is a critical step in tumorigenesis, since it is likely to confer a growth advantage on the tumor cells [9]. In 1968, Greenblatt and Shubik [10] and Ehrmann and Knoth [11] demonstrated that transplantation of tumor cells promotes blood vessel proliferation, even when a Millipore filter is interposed between the tumor and the host, suggesting that the neovascularization is mediated by diffusible factors produced by tumor cells. In 1971, Folkman proposed that antiangiogenesis might be an effective approach to treat human cancer [12]. Subsequently, several putative angiogenic factors were described, including aFGF, bFGF, EGF, TGF, etc. [7].

HISTORY OF VEGF

In 1983, Senger *et al.* described the identification, in the conditioned medium of a guinea-pig tumor cell line, of a protein able to induce vascular leakage in the skin. This was named "tumor vascular permeability factor" (VPF) [13]. The authors proposed that VPF could be a mediator of the high permeability of tumor blood vessels. However, these efforts did not yield the full purification of the VPF protein. The lack of amino acid sequence data precluded cDNA cloning and establishing the identity of VPF. Therefore, very limited progress in elucidating the role of VPF was possible during the following several years. In 1990, Senger *et al.* reported the purification and NH_2-terminal amino acid sequencing of guinea pig-VPF [14].

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) [15]. The NH_2-terminal amino acid sequence of VEGF did not match any known protein in available databases [15]. Subsequently, Connolly's group at Monsanto Co., reported the isolation and sequencing of VPF [16]. By the end of 1989, we had isolated cDNA clones encoding bovine $VEGF_{164}$ and three human VEGF isoforms: $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ [17]. The Monsanto group described a human VPF clone, which encoded a protein identical to $VEGF_{189}$ [18]. These studies indicated that, unexpectedly, a single molecule was responsible for both mitogenic and permeability-enhancing activities. The finding that VEGF is potent, diffusible and specific for vascular endothelial cells led to the hypothesis that this molecule might play a role in the regulation of physiological and pathological growth of blood vessels [15, 17, 19].

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MOLECULAR AND BIOLOGICAL PROPERTIES OF VEGF-A

VEGF belongs to a gene family that also includes VEGF-B, C, D, E, and placenta growth factor [20-23]. Multiple isoforms of VEGF, ranging from 121 to 206 amino acids, can be generated by alternative exon splicing [23]. These isoforms differ in their ability to bind heparin, which determines their bioavailability, and may play distinct roles in angiogenesis during development [24]. In addition, extracellular proteolysis regulates VEGF activity. Early studies showed that plasmin is able to cleave heparin-binding VEGF isoforms at the COOH-terminus to generate bioactive and diffusible fragments [25, 26]. More recently, Lee *et al.* reported that MMP3 is able to generate VEGF proteolytic fragments, which are biologically and biochemically very similar to those resulting from plasmin cleavage [27]. VEGF promotes growth of vascular endothelial cells derived from arteries, veins and lymphatics (for review [21, 28]. VEGF also induces a strong angiogenic response in a variety of *in vivo* models [17, 29]. VEGF-A was also shown to promote monocyte chemotaxis [30]. Subsequently, VEGF-A was reported to have hematopoietic effects, inducing colony formation by mature subsets of granulocyte-macrophage progenitor cells [31].

VEGF-A is also a survival factor for endothelial cells [32-35]. While in most circumstances VEGF functions as a paracrine mediator, autocrine roles for VEGF in the survival of hematopoietic stem cells and endothelial cells have been described [36, 37].

Three tyrosine kinase receptors bind members of the VEGF gene family: VEGFR-1 (Flt-1), VEGFR-2 (KDR) and VEGFR-3. Moreover, co-receptors, such as heparan sulphate proteoglycans and neuropilins, may facilitate activation of VEGFRs (reviewed in [28]). VEGF-B and PlGF bind selectively to VEGFR-1. VEGF-A is the main ligand for VEGFR-2 [28]. However, proteolytically-cleaved forms of VEGF-C and VEGF-D may also bind to and activate VEGFR-2 [38]. In contrast, VEGFR-3 is activated only by VEGF-C and VEGF-D [38]. VEGFR-1 and VEGFR-2 are expressed in vascular endothelial cells, monocytes, macrophages and hematopoietic stem cells. VEGFR-1 is also expressed in certain non-endothelial cell types [28]. In contrast to VEGFR- 1 and VEGFR-2, VEGFR-3 is critically involved in the regulation of lymphangiogenesis, and its expression in the adult appears to be largely restricted to lymphatic endothelial cells [38]. All VEGF-A isoforms can bind VEGFR-1 and VEGFR-2. Despite the fact that VEGF binds to VEGFR1 with ~ 10-fold higher affinity than VEGFR2, it is mainly VEGFR2 that mediates VEGF signaling in endothelial cells [39, 40]. Hence, many efforts have been made toward targeting the VEGF/VEGFR2 pathway for the treatment of cancer and other disorders such as age-related macular degeneration.

