Molecular Origins of Cancer

Tumor Angiogenesis

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The current era of research in antiangiogenic therapy for cancer began in earnest in 1971 with the publication of Folkman’s imaginative hypothesis, but 33 years would elapse before the first drug developed as an inhibitor of angiogenesis was approved by the Food and Drug Administration (FDA). This approval was based on the survival benefit observed in a randomized phase 3 trial of first-line treatment of metastatic colorectal cancer; in that trial, bevacizumab, a humanized monoclonal antibody directed against vascular endothelial growth factor (VEGF), was combined with conventional chemotherapy. Bevacizumab therapy also increased overall survival in the first-line treatment of advanced non–small-cell lung cancer when used in combination with standard chemotherapy. Two other antiangiogenic drugs, sorafenib and sunitinib, have also been approved by the FDA; these are oral small-molecule-receptor tyrosine kinase inhibitors (RTKIs). They target multiple receptor tyrosine kinases, including VEGF receptors and platelet-derived growth factor (PDGF) receptors. Sorafenib and sunitinib have been beneficial in the treatment of metastatic renal-cell cancer when used alone. Sorafenib monotherapy is also active in the treatment of hepatocellular carcinoma and was recently approved by the FDA for this indication.

The survival benefits of these treatments are relatively modest (usually measured in months), with the possible exception of the benefits for patients with renal-cell carcinoma. These treatments are also costly and have toxic side effects. These concerns raise the following questions with respect to improving antiangiogenic therapy: How do such drugs work, and how does bevacizumab increase the efficacy of chemotherapy? Several theories have been postulated, including the theory that antiangiogenic drugs improve chemotherapy by causing “vessel normalization” in tumors (see Appendix 1 of the Supplementary Appendix, available with the full text of this article at www.nejm.org). How do tumors become resistant to antiangiogenic drugs? Are there clinically useful markers that can predict the efficacy of this class of drug? Are there promising surrogate pharmacodynamic biomarkers that will help to determine the best dose of a particular agent? Will antiangiogenic RTKIs such as sunitinib or sorafenib consistently enhance the efficacy of chemotherapy? What accounts for the side effects of these agents?

Many recent discoveries have the potential not only to answer some of these questions but also to indicate new therapeutic targets and treatment strategies. The purpose of this review is to summarize a number of these discoveries, made mainly over the past 5 years, and to point out their potential clinical impact.

The VEGF and VEGF-Receptor Family in Tumor Angiogenesis

Much attention has been focused on the VEGF family of growth factors and the receptor tyrosine kinases that mediate their proangiogenic effects (Fig. 1). This family of structurally related molecules includes VEGF-A, VEGF-B, VEGF-C, VEGF-D,
and placental growth factor (PIGF).\textsuperscript{17,18} The major mediator of tumor angiogenesis is VEGF-A, usually referred to as VEGF. VEGF signals mainly through VEGF receptor 2 (VEGFR-2), which is expressed at elevated levels by endothelial cells engaged in angiogenesis and by circulating bone marrow–derived endothelial progenitor cells. The role of VEGF receptor 1 (VEGFR-1) is a mystery with respect to VEGF-mediated angiogenesis. It binds VEGF with approximately 10 times the affinity of VEGFR-2 binding, but its signal-transducing properties are extremely weak.\textsuperscript{19}

Most types of human cancer cells express VEGF, often at elevated levels; this is a likely consequence of the numerous and diverse genetic and epigenetic ways in which VEGF can be induced (Fig. 1).\textsuperscript{20} Hypoxia, a characteristic of solid tumors,\textsuperscript{21} is an important inducer of VEGF. Its effect is mediated through the hypoxia-inducible transcription factors 1α and 2α.\textsuperscript{21}

It is commonly held that VEGF action is attributable to a paracrine mechanism by tumor cells—that is, tumor cells produce VEGF but cannot respond to it directly since they do not have cellsurface VEGF receptors. In contrast, endothelial cells engaged in angiogenesis express numerous VEGF receptors, but they produce very little or no detectable VEGF. It is now clear, however, that VEGF in amounts sufficient to drive tumor angiogenesis originates from various host cells in the body such as platelets and muscle cells;\textsuperscript{22} such cells also include tumor-associated stromal cells.\textsuperscript{23,24} These findings explain, at least in part, why elevated VEGF levels in blood or even tumor tissue do not predict a benefit in patients receiving drugs that target the VEGF–VEGFR-2 pathway.\textsuperscript{25} The observation that tumor cells of many types,\textsuperscript{26-28} including those of hematologic tumors,\textsuperscript{26,27} express VEGF receptors (especially VEGFR-1) and also produce VEGF indicates that VEGF may sometimes act as a direct (cell-autonomous) autocrine growth factor for tumor cells. Furthermore, in some cases the VEGF receptors may be expressed not on the surface of the tumor cell but rather within the cell, where they promote cell survival by an “intracellular” mechanism; this has been shown for VEGFR-1 in breast-cancer cells.\textsuperscript{29} Hematopoietic stem cells also express both intracellular VEGFR-1 and VEGF, which can in some instances promote the growth and survival of these cells.\textsuperscript{30}

