Vascular Endothelial Growth Factor Signaling Pathway as an Emerging Target in Hematologic Malignancies

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ABSTRACT

Angiogenesis is important in a variety of physiologic and pathologic disorders. It is a central element in embryogenesis, ovulation, wound healing, diabetic retinopathy, and rheumatoid arthritis and in the establishment and spread of malignant tumors. Angiogenic factors include direct angiogens, indirect angiogens, and integrins. Direct angiogens stimulate the formation of new blood vessels directly. Indirect angiogens promote neovascular formation by paracrine stimulation of direct angiogens. Integrins mediate interactions between the developing vessels and components of the extracellular matrix. Vascular endothelial growth factor (VEGF) is a principal direct angiogen. By binding to 1 of 3 receptors (VEGFR-1, -2, or -3), it influences vasculogenesis during embryogenesis, physiologic and neoplastic angiogenesis, and lymphangiogenesis. Although the importance of angiogenesis in solid tumors has been recognized for some time, its exact significance in hematologic malignancies is less clear. Evidence now suggests that VEGF has a major role in the development and progression of hematologic malignancies such as acute leukemia, chronic leukemia, myelodysplasia, non-Hodgkin’s lymphoma, and multiple myeloma. Potential therapeutic interventions to interrupt the VEGF signaling pathway of malignancy include antibodies that neutralize the growth factor and small molecules that inhibit the receptor tyrosine kinase activity of VEGF receptors. The Oncologist 2001;6(suppl 5):24-31

INTRODUCTION

The development of cancer consists of multiple, sequential, and interrelated steps that lead to the generation of an autonomous clone with aggressive growth potential. These steps include sustained growth and unlimited self-renewal through a process of growth signal self-sufficiency, decreased sensitivity to growth suppressive signals, and resistance to apoptosis [1]. Genetic or cytogenetic events that initiate aberrant growth sustain cells in a prolonged “ready” state by preventing apoptosis. Tumor establishment and progression also require additional events that provide metastatic potential. Lastly, the stroma must be vascularized to support continued growth and spread [2]. This latter process is referred to as angiogenesis [1].

Angiogenesis is defined as the production of new blood vessels from an existing vascular network. It consists of a stepwise process of activation of existing endothelial cells, degradation of the extracellular matrix (ECM), and proliferation and migration of endothelial cells toward the angiogenic stimulus [3]. Degradation of the ECM components by matrix metalloproteinases (MMPs) allows the migrating cells to invade along a front and organize themselves into a three-dimensional matrix [3]. Subsequently, vessel patency is established when intra- and intercellular vacuoles coalesce [4]. Angiogenesis is a central element in physiologic processes such as embryogenesis, ovulation, and wound healing as well as in diseases such as diabetic retinopathy, rheumatoid arthritis, and cancer.

Tumors can exist for months or years without neovascularization. However, with clonal progression, subsets of the tumor population may undergo a switch to an angiogenic phenotype [2]. This switch involves a change in the local balance between pro- and antiangiogenic factors. Clones of the tumor with a proangiogenic phenotype may produce their own angiogenic growth factors, mobilize angiogenic substances from the ECM, and recruit host cells such as monocytes/macrophages to produce angiogenic molecules [2]. In addition, the tumor cell must acquire means to downregulate
physiologic regulators or inhibitors of angiogenesis such as angiostatin [5]. While it has been known for some time that induction of tumor angiogenesis is associated with tumor volume expansion, the role of this process in hematologic malignancies has, until recently, been unrecognized. Recently, however, it has become clear that angiogenic factors play an important role in the pathophysiology of lymphocytic and myelogenous leukemias, myelodysplastic syndromes, myeloproliferative diseases, multiple myeloma, and non-Hodgkin’s lymphomas (Table 1) [6-10]. Because of its broad relevance, neoplastic angiogenesis is now recognized as a biological target of paramount importance. This has raised expectations for the therapeutic potential of antiangiogenic molecules in cancer therapy. Recognition of the role of angiogenesis in hematologic malignancies broadens the spectrum of malignancies potentially amenable to antiangiogenic therapy. This article will discuss the role of vascular endothelial growth factor (VEGF) as an emerging target in hematologic malignancies.

