Introduction

It is just 40 years since Judah Folkman published his classic *New England Journal of Medicine* article entitled “Tumor angiogenesis: therapeutic implications” (1). In that article, he set forth 3 bold postulates: (i) angiogenesis is essential for tumor growth beyond minimal size; (ii) tumors secrete a “tumor angiogenesis factor” (TAF) that is responsible for inducing angiogenesis; and (iii) antiangiogenesis is a potential therapeutic strategy for treating cancer. Now, 4 decades later, after considerable debate and not a little controversy, all 3 of these postulates are widely accepted. Generally speaking, tumors do need to induce a new vascular supply if they are to grow, though some make do, at least for a time, by coopting the existing host vasculature (2). Folkman’s RNA-like TAF candidate proved to be a blind alley, but the discovery of vascular permeability factor/VEGF (VPF/VEGF or more simply VEGF-A), and the preparation of antibodies against it, provided an opportunity for testing Folkman’s hypotheses (3, 4). VEGF-A is generally agreed to be the most important TAF. It acts on vascular endothelial cells within minutes to induce vascular permeability and, longer term, reprograms gene expression, leading to endothelial cell proliferation and migration *in vitro*, and generation of new blood vessels *in vivo*. Moreover, a variety of drugs that target VEGF-A or its receptors effectively prevent the growth of many mouse tumors and tumor xenografts (2, 5–7). Among these drugs are antibodies against VEGF-A or its receptors, engineered proteins that mimic VEGFRs, and small-molecule receptor tyrosine kinase (RTK) inhibitors that preferentially target VEGFR-2 (VEGFR-2/flk-1/KDR) with high affinity.

Unfortunately, however, the striking benefits of anti-VEGF/VEGFR receptor (VEGFR) therapy observed in treating mouse tumors have not been translated to the clinic. All of these drugs have had only modest effects on human cancers and have not lived up to the great expectations that Folkman and many others anticipated. For example, bevacizumab (Avastin; Genentech), a humanized antibody against VEGF-A, prolongs the life of patients with advanced colon cancer by, on average, 4 to 5 months, and then only when combined with triple chemotherapy (8). In fact, a recent editorial has questioned whether bevacizumab was “boon or bust” because of its limited effectiveness, serious (though uncommon) side effects, and high cost (9). Other U.S. Food and Drug Administration–approved drugs that bind VEGF-A, or that target VEGF-A receptors, have fared no better (10). Thus, we are left with the question, why doesn’t anti–VEGF-A/VEGFR therapy work better against human cancers? But, before tackling that question, it could be helpful to address some others first: What are tumor blood vessels? How do they form? What does anti–VEGF-A/VEGFR therapy do to tumor blood vessels?

Anti-VEGF/VEGFR Therapy for Cancer: Reassessing the Target

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Abstract

Judah Folkman recognized that new blood vessel formation is important for tumor growth and proposed antiangiogenesis as a novel approach to cancer therapy. Discovery of vascular permeability factor VEGF-A as the primary tumor angiogenesis factor prompted the development of a number of drugs that targeted it or its receptors. These agents have often been successful in halting tumor angiogenesis and in regressing rapidly growing mouse tumors. However, results in human cancer have been less impressive. A number of reasons have been offered for the lack of greater success, and, here, we call attention to the heterogeneity of the tumor vasculature as an important issue. Human and mouse tumors are supplied by at least 6 well-defined blood vessel types that arise by both angiogenesis and arterio-venogenesis. All 6 types can be generated in mouse tissues by an adenoviral vector expressing VEGF-A164. Once formed, 4 of the 6 types lose their VEGF-A dependency, and so their responsiveness to anti-VEGF/VEGFR receptor therapy. If therapies directed against the vasculature are to have a greater impact on human cancer, targets other than VEGF and its receptors will need to be identified on these resistant tumor vessels. Cancer Res; 72(8); 1909–14. ©2012 AACR.
What Are Tumor Blood Vessels, and How Do They Form?

Despite the great sums of money that companies, the NIH, and charitable organizations have invested in anti-VEGF/VEGFR approaches to cancer therapy, relatively little attention has been paid to the types of new blood vessels that these drugs are expected to target. Tumor angiogenesis has often been regarded as if it were a single entity in which all tumor blood vessels were identical. However, it has long been known that tumor blood vessels are heterogeneous and differ considerably among themselves with respect to organization, structure, and function (11). Studying several important human carcinomas, we identified 6 distinctly different types of blood vessels (12, 13). Further, to avoid the complexity of the tumor environment, we developed a means of generating surrogate forms of tumor blood vessels in the absence of tumor cells, making use of a nonreplicating adenovirus (engineered by Richard Mulligan) to express the most common VEGF-A isoform, mouse VEGF-A164 (human VEGF-A165; ref. 14).

