Role of Angiogenesis Inhibitors in Cancer Treatment

Introduction

Currently, the field of tumor angiogenesis is undergoing more explosive growth than any other field in cancer research. More than 860 papers were published on various aspects of tumor angiogenesis in 1999, and scientists, clinicians, patients, and the media have closely scrutinized this research. Regrettably, trials of anti-angiogenic agents thus far have produced mostly toxicity data. Given the complexity of this process, the basic biology of angiogenesis also must be better understood before more effective anti-angiogenic therapy can be developed.

By definition, "angiogenesis" is the establishment of a neovascular blood supply derived from preexisting blood vessels, whereas "vasculogenesis" is the embryonic establishment of a blood supply from mesodermal precursors such as angioblasts or hemangioblasts. "Tumor angiogenesis" more accurately refers to a combination of angiogenesis and vasculogenesis, in which the main blood supply to a tumor is derived from preexisting blood vessels, but circulating endothelial cell precursors may contribute to the growing endothelial cell mass.

Angiogenesis is an essential step in both the growth of primary tumors and metastasis.[1,2] A neovascular blood supply is also essential for increasing the chance that tumor cells will gain access to the circulation and subsequently begin the process of growth at a different site. Once a tumor establishes an invasive phenotype in the organ of metastasis, it must then establish its own neovascular blood supply for growth. Numerous investigators have established the association between tumor angiogenesis and tumor metastasis.[3]

Balance Between Stimulatory and Inhibitory Angiogenic Factors

Pathologic angiogenesis occurs when the effect of stimulatory molecules outweighs the effect of inhibitory molecules (Table 1). Intensive study led to the realization that the angiogenic process involves more than simple proliferation of endothelial cells; rather, it requires endothelial cells to divide, invade the basement membrane, migrate, and undergo differentiation and capillary-tube formation (Figure 1). This process is driven by angiogenic molecules and also by other factors, such as degradative enzymes, which mediate the above processes. Interestingly, tumor angiogenesis and tumor-cell invasion are very similar processes.

Growth Factors

The best characterized of the stimulatory angiogenic factors is the vascular endothelial growth factor (VEGF). VEGF has also been associated with the aggressive phenotype in numerous solid malignancies.[4-9] VEGF is a 32- to 44-kDa protein secreted by nearly all cells. VEGF is expressed as four isoforms derived from alternate splicing of the mRNA.[10] The smaller isoforms, VEGF-121 and VEGF-165 (the numbers denote the number of amino acids), are secreted from cells. The larger isoforms, VEGF-189 and VEGF-205, are cell-associated, and their functions are not well known at this time.

One distinguishing factor of VEGF is its ability to induce vascular permeability. In fact, this factor was originally named the vascular permeability factor (VPF) and was subsequently found to be homologous to VEGF. [11-13] The extent of vascular permeability induced by VEGF is 50,000 times
that of histamine, the gold standard for induction of permeability. This action by VEGF allows proteins to diffuse into the interstitium and to form the lattice network onto which endothelial cells migrate.

Endothelial Growth Factor-Receptor Family

Receptors for VEGF are expressed almost exclusively on endothelial cells. VEGF receptors have been found on cells of neural origin, Kaposi’s sarcoma cells, hematopoietic precursor cells, and other rare tumor cell types.[14,15] The current nomenclature for the VEGF receptors lists three receptors: VEGFR-1/Fit-1, VEGFR-2 KDR/Flk-1, and VEGFR-3/Flt-4. These tyrosine-kinase receptors require dimerization to induce intracellular signaling upon binding to specific ligands. The receptors for VEGF may mediate distinct functions within the endothelial cell; for example, VEGFR-1 may be important in migration, whereas VEGFR-2 may be important in the induction of permeability and cell proliferation.