**Physiologic Role of VEGF**

Growth factors that stimulate angiogenesis can be divided into direct and indirect angiogens or angiogenic molecules (Table 2). Direct angiogens include VEGF, basic fibroblast growth factor (bFGF), and hepatocyte growth factor (HGF) (Table 2) [11, 12]. Indirect angiogens, whose vascular effects derive from the paracrine stimulation of direct angiogenic molecules, include interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), platelet-derived growth factor (PDGF), and transforming growth factor-β (TGF-β) [13]. They promote blood vessel growth by upregulating the expression of direct angiogens such as VEGF, IL-8, or other peptides [11, 13-15]. Angiopoietins are angiogenic proteins that function during angiogenesis, not vasculogenesis, the process of de novo formation of vessels rather than outgrowth from existing structures [3]. Integrins are heterodimeric transmembrane proteins that mediate cellular adhesion and regulate cell survival, proliferation, and migration. They are expressed on endothelial cells and mediate adhesion to a variety of ECM proteins including vitronectin, fibronectin, laminin, collagen, fibrinogen, and von Willebrand factor [16]. Following exposure to direct angiogens, the expression of integrin αβ3, as well as αβ1 and αβ5, is upregulated [3].

VEGF plays an essential role in vasculogenesis during embryogenesis, physiologic angiogenesis, and the neovascularization of malignancy. VEGF is a 40-45 kDa homodimer released by a variety of benign and malignant cell types [3]. As a result of alternative splicing of mRNA, five different VEGF monomers of varying amino acid lengths (denoted by subscript numbers) are produced: VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206 [17, 18].

**Table 1. Hematologic malignancies with angiogenic components**

- Multiple myeloma
- Leukemia (acute and chronic lymphocytic leukemia, acute and chronic myelogenous leukemia)
- Non-Hodgkin’s lymphoma
- Myelodysplastic syndromes
- Chronic myelomonocytic leukemia
- Myeloproliferative disorders

**Table 2. Angiogenic molecules**

<table>
<thead>
<tr>
<th>Type</th>
<th>Factor</th>
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<tr>
<td>Direct angiogen</td>
<td>Vascular endothelial growth factor (VEGF)</td>
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<td></td>
<td>Basic fibroblast growth factor (bFGF)</td>
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<td></td>
<td>Hepatocyte growth factor (HGF)</td>
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<tr>
<td>Indirect angiogen</td>
<td>Interleukin-6 (IL-6)</td>
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<tr>
<td></td>
<td>Tumor necrosis factor-α (TNF-α)</td>
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<td></td>
<td>Platelet-derived growth factor (PDGF)</td>
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<td></td>
<td>Transforming growth factor-β (TGF-β)</td>
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<tr>
<td>Angiopoietin</td>
<td>Angiopoietin-1</td>
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<tr>
<td>Angiogenin</td>
<td>Angiogenin</td>
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<tr>
<td>Angiotropin</td>
<td>Integrin-αβ3</td>
</tr>
<tr>
<td>Integrin</td>
<td>Integrin-αβ1</td>
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<td>Integrin-αβ5</td>
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VEGF mRNA transcription is induced by hypoxia and a variety of growth signals and cytokines [15, 19]. Endothelial cells stimulated by VEGF migrate and proliferate, develop increased vascular permeability, elaborate MMPs, and divide and recruit supporting elements such as pericytes. Vasculogenesis by VEGF also occurs indirectly. Following exposure of human endothelial cells to VEGF, cells upregulate expression of α4-, α5-, and β3-integrins [20]. This facilitates the adhesion and transmigration of monocytes to and through endothelial cells. Extravasated monocytes could then contribute to angiogenesis by secreting growth factors and cytokines [20].