Results in mice bearing a mutant VEGF gene only in vascular endothelial cells suggest that very low levels of autocrine-acting VEGF mediate endothelial-cell survival and vascular homeostasis by signaling through intracellular VEGFR-2.\textsuperscript{31} Such mice have severe cardiac defects and are subject to gastrointestinal perforations and thrombotic
events; these adverse effects are sometimes observed in patients treated with bevacizumab.¹¹,¹²
These observations have potential clinical implications with respect to the use of small-molecule antiangiogenic RTKIs or monoclonal antibodies. Thirty. The RTKIs, by virtue of their ability to penetrate cells, could cause certain toxic side effects, thirty-one which can also be caused by targeting other receptor tyrosine kinases such as c-kit and fms-like tyrosine kinase 3, but they may also be more effective than antibodies when used to treat tumors with functional intracellular autocrine VEGF receptors.

There is growing evidence of the role, under certain circumstances, of neuropilins in tumor angiogenesis. Thirty-two, thirty-three These transmembrane receptors lack tyrosine kinase activity, bind semaphorin 3A, and are normally involved in axon guidance. However, neuropilins also bind VEGF₁₆₅, a splice variant of VEGF-A, and thereby modulate angiogenesis; they can act as coreceptors for VEGF-2. Consequently, neuropilins are emerging as potentially promising antiangiogenic targets. Thirty-four

Another advance is the development of monoclonal antibodies to PlGF, thirty-five a growth factor that binds to VEGFR-1. These antibodies have few side effects in mice because PlGF, unlike VEGF, is expressed minimally or not at all by most normal cells and tissues. Thirty-five Moreover, anti-PlGF antibodies can act in concert with antibodies targeting the VEGF pathway of tumor angiogenesis. Thirty-five

Circulating VEGF and a soluble form of VEGFR-2
have been used as surrogate markers of antiangiogenic drug activity. The measurement of these molecules in blood has been used as a preclinical means of establishing the optimal biologic dose of drugs that target VEGFR-2, including antibodies and small-molecule RTKIs. Such drugs can cause dose-dependent alterations in the levels of circulating VEGF or soluble VEGFR-2, and in mice these levels correlate with antitumor activity. The assessment of circulating VEGF in a complex with a VEGF antagonist such as the soluble “decoy” receptor drug called the “VEGF trap” (aflibercept) is also a promising approach for predicting the blockade of angiogenesis.

Another receptor tyrosine kinase signaling pathway is mediated by tie-2, a receptor tyrosine kinase expressed principally on the vascular endothelium. There are two major ligands for tie-2, angiopoietin-1 (ang-1) and angiopoietin-2 (ang-2). Ang-1 acts as an agonist, whereas ang-2 acts as an antagonist, but it can promote angiogenesis, especially in cooperation with VEGF. These angiopoietins act in concert with VEGF to stabilize and mature new capillaries. Blockade of this tie-2 pathway has been more difficult than blockade of the VEGF pathway, in part because of the complexity of agonistic and antagonistic ligands for the same receptor and the problems in finding effective and specific drugs against tie-2 or the angiopoietins. However, antibodies and peptidelike antibodies (“peptibodies”) against ang-2 have recently been developed; these can block tumor angiogenesis and tumor growth in preclinical models.

Research on this signaling system has also highlighted the importance of the pericyte, an accessory cell that is closely associated with blood vessels. Not only endothelial cells but also pericytes secrete ang-1. Moreover, pericytes express PDGF receptors, which may be relevant to the antiangiogenic effects of certain small-molecule RTKIs that target PDGF receptors.