Recently, the angiopoietin family of ligands has been found to play an important role in homeostasis of tumor vasculature. The angiopoietins are proteins involved in angiogenesis that bind to the endothelial-cell-specific tyrosine kinase receptor Tie-2. Angiopoietin-1 (Ang-1) acts as an agonist and is involved in endothelial-cell differentiation and stabilization.[16] In contrast, Ang-2 binds to Tie-2 and blocks the binding of Ang-1 to this receptor.[17,18] This blockade leads to endothelial cell destabilization and vascular regression.[19]

New Theories of Angiogenesis

A recent theory of tumor angiogenesis suggests that this process involves the co-option of preexisting blood vessels in addition to vascular regression and subsequent neovascularization.[19] Initially, tumors co-opt existing blood vessels within an organ for their nutrient blood supply. Shortly thereafter, the existing vasculature becomes destabilized, most likely through the release of Ang-2 by endothelial cells. This loss of vascular integrity leads to relative hypoxia within the tumor, which, in turn, leads to upregulation of VEGF in the tumor cells. These events then lead to a robust angiogenic response. At that stage, the newly developed endothelial cells require stabilization, achieved through release of Ang-1 by endothelial cells and possibly through continued response to VEGF. Thus, the process of angiogenesis depends on the temporal coordination of factors that regulate pathways for the establishment of stable conduits that provide a nutrient blood supply to the tumor.

Numerous nonspecific angiogenic molecules and factors affect the growth of cell types other than endothelial cells. These include fibroblast growth factors (acidic and basic); transforming growth factor-alpha and epidermal growth factor (EGF), both of which bind to the EGF receptor; platelet-derived growth factor (PDGF); platelet-derived endothelial cell growth factor (PD-ECGF); angiogenin; and the CXC chemokine IL-8, macrophage inflammatory protein (MIP), PF-4, and growth-regulated oncogene (GRO)[20] (Table 1).

These factors are known to be angiogenic in in vivo models but are not specific for endothelial cells. However, as noted earlier, angiogenesis is not driven by a single molecule or family of molecules, but rather depends on the cooperation and integration of various factors that lead to endothelial cell proliferation, migration, invasion, differentiation, and capillary-tube formation. It has yet to be determined whether inhibiting the activity of a single angiogenic factor will lead to vascular compromise of significant duration. More likely, the redundancy in the angiogenic process will select for other angiogenic factors when a specific angiogenic factor is targeted.

Upstream Regulation of Angiogenic Factors

In formulating antiangiogenic regimens, it is essential to understand the cascade of events that leads to upregulation of angiogenic factor expression and secretion. Signals that upregulate angiogenic factors include extracellular signals, intrinsic upregulation of signal transduction activity, and loss of tumor suppressor genes. Examples of these signals are discussed below.
External signals that lead to the induction of angiogenic factor expression include environmental stimuli such as hypoxia or a decrease in pH.[21-23] Hypoxia is the most potent stimulus for inducing angiogenic factors, especially VEGF. Hypoxic induction of VEGF is probably mediated through Src kinase activity, which then leads to downstream induction of signaling cascades and eventually to an increase in the activity of hypoxia-inducible factor-1 (HIF-1) alpha.[24,25] This factor then increases the transcription of the VEGF gene, which in turn leads to the induction of angiogenesis. Other external factors that increase the angiogenic response include various cytokines and growth factors. Insulin growth factors -I and -II, epidermal growth factor, hepatocyte growth factor, interleukin-1, and platelet-derived growth factor have all been shown to upregulate VEGF.[26-28] Thus, anti-angiogenic therapy could involve downregulation of upstream targets of the angiogenic factors rather than targeting the angiogenic factors themselves.[24,29]