**VEGF Receptor Signaling**

Activity of VEGF-A, the prototype VEGF species, is mediated by interaction with high-affinity receptor tyrosine kinases (RTKs) expressed on most endothelial cells [3]. Three major VEGF RTKs have been identified: VEGF receptor 1 (VEGFR-1) (Flt-1), VEGFR-2 (KDR), and VEGFR-3 (Flt-4). These high-affinity receptors transduce signals that affect normal endothelial cell proliferation, differentiation, migration, and metabolism [3, 21].

VEGFR-1, or Fms-like tyrosine kinase-1 (Flt-1), is a member of the type III receptor tyrosine kinase family [15]. Although it is expressed primarily on endothelial cells,
VEGFR-1 is also present on smooth muscle cells and monocytes. Activation of VEGFR-1 by VEGF results in cell migration and contributes indirectly to migration of primitive vascular buds through the ECM by enhancing production of MMPs by both endothelial and associated smooth muscle cells [22, 23]. VEGFR-1 ligation also mediates monocyte recruitment and expression of tissue factor by both monocytes and endothelial cells [24].

VEGFR-2, also known as KDR or fetal liver kinase-1 (Flk-1), is essential for embryonic vasculogenesis and definitive hematopoiesis. Mice deficient in VEGFR-2 die in utero because of deficient vascular and hematopoietic development [25, 26]. Outside of the embryo, this receptor is exclusively expressed in endothelial cells and primitive hematopoietic stem cells, playing a role in endothelial cell proliferation, differentiation, and vasculogenesis [15, 27]. In malignancies, VEGF binding to VEGFR-2 triggers the process of neovascularization that leads to growth and metastasis of tumors. Activation of both VEGFR-1 and -2 is required for the assembly of vinculin in focal adhesion plaques, a second distinct signaling system for cell migration [19].

Production of nitric oxide and prostacyclin (PGI2) by endothelial cells playing a role in mediating the effects of VEGF. This occurs through the recruitment of c-Src following VEGFR-2 ligation [28]. VEGFR-2 signaling is influenced by cadherin-5 mediated cell-cell interactions that lead to downregulation of endothelial cell proliferation in areas of high vascular density [29]. VEGFR-2 signaling also acts through a cadherin/b-catenin pathway to loosen cell-cell contacts [23]. This serves to modulate transendothelial permeability and to allow sprouting and cell migration during angiogenesis.

Ligation of VEGFR-2 promotes cell survival by inhibiting apoptosis through a phosphatidylinositol 3′-kinase/Akt signal transduction pathway [30]. Inactivation of pentaerythritol tetranitrate (PTEN) and stimulation of the phosphatidylinositol 3′-kinase/Akt signal transduction pathway provide a mechanism to link VEGFR-2 ligation and integrin activation. Incubation of endothelial cells with VEGF results in upregulation of integrins and leads to enhanced cell adhesion, migration, and soluble ligand binding. These phenomena may help explain why VEGF is essential for engraftment of leukemic myeloblasts in severe combined immunodeficient mice [12, 31].

VEGFR-3, the most recently described member of the VEGFR family, was previously designated Flt-4. This receptor is restricted predominantly to endothelial cells lining lymphatic channels [32].

**VEGF in Hematologic Malignancies**

The capacity for hematologic malignancies to disseminate within the bone marrow and gain access to the peripheral circulation, areas in which blood supply is already abundant, possibly obscured the link between endothelial growth factors and hematopoietic malignancies. Nevertheless, bone marrow stromal elements have been recognized to play an important role in hematopoiesis for more than two decades [33, 34]. The observation that VEGFR-2 deficient mice expire in utero formed the basis for subsequent investigations confirming the presence of a common endothelial cell and hematopoietic progenitor that harbors the VEGFR-2 receptor [35-38].

VEGF was first isolated from the HL-60 myeloid leukemia cell line [39]. Recent studies have demonstrated VEGF expression in a wide range of cell lines derived from human hematopoietic malignancies associated with secretion in concentrations within its range of biological activity [33]. The detection of VEGFR-1 expression in approximately half of these cell lines suggests that VEGF may stimulate tumor cells through an autocrine mechanism in hematologic malignancies [19, 33]. VEGF may also potentiate trophic signals in hematopoietic malignancies through the paracrine induction of other cytokines and hematopoietic growth factors. For example, human vascular endothelial cells stimulated with recombinant human VEGF elaborate G-CSF, macrophage-CSF, kit ligand, and IL-6 [40, 41].