Studies have implicated what appears to be a pivotal new angiogenesis signaling pathway, notch–deltalike ligand (Dll) 4 signaling (Fig. 2). Notch cell-surface receptors (i.e., notch 1, 2, 3, and 4) are expressed by various cell types and are generally involved in cell fate, differentiation, and proliferation. These receptors interact with transmembrane ligands (jagged 1, jagged 2, and Dll1, Dll3, and Dll4) on adjacent cells. Vascular endothelial cells express notch 1 and notch 4 receptors and the jagged 1, Dll1, and Dll4 ligands; among these, Dll4 is expressed exclusively by endothelial cells. Experiments involving gene disruption in mice have shown that notch–Dll4 signaling is essential for vascular development in the embryo — knockout of only one Dll4 allele is lethal to the embryo; VEGF haploinsufficiency has a similar effect.

This finding may suggest that the notch–Dll4 signaling system is a major stimulator of angiogenesis and could be an appealing drug target, since Dll4 is up-regulated in the tumor vasculature, in part by VEGF. Paradoxically, drugs that target Dll4, including neutralizing antibodies, actually increase tumor angiogenesis, but most of the newly formed blood vessels are abnormal and functionally compromised in ways that drastically reduce blood flow. As a result, tumor hypoxia is
increased by up to seven times the normal level, thereby retarding tumor growth. Apparently, angiogenesis induced by VEGF up-regulates Dll4 in the endothelial cells of developing blood vessels and in doing so allows Dll4 to act as a negative feedback mechanism to prevent an excess of chronic, functional angiogenesis.

Another mechanism that inhibits excessive angiogenesis involves vasohibin, a protein in activated endothelial cells that is induced by stimulators of angiogenesis such as VEGF. Vasohibin is thus a specialized member of a large and growing number of endogenous angiogenesis inhibitors such as angiostatin, endostatin, thrombospondin-1, and tumstatin.

The therapeutic potential of anti-Dll4 drugs is an issue of considerable interest. The question of whether they need to be combined with chemotherapy, radiation therapy, or other antiangiogenic drugs is important, because a therapy that induces...
such profound vessel dysfunction in tumors and elevated levels of tumor hypoxia could be counterproductive when added to radiation therapy or chemotherapy. On the other hand, the increase in proliferating endothelial cells induced by anti-Dll4 drugs could result in a more potent vascular targeting effect mediated by chemotherapy and perhaps radiation therapy.

**Angiogenesis and Circulating Bone Marrow–Derived Cells**

Many cell types can be mobilized from the bone marrow and can home to sites of new blood-vessel formation, where they amplify the angiogenic process\(^5^2\) (Fig. 3). These cells include various hematopoietic-cell (CD45+) populations, many of which are monocytic or myeloid cells that express such endothelial-cell markers as VE-cadherin, VEGFR-1, VEGFR-2, and tie-2.\(^5^3\)\(^-\)\(^5^7\) They also express chemokine receptors such as CXC chemokine receptor 4 (CXCR4), which binds stromal-cell–derived factor 1 (SDF-1, also called CXCL12), a chemokine that attracts lymphocytes and certain other cell types. Neutrophils\(^5^8\) and macrophages\(^5^9\) can also have proangiogenic properties.

In addition, there is a nonhematopoietic (CD45−) bone marrow cell population, the circulating endothelial progenitor cells. In contrast to perivascular cells, which function through paracrine mechanisms such as local secretion of VEGF,\(^5^7\) circulating endothelial progenitors are thought to merge with the wall of a growing blood vessel, where they differentiate into endothelial cells\(^6^0\) (Fig. 3).