Once a growth factor or a cytokine binds to its receptor, a cascade of intracellular signaling events is initiated. Two specific signal transduction pathways are well known to mediate the upregulation of angiogenic factors: the PI-3 kinase/Akt signal transduction pathway, which eventually leads to stabilization of HIF-1 alpha,[30,31] and the mitogen-activated protein (MAP) kinase pathway, in which activation of extracellular signal-regulated kinases-1/2 (Erk-1/2) activates factors that increase transcription of the VEGF gene.[32] Activated Ras and Src also have been demonstrated in in vivo models to be associated with increased VEGF production and angiogenesis.[33] Again, therapeutic strategies that target the upstream effector molecules in angiogenesis may be a rational means of preventing angiogenesis. Inhibitors of signal transduction molecules have been demonstrated to inhibit angiogenesis in in vivo tumor models.[24]

Protein products of tumor suppressor genes such as the von Hippel-Lindau (VHL) or p53 genes also regulate angiogenesis. The wild-type VHL protein represses transcriptional regulation of the VEGF gene.[34-36] A loss of heterozygosity with a mutation in the remaining VHL allele leads to loss of transcriptional control of the VEGF gene and overexpression of VEGF. Mutant p53 has also been associated with an increase in angiogenesis.[37] Reinsertion of the wild-type p53 gene into cells with mutant p53 can downregulate VEGF expression and angiogenesis.[29] Thus, the process of angiogenesis is driven by external forces (including environmental stimuli), aberrations in internal signaling, and alterations in tumor suppressor gene function.

**Anti-Angiogenic Therapy**

**Overall Expectations**

The knowledge that angiogenesis is essential for tumor growth and formation of metastases has led to one of the largest research efforts ever undertaken in an attempt to discover effective anti-angiogenesis compounds. Angiogenesis is not only a pathologic process but also is essential for homeostasis. Physiologic angiogenesis is important in reproduction, wound healing, and menses, as well as in coronary artery and peripheral vascular diseases. Thus, therapeutic efficacy requires a balance in which angiogenesis in tumors is limited while the host is protected from toxic effects.

In addition to potential toxicity from anti-angiogenic therapy, therapy duration and criteria for efficacy are other issues to be considered. Because most anti-angiogenic therapy is intended to decrease the development of new blood vessels, the traditional end points for treatment success or failure must be redefined. For example, a desirable response for standard chemotherapy is a 50% decrease in the cross-sectional area of a tumor; in contrast, the desired end point after anti-angiogenic therapy might be inhibition of further tumor growth. Thus, criteria for the effectiveness of anti-angiogenic therapy, whether in the clinic or in the laboratory, must be considered from a new perspective relative to those for conventional therapies.

Although some reports exist of tumor regression in experimental models of angiogenesis,[38,39] such findings are rare. The vast majority of studies in this field demonstrate that anti-angiogenic therapy leads to inhibition of tumor growth rather than regression of established tumors.[40,41] The ability to interpret experimental studies appropriately is critical to ensure that extrapolations to the clinical setting are not fraught with unrealistic expectations. For example, a typical growth curve for a subcutaneously implanted tumor may demonstrate that anti-angiogenic therapy significantly
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decreases the growth of a tumor. In this preclinical model, this "positive" result may lead to clinical trials of that same agent. In the clinic, however, inhibition of tumor growth can be interpreted as "progressive disease" and the therapy thus considered a failure, particularly if tumor-imaging studies are done at short intervals. Anti-angiogenic therapy may well require a longer period of administration than does chemotherapy to obtain a desirable response.

Effective anti-angiogenic therapy will probably need to be delivered on a chronic basis. Chronic administration will require that the agent be delivered easily (perhaps by the oral route) and have few cumulative long-term side effects. As noted in the previous paragraph, the effect of anti-angiogenic therapy may require longer evaluation intervals. One must also consider that the goal of standard anti-angiogenic therapy is to decrease blood vessel formation, not cause tumor regression. Therefore, uniform criteria should be developed for determining the effectiveness of anti-angiogenic therapy (eg, time to progression, survival, and quality of life); these criteria will probably differ from current criteria for tumor response to cytotoxic agents.