**VEGF Activity in Hematologic Malignancies**

Hematologic malignancies may show a tropic response to a variety of autocrine and paracrine molecules (Fig. 1) [12]. In many hematologic malignancies, markers of angiogenesis may have prognostic significance (Table 3).

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**Figure 1. Tropic response to angiogenic molecules in hematologic malignancies: paracrine and autocrine stimulation of progenitor cells by angiogenic growth factors.** Basic fibroblast growth factor (bFGF); Fas ligand (FL); granulocyte/granulocyte-macrophage colony-stimulating factor (G/GM-CSF); hepatocyte growth factor (HGF); interferon-γ (IFN-γ); interleukin-1β (IL-1β); interleukin-6 (IL-6); interleukin-8 (IL-8); matrix metalloproteinase (MMP); stromal cell-derived factor (SDF); tumor necrosis factor-α (TNF-α); vascular endothelial growth factor (VEGF).
This section will explore the experimental and clinical evidence for VEGF in a variety of malignancies of the hematopoietic system.

**Acute Myelogenous Leukemia**

Bone marrow biopsies from patients with acute myelogenous leukemia (AML) display increased neovascularity that is reversible upon disease suppression. In a study of 20 patients with AML, microvessel density (MVD) was significantly greater in leukemic bone marrow biopsy samples from untreated patients than in those from normal controls ($p < 0.001$) [6]. This difference was independent of the French-American-British (FAB) classification of the tumor. In addition, fresh blasts from these patients expressed VEGF mRNA. Analysis of leukemic blasts from 28 patients with de novo AML and five patients with secondary AML demonstrated VEGF-specific transcripts by polymerase chain reaction in 20 and 3 patients, respectively [40]. In addition, supernatants of fresh leukemic cells contained significantly more VEGF than specimens from normal donors [8, 40].

The presence of VEGFR-1 and -2 on many of these cells implies that expansion of the leukemic population may be facilitated by an autocrine loop [41]. The ability of VEGF to produce a dose-dependent increase in GM-CSF secretion from cultured human umbilical cord endothelial cells suggests that paracrine mechanisms may also be active. In newly diagnosed patients with a range of FAB types of AML, there is a direct relationship between increasing cellular VEGF content and shorter survival ($p = 0.01$) [42]. Furthermore, the lack of a relationship between VEGF levels and age, cytogenetics, performance status, or antecedent hematologic disorder suggests that this measure of myeloblast angiogenic potential is an independent predictor of outcome [42].

**Acute Lymphocytic Leukemia (ALL)**

Bone marrow biopsies from patients with acute lymphocytic leukemia (ALL) display increased neovascularity that is reversible upon disease suppression. In a study of 20 patients with ALL, the median number of blood vessels in the bone marrow was also significantly higher than in age-matched normal marrow ($p = 0.005$) [8]. Interestingly, bFGF, another potent angiogenic molecule, but not VEGF, was elevated in the serum of these patients [43]. However, Bellamy et al. demonstrated that some cell lines from patients with ALL express VEGF as well as VEGFR-1 [33].

**Chronic Lymphocytic Leukemia (CLL)**

B-CLL cells produce biologically active VEGF, both in vitro and in vivo [9]. Although Aguayo et al. were unable to demonstrate an increase in MVD in bone marrows of patients with CLL, lymph nodes infiltrated by CLL cells showed a significant increase in vascular densities [8, 33, 44]. Expression of VEGFR-2 and increased serum levels of VEGF are associated with progressive disease and shortened survival in patients with CLL [45, 46].