The study of circulating endothelial progenitors has generated considerable interest and controversy.\(^7^2\) The various cell-surface markers used to describe such cells (which can be expressed by other cell types) and the methods used to detect them probably contribute to the widely divergent reports of the levels of incorporation of these cells into new blood vessels\(^5^2\); these levels range from highs of 20 to 50% or more\(^6^4\)\(^,\)\(^6^2\) to lows of 5% or less\(^5^2\)\(^,\)\(^6^3\) the lower levels being more common. These differences have cast doubt on the general role of circulating endothelial progenitors in tumor angiogenesis, but in virtually all studies of these cells in tumors, untreated tumors were analyzed, or, as in one clinical study, the treatment was completed long before the tumors were analyzed.\(^6^3\) In contrast, in preclinical studies, acute and marked mobilization of the progenitors from the bone marrow has been shown to occur immediately after treatment with vascular disrupting agents (VDAs).\(^6^4\) These potent drugs, mostly microtubule inhibitors, can rapidly shut down the abnormal tumor vasculature, thereby causing massive tumor hypoxia and necrosis.\(^6^5\)\(^,\)\(^6^6\) Invariably, a viable rim of tumor tissue remains.\(^6^5\)\(^,\)\(^6^6\) Within hours after VDA treatment, the number of circulating endothelial progenitors increases, and they invade and colonize the viable rim of the tumor, thereby contributing to the rapid regrowth of the tumor.\(^6^4\) However, this process can be blocked by an anti-VEGFR-2 antibody.\(^6^4\) In addition to their role in VDA treatment, the contribution of circulating endothelial progenitors may be critical during the very early stages of the development of a tumor, but the population of these cells in the vasculature is progressively diluted by differentiated endothelial cells.\(^6^7\) Furthermore, the incorporation of relatively few endothelial progenitor cells (e.g., approximately 12%) into new blood vessels can have major promoting effects on functional tumor growth such as the progression of microscopic metastatic lesions to macroscopic ones.\(^6^8\)

The contribution of circulating bone marrow–derived cells to angiogenesis has a number of clinical implications. For example, a chemotherapy drug such as cyclophosphamide, administered at maximum tolerated doses, can mobilize circulating endothelial progenitors,\(^6^9\)\(^,\)\(^7^0\) which could conceivably contribute to regrowth of the tumor. Conversely, the closely spaced, regular administration of relatively less toxic doses of chemotherapy drugs, with no prolonged breaks — metronomic chemotherapy\(^7^1\) — can prevent mobilization of circulating endothelial progenitors and can act against not only the endothelial progenitors\(^7^2\) but also differentiated endothelial cells in the tumor neovasculature.\(^7^3\)\(^,\)\(^7^4\) Several clinical trials of metronomic chemotherapy are ongoing (Appendix 2 of the Supplementary Appendix).\(^7^5\)

The use of hematopoietic growth factors in patients with cancer who receive high-dose or dose-dense chemotherapy merits consideration here because recombinant granulocyte colony-stimulating factor (G-CSF) can mobilize not only endothelial-cell progenitors,\(^7^6\) but also CD11b+ granulocyte differentiation antigen (Gr1)+ myeloid suppressor cells that can promote angiogenesis\(^7^7\)\(^,\)\(^7^8\) (Fig. 3). In preclinical studies, the number of mobilized progenitors can be used as a surrogate pharmacodynamic marker to help establish
the optimal dose of metronomic chemotherapy, and enumeration of apoptotic circulating endothelial cells has been used to predict anti-angiogenic drug activity induced by metronomic chemotherapy. However, these methods remain to be validated in larger prospective clinical studies. Finally, since many of the cells shown in Figure 3 express VEGFR-1 and CXCR4, there are prospects for using drugs that target these receptors to inhibit angiogenesis mediated by relevant bone marrow–derived cells.

Intrinsic resistance and acquired resistance to anti-angiogenic drugs are clinically significant problems. Preclinical studies have begun to shed light on the mechanisms of such resistance. With respect to intrinsic resistance, it has been shown that a tumor cell line from mice that is resistant to anti-VEGF antibodies becomes colonized by bone marrow–derived cells (CD11b+Gr1+ myeloid-suppressor cells) when transplanted into mice. Moreover, when normally sensitive tumor cells are mixed with these cells that are resistant to anti-VEGF antibodies and transplanted into other mice, the transplanted tumors resist anti-VEGF antibodies. Intrinsic resistance can also occur as a result of tumor cells using existing blood vessels in vasculature-rich organs such as the lungs, or simply as a result of the absence of VEGF or VEGF receptors in metastatic tumors growing in certain organ sites when drugs that target the VEGF pathway are used.

Acquired resistance to anti–VEGFR-2 antibodies can be caused by the redundancy of angiogenesis stimulators. An example is the up-regulation of other angiogenic factors or receptors when VEGFR-2 is inhibited. Additionally, tumor cells may develop alternative signaling pathways that promote angiogenesis. These mechanisms can be targets for novel therapeutic strategies.
of the angiogenesis stimulator basic fibroblast growth factor (bFGF) within the tumor after treatment with anti–VEGFR-2 antibody therapy; this effect is probably caused by the elevated levels of hypoxia induced by the drug treatment.\textsuperscript{87} Clinically, treatment with bevacizumab increases circulating PlGF, which could cause drug resistance.\textsuperscript{88}