Overall Strategies

Despite the simplified view that anti-angiogenic therapy simply interferes with the blood supply to a tumor, developmental strategies for anti-angiogenic therapies are quite diverse and distinct. Anti-angiogenic strategies can be classified into four major categories: (1) Those that decrease the activity of specific angiogenic factors; (2) those that decrease the activity of endothelial survival factors; (3) those that increase the activity of naturally occurring anti-angiogenic agents, ie, angiostatin, endostatin, thrombospondin, etc; and (4) those that indirectly downregulate activity of angiogenic and survival factors. (Matrix metalloproteinase inhibitors are not specific anti-angiogenic agents and will not be discussed in this article.)

Anti-VEGF Approaches

Because VEGF has been linked to the angiogenesis and aggressiveness of many disease types, this prototype molecule will be used in describing strategies to decrease the activity of angiogenic factors. Anti-VEGF strategies include neutralizing antibodies, ribozymes, soluble receptors, or antisense infection. Other anti-VEGF strategies include neutralizing antibodies to the receptors for VEGF, and the use of tyrosine kinase inhibitors that block downstream signaling, even upon binding of the ligand to its receptor. Currently, several of the above-mentioned strategies are being assessed in clinical trials, and all of the strategies have shown promise in the preclinical trials.

One of the earliest strategies used to inhibit VEGF activity involved the use of neutralizing antibodies to VEGF where the antibody is a hybrid and the variable region recognizes the epitope, whereas the Fc is humanized and is not recognized as foreign by the host. This region should also interact with human Fc receptor-bearing effector cells and/or human complement. This strategy is similar to that used for anti-VEGF receptor antibodies. The antibodies must be administered by the intravenous route.

The other commonly used strategy for inhibiting VEGF activity is the use of tyrosine kinase inhibitors.[42] These are small molecules that prevent kinase activation upon binding of its ligand to its receptor. Although these compounds are claimed to be selective for their specific targets, in reality these tyrosine kinase inhibitors do have some cross-reactivity with other receptors and require a much higher dose to achieve an effect. Thus, for all essential purposes, these tyrosine kinase inhibitors are selective. These inhibitors can be administered by the intravenous route, although the newer generation of tyrosine kinase inhibitors can be given orally. This is of great advantage to patients who may need to take anti-angiogenic therapy chronically.

Anti-VEGF and Apoptosis

Studies from our laboratory have examined anti-VEGF receptor antibodies and tyrosine kinase inhibitors in mouse models of colon cancer and liver metastasis.[41,43] Interestingly, the compounds were similarly effective in decreasing hepatic tumor burden, vessel count, and proliferative index of the tumor cells. An unexpected finding from these studies was the increase in the number of tumor cells undergoing apoptosis in the treated metastases. We investigated this phenomenon to
determine if endothelial cell apoptosis was the preemptive cause of tumor cell apoptosis. We established a technique of double-staining to identify endothelial cells and superimpose terminal deoxynucleotidyl transferase-mediated UTP end labeling (TUNEL)-positive cells that would selectively identify those endothelial cells undergoing apoptosis.[41] Results showed an increase in the wave of endothelial cell apoptosis that preceded an increase in the number of tumor cells undergoing apoptosis.[43] These data suggest that endothelial cell apoptosis occurs before tumor cell apoptosis, again demonstrating the selectivity of VEGF and its activity for endothelial cells. This also implicates VEGF as a survival factor for tumor endothelium.

Because anti-VEGF therapy leads to an increase in tumor and endothelial cell apoptosis, one would surmise that this therapy could lead to a decrease in tumor size. There are reports of studies in subcutaneous xenograft models where tyrosine kinase inhibitors to the VEGF receptor and other angiogenic factor receptors can cause regression of established tumors.[42,44] However, in our model of colon cancer liver metastasis, tumor growth was inhibited but eventually the continued tumor growth led to the animals’ deaths. This probably is because tumors contain redundant mechanisms for angiogenesis, and anti-angiogenic therapy directed at a specific factor may lead to selection of cells whose angiogenesis is driven by a different factor.[2,45]

**Endothelial Cell Proliferation**

A second anti-angiogenic strategy involves agents that decrease the activity of endothelial cell survival factors. Typically, angiogenesis is simply thought of as the development of a new vasculature within tumors where endothelial cells migrate, proliferate, invade the basement membrane, and differentiate to form a primitive capillary network. However, the tumor microenvironment is caustic, with low pH and low oxygen tension. Therefore, to survive, these fragile endothelial cells must be exposed to endothelial cell survival factors that prevent apoptosis in adverse conditions.