**Chronic Myelogenous Leukemia (CML)**

The bone marrow MVD in patients with CML is approximately twice that of controls ($p = 0.003$) [8]. This correlates with production of VEGF in these patients. Median levels of VEGF in plasma samples from these patients are approximately threefold greater than those of control specimens [47]. The potential prognostic relevance of these findings remains undefined.

**Non-Hodgkin’s Malignant Lymphomas (NHL)**

While MVD is higher in the involved lymph nodes of patients with B-CLL (i.e., small lymphocytic lymphoma), the number of blood vessels does not correlate with the grade of the tumor [44]. In fact, areas involved by follicular center cell lymphomas actually have lower MVDs than the surrounding normal lymph nodal tissue. However, VEGF has been implicated in the overall disease course of patients with these common tumors. For example, VEGF levels are significantly lower in patients in complete remission after a median follow-up of 21 months, compared with those with progressive disease ($p = 0.016$) [48]. Furthermore, event-free survival was significantly higher when patients had baseline VEGF levels that were below the median of 147 pg/ml ($p = 0.018$) [48]. Although cellular expression of VEGF is common in NHL, receptor co-expression appears limited to intermediate- or high-grade histology [42].
Multiple Myeloma

IL-6 is a plasma cell growth factor. Plasma levels of IL-6 directly correlate with tumor burden, bone destruction, and other measures of tumor activity in myeloma [49]. The source of the cytokine is believed to be both autocrine and paracrine. Irrespective of the site of origin, elevated levels of IL-6 induce VEGF expression in endothelial cells [13, 50]. As a result, levels of VEGF are elevated in the bone marrow of patients with multiple myeloma and correlate with the stage of the disease [50]. VEGF may also serve as a paracrine growth factor for the malignant plasma cells [51]. This sets up a positive feedback loop that may contribute to disease progression. In the laboratory, antibodies to IL-6 reduced the number of plasma cells in bone marrow cell culture as well as the number of endothelial cells [10].

Experimental and clinical studies suggest that myeloma cell growth is dependent upon endothelial cell proliferation within the bone marrow [10]. This may be related to paracrine stimulation from IL-6 as well as other endothelial cell-derived cytokines, such as bFGF and MMPs. In patients with multiple myeloma, there is a strong correlation among increased angiogenesis, MVD, and survival. In the Mayo Clinic experience, patients with ≤50 microvessels/field survived 5.1 years, while those with more than 50 survived only 2.6 years (p = 0.004) In addition, angiogenesis grade, evaluated by semiquantitative visual examination of bone marrow samples, correlated with high plasma cell labeling indices, another marker of disease severity [52].

Myelodysplasia

Myelodysplastic syndromes (MDSs) are preleukemic disorders, particularly in patients who present with measurable leukemia burden or unfavorable karyotype. Among the FAB subtypes, MVDs were significantly higher in refractory anemia with excess blasts in transformation (RAEB-t), chronic myelomonocytic leukemia (CMML), or the fibrosis subtypes, compared with refractory anemia with excess blasts (RAEB), refractory anemia, or refractory anemia with ring sideroblasts [53]. Plasma levels of VEGF are elevated in MDS to an extent comparable to those in AML, but less than reported in CLL and CML [8]. Cytologically, VEGF is expressed by immature myeloid elements, particularly leukemic monocytoid precursors but not erythroid elements or lymphocytes [41]. Foci of abnormal localization of immature precursors often indicate transformation to a higher grade of MDS. These foci of myelomonocytic precursors coexpress VEGF and VEGFR-1, suggesting the possibility of an autocrine mechanism; this is supported by in vitro VEGF neutralization studies [41].

Idiopathic Myelofibrosis

Angiogenesis is increased in the bone marrow of patients with idiopathic myelofibrosis. Grade 3 or 4 increases in bone marrow neovascularity were observed by visual examination in 70% of 114 patients with this disease [54]. The degree of MVD was higher than in patients with polycythemia vera, essential thrombocytemia, or controls. Increased MVD correlated with spleen size and was a significant and independent risk factor for overall survival [54]. Neoangiogenesis was independent of the degree of reticulin fibrosis. This suggests that neovascularization is an integral component of this disease and may offer a target for therapeutic intervention.