When injected into the tissues of immunodeficient mice, Ad-VEGF-A164 generates each of the 6 vessel types we had found in human cancers and does so by 2 distinct but interrelated processes, angiogenesis and arterio-venogenesis. On the angiogenesis side, preexisting venules transform over the course of a few days into ‘mother’ vessels, which are greatly enlarged, pericyte-poor vessels that result from proteolytic degradation of venule basement membranes and from pericyte detachment (Fig. 1; refs. 12–15). Freed of these restraints, normally cuboidal venules undergo dramatic enlargement that is made possible by extensive endothelial cell thinning; in this process, endothelial cells discharge the cytoplasmic vesicles and vacuoles that compose their vesiculo-vacuolar organelles to the cell surface, providing the greatly expanded plasma membrane that is needed to line a 4- to 5-fold expanded lumen (14, 16). Mother vessels are highly permeable to plasma proteins, largely by transcellular routes that involve residual vesicles and newly formed fenestrae. Over the course of days to weeks, mother vessels evolve into daughter vessels of 3 types: (i) glomeruloid microvascular proliferations (GMP), poorly organized structures that, as their name implies, resemble renal glomeruli macroscopically. GMP result from intraluminal proliferation of endothelial cells and pericytes and the accumulation of macrophages; (ii) vascular malformations, thought to represent mother vessels that have reacquired a smooth muscle cell coat; and (iii) typical capillaries that form as mother vessels sprout inwards instead of outwards, projecting cytoplasmic processes into their lumens that form interconnecting intraluminal bridges that subsequently separate mother vessels into smaller vessels by intussusception. Vascular malformations and capillaries, unlike mother vessels and GMP, are stable vascular structures that persist indefinitely. Arterio-venogenesis proceeds in parallel with angiogenesis as arterioles, arteries, and veins undergo extensive remodeling.
and enlargement with proliferation of endothelial cells and smooth muscle cells (Fig. 1; refs. 13, 14).

**What Does Anti-VEGF/VEGFR Therapy Do to Tumor (and Normal) Blood Vessels?**

Folkman conceived of antiangiogenesis primarily as a means of preventing angiogenesis and, thereby, limiting further tumor growth. However, it was soon found that anti-VEGF/VEGFR therapies not only prevented angiogenesis, but additionally caused considerable pruning of pre-existing vessels (5, 17). All of the drugs used, whether targeting VEGF-A or its receptors, had similar effects. Endothelial fenestrations, when present, disappeared, vascular sprouting and permeability were suppressed, and blood flow and vessel patency were extensively reduced. Overall, vascular density decreased (depending on the tumor type by as much as 70%). Changes were also noted in the normal vasculature, particularly a loss of endothelial cells fenestrae in renal glomeruli and several endocrine organs (5, 17). These findings suggested an explanation for at least some of the side effects that have been found in patients receiving anti–VEGF-A/VEGFR drugs (9): they also provided strong evidence that VEGF-A was necessary for the maintenance of the normal adult vasculature. Some tumor cells, as well as tumor-infiltrating macrophages, express VEGFRs, particularly VEGFR-1, and so anti-VEGF/VEGFR agents may act on these cells as well (18). This action is more likely to be the case for small-molecule RTKs, because large molecules such as antibodies are unlikely to penetrate the vascular barrier efficiently.

**Explanations Commonly Offered for the Failure of Anti-VEGF/VEGFR Therapy to Work Better in Cancer Patients**

A number of explanations have been offered to explain the modest effectiveness of anti–VEGF-A/VEGFR therapy in cancer patients vis-à-vis their more potent effects in tumor-bearing mice (19). All of these explanations are likely to have some validity. One obvious possibility is that cancer patients are often elderly and very ill, in contrast with the young, relatively healthy tumor-bearing mice treated in the laboratory. Further, higher dosing is possible in mice, who cannot complain about side effects. A second likely reason for the limited effectiveness of anti-VEGF/VEGFR therapy is that it does not result in the killing of all tumor cells; residual tumor cells rendered hypoxic by a compromised blood supply are stimulated to make increased amounts of VEGF-A that may overwhelm anti-VEGF/VEGFR therapy, especially when accompanied by increased expression of matrix components that bind and sequester VEGF-A, protecting it from anti-VEGF drugs (20). Hypoxic tumor cells also begin to make a plethora of other growth factors and cytokines, which have the capacity to replace VEGF and stimulate new blood vessel growth. Included among these are FGF, PDGF-B, PDGF-C, HGF, EGF, IL-8, IL-6, Ang-2, SDF1α, PDGF-C, CXCL6, and others, as well as their receptors. Other possibilities are recruitment from bone marrow of vascular progenitor cells and proangiogenic myelocytes that can serve as a rich source of growth factors (19, 21).