ROLE OF VEGF-A IN TUMOR ANGIOGENESIS IN MOUSE MODELS

The existence of numerous angiogenic factors, suggested that blocking single angiogenic molecules might have very limited effect on tumor growth (reviewed in [7]). However, experiments with neutralizing antibodies and other inhibitors demonstrated that blockade of the VEGF pathway is sufficient to significantly suppress angiogenesis associated with solid tumor growth in many models. Subcutaneous and orthotopic models have been used to test the effects of inhibitors of the VEGF/VEGFR pathway on the growth of a variety of tumor cell lines. Mab A4.6.1 (the murine precursor of bevacizumab) was first shown to suppress the growth of human rhabdomyosarcoma, glioblastoma, and leiomyosarcoma cells implanted in immunodeficient mice [41]. Since then, Mab A4.6.1/bevacizumab has been tested on a wide range of human tumor cells implanted subcutaneously or orthotopically [42]. Together, these studies demonstrate that Mab A4.6.1/bevacizumab is effective in reducing tumor vessel density and suppressing tumor growth, even as a single agent, regardless of tumor location and route of administration.

A confounding factor in assessing the efficacy of Mab A.4.6.1 (or bevacizumab) in human xenograft models is the species-specificity and inability of this antibody to neutralize murine VEGF [43]. Several studies have shown that the extent of stromal cell recruitment is tumor-dependent and the VEGF produced by host cells can be a major driver of tumor angiogenesis, such that the efficacy of Mab A.4.61 in human tumor xenografts is inversely related to the degree of stromal recruitment [44-47]. The availability of cross-reactive, phage-derived antibodies, which neutralize mouse and human VEGF [48], has enabled more complete VEGF blockade studies, not only in xenografts, but also in genetic mouse models. Using such cross reactive antibodies, Shojaei *et al.* examined the differences among various syngeneic murine tumor cell lines in terms of responsiveness to VEGF blockade [49]. They found that tumor cells that are relatively insensitive to VEGF blockade exhibit a greater ability to recruit $CD11b^{+}Gr^{+}$ myeloid cells compared to the sensitive ones. Subsequent studies identified the secreted protein Bv8 as a myeloid cell-derived mediator of tumor angiogenesis [50, 51]. Recent studies indicate that not only frankly malignant tumors, but also benign or premalignant tumors may be sensitive to anti-VEGF therapies. Inhibition of VEGF-A has been shown to suppress the angiogenic switch, resulting in a substantial increase in survival, in the $Apc^{+/min}$ mouse model of intestinal polyposis [52]. Furthermore, Korsisaari *et al.* tested the efficacy of anti-VEGF treatment in a mouse model of multiple endocrine neoplasia type 1(Men1) [53]. They found that tumors in animals that received anti-VEGF treatment were growth-arrested, resulting in reduced serum prolactin levels and increased lifespan of mice [53].

CLINICAL TRIALS WITH VEGF INHIBITORS IN CANCER PATIENTS

Several VEGF inhibitors have been developed as anti-cancer agents including a humanized anti-VEGF-A monoclonal antibody (bevacizumab; Avastin®) [54, 55], various small molecules inhibiting VEGFR-2 signal transduction [56], and a VEGF receptor chimeric protein [57]. For recent reviews, see [4, 58-62].

The clinical benefit of bevacizumab is being evaluated in a variety of tumor types and lines of therapy, in combination with chemotherapy and several biologicals. 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 [63]. Median survival and progression-free survival were increased by the addition of bevacizumab [63]. Although bevacizumab was generally well tolerated, some serious and unusual toxicities were observed including gastrointestinal perforation and arterial thromboembolic complications. Hypertension requiring medical intervention with standard antihypertensive therapy developed in 11% of bevacizumab-treated patients and is now recognized as a class effect of VEGF blockers [60]. Also, bevacizumab combined with weekly paclitaxel in women with previously untreated metastatic breast cancer, provided a significant improvement in the primary endpoint of progression-free survival [64]. Combining bevacizumab with paclitaxel and carboplatin in patients with previously untreated, nonsquamous, non-small-cell lung carcinoma (NSCLC) provided a significant improvement in the primary endpoint of overall survival [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, markedly reducing the rate of serious bleeding associated with bevacizumab [65]. Also, combining bevacizumab with 5-fluorouricil, leucovorin, and oxaliplatin (FOLFOX) in patients with previously treated metastatic colorectal cancers provided a significant improvement in the primary endpoint of survival [67]. Most recently, bevacizumab has been approved by the FDA also for the therapy of renal cell carcinoma (in combination with interferon-alfa) and glioblastoma multiforme.