Preclinical Studies of Anti-VEGF in Hematologic Malignancies

The development of a malignancy involves a series of complex interactions between positive and negative regulatory factors as well as interactions between the tumor, its vasculature, and other elements of the ECM [55]. Abnormalities of angiogenesis are common in hematologic malignancies. With the exception of ALL, VEGF levels are elevated in AML, CLL, CML, MDS, CMML, and multiple myeloma [8, 50]. Therefore, compounds that neutralize the activity of VEGF represent attractive agents for therapy in these diseases. Some potential effects resulting from downregulation of VEGF production include inhibition of hematopoiesis-promoting cytokines and growth factor production, promotion of differentiation of immature precursors, inhibition of MMP production, and induction of apoptosis in receptor-competent cells [41]. Experimentally, VEGF neutralization suppresses the production of TNF-α, GM-CSF, and IL-1β by bone marrow stroma derived from MDS bone marrow (Fig. 2) [41]. In cell culture studies, antibodies to VEGF inhibited leukemia colony-forming activity from the majority of patients with CMML and RAEB-t, whereas VEGF stimulated formation of leukemic colonies [41].

As receptors for VEGF, VEGFR-1 and -2 are logical targets for control of angiogenesis in benign and malignant disorders including those of the hematopoietic system. Substituted 3-[(3- or 4-carboxyethyl)pyrrol-2-yl)methylidene]indolin-2-ones are RTK inhibitors [56]. These small, selective, nonimmunogenic synthetic molecules have a favorable toxicity profile, and are resistant to enzymatic inactivation [57]. Two novel compounds in this class are SU5416 and SU6668. The former selectively inhibits only VEGFR and c-kit, while the latter also inhibits bFGF and PDGF and, therefore, possibly tumor cells that express these targets [58]. Both of these compounds have been shown to inhibit colon cancer metastases, microvessel formation, and cell proliferation while increasing tumor cell
and endothelial cell apoptosis. Importantly, neither is myelo-suppressive [59]. By inhibiting VEGF, these novel compounds can downregulate angiogenesis and its associated features as discussed above in patients with hematologic malignancies. For example, SU5416 has been shown to inhibit the clonogenic response to VEGF in the human leukemia cell line KG1 [60]. In addition, both compounds inhibit ligand-dependent phosphorylation of c-kit. c-kit, the RTK whose ligand is stem cell factor, is expressed uniformly by myeloblasts but is absent from normal peripheral blood elements. The combination of angiogenesis inhibition with c-kit inhibition offers a particularly novel approach to the therapy of AML.

CONCLUSIONS

Angiogenesis and hematopoiesis are linked at the level of the putative hemangioblast, a progenitor cell that is involved in both vasculogenesis and blood cell formation. VEGF-induced angiogenesis resulting from autocrine and/or paracrine regulation plays an important role in hematologic malignancies and in the progression of myelodysplasia. Multiple mechanisms are involved. These include paracrine stimulation of cytokines and hematopoietic growth factors such as IL-6 and GM-CSF by the proliferating endothelium and its associated stroma. Synthesis of MMPs, as well as VEGF-induced alterations in integrin expression, may also contribute to the pathophysiology of the disease. The scientific background presented in this article indicates that angiogenesis is not only relevant in these disorders but is an intriguing target for therapeutic intervention.

REFERENCES


Figure 2. Effect of VEGF on cytokine elaboration in bone marrow stroma from a patient with RAEB. VEGF-neutralizing antibody (anti-VEGF) 1 µg/ml of recombinant human-VEGF (rHu-VEGF) 50 ng/ml for 24 hours. Cytokine concentrations in stroma supernatants were analyzed by enzyme-linked immunosorbent assays (ELISA). *Statistically significant differences in concentration as compared with control (p < 0.05). Tumor necrosis factor-α (TNF-α); granulocyte-macrophage colony-stimulating factor (GM-CSF); interleukin-1β (IL-1β). Reproduced with permission [33].
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