In humans, sunitinib can induce high levels of circulating PlGF and VEGF that revert to normal levels during drug-free periods;\textsuperscript{7} these results can be reproduced in mice, but the presence of a tumor is not necessary for the effect.\textsuperscript{36} G-CSF and SDF-1, which can mobilize circulating endothelial progenitors\textsuperscript{52,76} and possibly other proangiogenic accessory cells,\textsuperscript{57} are also up-regulated in healthy mice treated with sunitinib. Moreover, it is possible that two or more such induced growth factors\textsuperscript{89} could act in a synergistic manner to promote tumor angiogenesis, as has been shown for bFGF and an isoform of PDGF.\textsuperscript{90} Such effects could contribute to rapid vascular regrowth in tumors after discontinuation of certain antiangiogenic treatments.\textsuperscript{90}

Acquired resistance can also develop through the selection and overgrowth of mouse tumor cells with mutations in genes such as \(Tp53\), which cause relative resistance to hypoxia.\textsuperscript{91} These variants may be less dependent on the oxygen supplied by the newly formed blood vessels than are tumor cells without the mutations.\textsuperscript{91} Rapid vascular remodeling of tumor-associated vessels as a consequence of antiangiogenic therapy is another cause of resistance.\textsuperscript{92} The mature remodeled vessels are resistant to antiangiogenic drugs, which usually target relatively immature vessels.\textsuperscript{93} These aforementioned mechanisms suggest various possible strategies to delay or possibly even reverse acquired resistance.\textsuperscript{87}

### ANGIOGENESIS AND CANCER STEM CELLS

Studies have identified in tumors a minor population of cells with the characteristics of “tumor-initiating” cancer stem cells.\textsuperscript{94–96} These cells are thought to drive tumor growth and to constitute the seeds of resistance to treatment. The cancer stem-cell hypothesis is based largely on the results of transplantation of selectively enriched populations of cells into immunodeficient mice. Transplantation of extremely small numbers of such putative human cancer stem cells results in a high rate of tumor “takes,” whereas transplantation of much larger numbers of cancer cells lacking stem-cell characteristics does not.\textsuperscript{94,95} It has been suggested that conventional chemotherapy and other types of drugs attack the latter cells but not the cancer stem cells.\textsuperscript{96} The potent tumorigenic properties of cancer stem cells suggest that they may be strongly proangiogenic, and there is some evidence of this feature,\textsuperscript{97} which may help to explain some of the effects of tumor-inhibiting antiangiogenic drugs.

In addition, putative cancer stem cells in brain tumors reside in close proximity to blood vessels in a “vascular niche.”\textsuperscript{98} Treatment of orthotopic transplanted gliomas in mice with antibodies to VEGF disrupts the vascular niche and targets the stem-cell population.\textsuperscript{98} This population expresses high levels of VEGF and thus would be expected to be sensitive to anti-VEGF treatment.\textsuperscript{98} Low-dose metronomic chemotherapy may target the population of cancer stem cells or cells that are like cancer stem cells,\textsuperscript{99} especially when it is combined with an antiangiogenic drug such as anti–VEGFR-2 antibodies.\textsuperscript{74}

### SUMMARY

The increasing use of antiangiogenic drugs for the treatment of cancer has emerged from decades of extensive basic and clinical research. The clinical benefits of such drugs, however, are relatively modest. Improvements are likely to come from a more thorough understanding of the molecular and cellular mechanisms governing tumor angiogenesis and the response to antiangiogenic therapies. A number of recent advances promise to bring about such improvements. These include new findings in the VEGF and the VEGF-receptor family, discovery of the notch–Dll4 signaling pathway in tumor angiogenesis, elucidation of the proangiogenic role of circulating bone marrow–derived cells, identification of the mechanisms of resistance to antiangiogenic drugs, and observations that suggest a role of angiogenesis in the survival and growth of cancer stem cells.

Many of these discoveries and others suggest strategies for improving the clinical benefits of antiangiogenic therapy. These strategies include the development of better preclinical models to study the biology of tumor angiogenesis and antiangiogenic therapies (see Appendix 3 of the Supplementary Appendix). Such improvements will
also be critical in the use of long-term antiangiogenic therapy in the adjuvant setting in patients with early-stage disease. With respect to the treatment of metastatic disease, the magnitude and diversity of targets for antiangiogenic approaches suggest numerous possibilities for antiangiogenic drug combinations that should be much more effective than monotherapy in treating cancer.

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REFERENCES


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