Besides bevacizumab, several other types of VEGF inhibitors are being developed. Among these, a variety of small molecule RTK inhibitors targeting the VEGF receptors are at different stages of clinical development. The most advanced are Sunitinib (Sutent®) and sorafenib (Nexavar®). Sunitinib inhibits tyrosine phosphorylation of several RTKs including VEGFRs, PDGFR, c-kit and Flt-3. Sunitinib is FDA-approved for the treatment of Gleevec-resistant, gastro-intestinal stromal tumor (GIST) [68] and for metastatic renal cell carcinoma [69]. Sorafenib is a raf kinase inhibitor that also inhibits VEGFR-2 and -3, PDGFR-β, Flt-3 and c-kit [70]. Sorafenib has been approved by FDA for advanced renal cell carcinoma (RCC) [71] and inoperable hepatocellular carcinoma [72].

ROLE OF VEGF-A IN INTRAOCULAR NEOVASCULAR SYNDROMES

VEGF-A mRNA expression is correlated with neovascularization in several animal models of retinal ischemia [32, 64]. This is consistent with the fact that VEGF-A gene expression is up-regulated by hypoxia [73]. 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 [74, 75]. Subsequently, animal studies using various VEGF inhibitors, including soluble VEGF receptor chimeric proteins [76], anti-VEGF-A monoclonal antibodies [77] and small molecule VEGF RTK inhibitors [78], have directly demonstrated the role of VEGF as a mediator of ischemia-induced, intraocular neovascularization.

Age-related macular degeneration (AMD) is the most common cause of severe, irreversible vision loss in the elderly [79]. AMD is classified as non-exudative (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 [80]. Verteporfin (Visudyne®) photodynamic therapy (PDT) [81], has been approved by the FDA only for predominantly classic lesions, in which 50% or more of the lesion consists of classic, choroidal neovascularization (CNV). Pegaptanib sodium (Macugen®), an aptamer that binds to the $VEGF_{165}$, but not to $VEGF_{121}$ or the proteolytic fragments of VEGF-A [82], was 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.

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 products $VEGF_{110}$ or $VEGF_{113}$ [27, 83, 84]. Ranibizumab has been 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 (less than 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 [85]. A significantly greater proportion of ranibizumab-treated subjects avoided moderate vision loss than the sham-injected subjects. Moreover, on average, ranibizumab-treated subjects gained vision at one or two years compared with baseline, while sham-injection subjects lost vision. A significantly larger percentage of subjects treated with ranibizumab gained ≥ 15 letters than did the sham-injection group.

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 [86]. In addition, on average, ranibizumab-treated subjects gained vision at one year compared with baseline, while verterporfin 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. In June 2006, ranibizumab was approved by the FDA for the treatment of all subtypes of neovascular AMD [84].

CONCLUSIONS AND PERSPECTIVES

Research conducted over the last two decades has established that VEGF plays an essential role in the regulation of embryonic [87, 88], postnatal physiological angiogenesis processes, including normal development [89, 90] and cyclical ovarian function [91]. A variety of animal models have generated much information on the biology of VEGF and the therapeutic potential of VEGF/VEGFR inhibitors in cancer. The findings obtained in xenografts have been substantially confirmed and extended in genetic models.

There is also clear evidence that targeting VEGF-A is a meaningful approach for the treatment of cancer and age-related macular degeneration. However, further studies are required to establish optimal dosages and therapeutic regimens. It appears likely that cancer therapy will be combinatorial in most cases. VEGF inhibitors have been approved by the FDA for the treatment of patients with highly advanced malignancies, although preclinical studies suggested that such agents are likely to be most effective when the tumor burden is low. Several adjuvant trials with bevacizumab in breast, colorectal and non-small-cell lung cancer patients are presently ongoing to test the hypothesis that patients with less advanced tumors may show greater responsiveness to such therapy. A particularly active area of research concerns the elucidation of the mechanisms of refractoriness or acquired resistance to anti-VEGF therapy. Tumor cell-intrinsic or treatment-induced expression of angiogenic factors has been implicated [92, 93]. 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 VEGF-dependent angiogenesis [49, 94]. Further work is needed to determine whether these findings are clinically relevant.

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