Endothelial cell survival factors include pericytes that may stabilize endothelium and function either by cell-to-cell contact or secretion of endothelial cell survival factors, such as VEGF or Ang-1. In the absence of pericytes, VEGF and Ang-1 are necessary for endothelial cell survival.[46] These factors can be secreted by endothelial cells, tumor cells, or nonmalignant cells within the microenvironment. VEGF inhibits endothelial cell apoptosis by activation of various survival pathways, including the Akt pathway, IAP, A1, and the MAP kinase pathway.[47] Angiopoietin-1 binds to the specific endothelial cell receptor, Tie-2, and activates the Akt pathway that mediates survival in many cell types.[48]

Another very important mechanism for endothelial cell survival is the binding of integrins on the endothelial cell surface to the extracellular matrix. At first, integrins were thought to be important only in cell-to-cell contact and binding to the extracellular matrix, but it is now known that integrins may mediate intracellular signaling, either alone or in combination with other receptors.[49] The integrins alpha 5, beta 3 and alpha 5, beta 5 have been shown to act as survival factors for endothelial cells; disruption of the binding between integrins and the extracellular matrix may lead to endothelial cell death.[50-52] It is likely that integrins lead to activation of focal adhesion kinase, resulting in downstream signaling, which initiates endothelial cell survival mechanisms.[52]

Several agents are currently being evaluated that inhibit integrin activation.[50,53] Specifically, small molecules have been developed that may inhibit integrin activation, and antibodies have been synthesized that block its binding to the extracellular matrix. Some of these compounds are in early clinical trials, and other compounds are being evaluated in preclinical trials.

**Natural Anti-Angiogenic Agents**

Another anti-angiogenic strategy is one that increases the activity of naturally occurring anti-angiogenic agents, including angiostatin, endostatin, and thrombospondin. A great deal of publicity has surrounded the discovery of angiostatin and endostatin, as these agents were first discovered as fragments of larger molecules (angiostatin is a fragment of plasminogen, endostatin is a fragment of collagen XVI-II). [38,54] The exact mechanism by which these two compounds lead to a decrease in angiogenesis is not yet clearly understood. In addition, the naturally occurring angiogenic antagonist thrombospondin is being evaluated in preclinical trials.
Another anti-angiogenic compound that has received more attention for its other activities is the interferon family of proteins.[55-58] One of these cytokines, interferon-alpha, was shown to cause regression of life-threatening childhood hemangiomas in a study published in the early 1990s.[57] Further laboratory studies have demonstrated that interferon-alpha and interferon-beta can downregulate basic fibroblast growth factor (b-FGF) levels, and regulation of basic FGF by the interferons has also been demonstrated in other tumor systems.[59] More recently, reports have demonstrated the efficacy of interferon-alpha in regression of other tumors in children.[56] The efficacy of interferon therapy may be dependent on chronic low-dose therapy as opposed to higher-dose therapy, which is often associated with intolerable side effects.

Finally, another category of anti-angiogenic therapy indirectly downregulates the activity of angiogenic or endothelial cell survival factors. VEGF and other angiogenic factors are often upregulated in response to stress, such as hypoxia, low pH, or cytokines. Strategies that down-regulate the upstream signaling pathways to VEGF and other angiogenic factors may indirectly downregulate VEGF activity and angiogenesis. Studies done in our laboratory as well as others have demonstrated that several growth factor receptors are involved in induction of VEGF upon binding of its ligand to its receptor (epidermal growth factor-receptor [EGF-R], insulin-like growth factor-1-receptor [IGF-R1]).[26,60] Strategies to inhibit the activity of these receptors lead to a decrease in in vivo VEGF production and angiogenesis, which in turn leads to decreased tumor growth.

It is also known that tumor suppressor genes, such as p53 and VHL, repress transcription of VEGF. We have shown that infection of a wild-type p53 gene can lead to downregulation of VEGF in a colon cancer cell line with a mutated p53 gene and inhibit angiogenesis in vivo.[29] It is possible that anti-VEGF therapy may be beneficial in patients with von Hippel-Lindau syndrome, which is almost certainly due to overexpression of VEGF in the formation of multiple vascular tumors.[61]

**Metronomic Therapy**

Recently, several publications have advocated the use of low-dose continuous chemotherapy as anti-angiogenic therapy. The premise behind this strategy is that endothelial cells are susceptible to chemotherapy, much as bone marrow progenitor cells are susceptible. Standard chemotherapy protocols include a rest period between regimens in order that the bone marrow can recover. However, dividing tumor endothelial cells that may have been injured during the chemotherapy are also able to recover and reestablish blood conduits during this recovery period. Thus, with continuous, low-dose chemotherapy, the dividing tumor endothelial cells are damaged by the chemotherapy but are not given sufficient time to recover and reestablish tumor vessels. The low dose prevents significant toxicity to bone marrow progenitor cells.

In tumor cells resistant to cyclophosphamide in vitro, Browder and colleagues showed that repetitive delivery of low-dose cyclophosphamide decreased angiogenesis and tumor growth.[62] When this dosing regimen was used in combination with TNP-470, tumors were eradicated. It was thought that this strategy provided sustained apoptosis of endothelial cells.

In a modified approach, Klement and associates delivered daily doses of vincristine with an antibody to VEG-FR-2 and again found marked tumor growth inhibition.[63] This strategy was formulated to inhibit the activity of VEGF survival function while damaging tumor endothelium with chemotherapy. While this "metronomic" therapeutic approach seems promising, it must be realized that low-dose continuous chemotherapy has been administered for numerous diseases with disappointing results. In fact, the basis of the development of some of the newer oral analog of fluorouracil and its derivatives is to deliver chronic chemotherapy by a convenient method (oral) with less toxicity and potentially better outcomes. At present, this dosing regimen appears to be equal, but not superior, to conventional chemotherapy delivered intravenously regarding response and survival of patients with unresectable disease.

**Conclusions**
As stated previously, most local tumors can be treated by surgery or radiation, or both. However, the true challenge in oncology lies in treating metastatic cancers. The host microenvironment plays a major role in modulating gene expression in tumors growing at different sites, and this holds true for angiogenic factor expression as well. In our laboratory, we have found that VEGF expression is actually higher in liver metastasis than in primary tumors. It would be naive for oncologists to think that anti-angiogenic activity would be equally efficacious in different tumors growing at different sites. In addition, the endothelium is phenotypically distinct at different sites, and therefore each tumor may not only express different angiogenic factors, but the endothelium may have different angiogenic factor receptors.[64]

Future Directions

It is foreseeable that in the future we will need to obtain biopsies of tumors growing at various metastatic sites and analyze expression of various genes within these biopsies (Figure 2). The revolution of microarray technology may allow us rapidly to identify angiogenic factors that may be driving angiogenesis in specific tumors at specific sites. At that point, we then may be able to direct appropriate anti-angiogenic therapy toward specific targets. It is also possible that continued antiangiogenic therapy against a specific target may lead to selection of clones whose angiogenesis is driven by a different tumor. Therefore, it may be important to "restage" patients with repeat biopsy of these tumors to determine adequately the angiogenic profile of tumors within the course of their growth, and ideally, response to anti-angiogenic regimens.

References:


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