Another explanation is that of “vascular normalization,” a limited period of time during which tumors treated with anti-VEGF/VEGFR therapy exhibit a reduction in tumor vessel tortuosity, hyperpermeability, interstitial tissue pressure, and edema (6). The mechanisms responsible for vascular normalization are not yet fully understood and could reflect a return to normality of abnormal tumor blood vessels and/or pruning of the most abnormal vessels, leaving behind those that are less abnormal. Whatever the mechanism, vascular normalization persists for only a short time, in both mouse and human cancers, and could have Janus-like effects on tumor growth. On the one hand, more normal blood vessels provide improved blood flow and so might be expected to provide a window of opportunity for improved drug delivery; however, a better blood supply could also favor tumor growth (for a review of this issue, see refs. 6, 10).

**Another Possible Explanation for the Limitations of Anti-VEGF/VEGFR Therapy: Differential Sensitivity of Tumor Blood Vessel Subtypes to Anti-VEGF/VEGFR Therapy**

Many of the early, highly successful studies targeting VEGF or its receptors in mice followed protocols in which therapy was initiated concurrently with or soon after the transplant of tumor xenografts. Obviously, however, this treatment pattern does not mimic the clinical situation in which tumors have been present for months or years prior to discovery and onset of therapy. Bergers and Hanahan (19, 22) recognized this distinction, raising an important red flag that called attention to the limitations of anti-VEGF/VEGFR therapy in treating established tumors. They found that VEGFR inhibitors were highly effective in preventing the development of the spontaneous Rip-Tag tumor and in inhibiting its early growth, but that they were of much less benefit in regressing tumors with an established vasculature (19, 22). Thus, in mice as in patients, anti-VEGF/VEGFR therapy was found to be less effective in advanced disease. Bergers and Hanahan (19) attributed the failure of late therapy to the maturing of the vasculature with increased pericyte coverage and found that addition of an RTK inhibitor that targeted PDGFR-β (highly expressed on pericytes) improved anti-VEGFR therapy, though in other systems this has not been the case. Many other reports indicate that “immature” vessels, variously defined, are preferentially susceptible to anti-VEGF/VEGFR therapy (19, 22–24). However, there are exceptions to this pattern of differential sensitivity. For example, in some tumors nearly all blood vessels are pericyte or smooth muscle cell coated and yet undergo extensive pruning in response to anti-VEGF/VEGFR therapy (5).

**Effects of Rapamycin and Aflibercept (VEGF Trap) on Tumor Surrogate Blood Vessels Induced by Ad-VEGF-A**

In 2 recent articles, we investigated the sensitivity of tumor surrogate blood vessels to anti–VEGF-A drugs (23, 25). When...
injected into the skin of immunodeficient mice, Ad-VEGF-A\textsuperscript{164} replicates at successive intervals large numbers of each of the 6 types of blood vessels that we had identified in human cancers. Therefore, by initiating treatment at different intervals before or after Ad-VEGF-A\textsuperscript{164} injection, we were able to evaluate the effects of drugs that targeted VEGF-A indirectly (rapamycin; ref. 25) or directly [alfibercept (VEGF Trap); ref. 23]. Rapamycin, acting on mTOR downstream of the VEGF-A–VEGFR-2 signaling pathway, prevented both angiogenesis and arterio-venogenesis and regressed early blood vessels (mother vessels, GMP). VEGF Trap, a human soluble decoy receptor protein with high affinity for all of the VEGF-A isoforms, as well as for VEGF-B and placental growth factor (26), had similar effects (Fig. 1). Up to about 1 month, mother vessels, GMPs, early vascular malformations, and developing feeder arteries and draining veins regressed, at least to some extent, and vascular volume and vascular leakage were strikingly reduced. However, like rapamycin, VEGF Trap became progressively less effective when administered at later times after Ad-VEGF-A\textsuperscript{164} injection, when the great majority of blood vessels induced by angiogenesis (vascular malformations) and arterio-venogenesis (feeder arteries, draining veins) had developed fully. Thus, although all 6 types of tumor surrogate blood vessels were induced by VEGF-A\textsuperscript{164}, not all remained sensitive to anti–VEGF-A therapy. Results consistent with ours were recently reported in a model of angiogenesis induced by HIF-1α-overexpressing keratinocytes; antibodies against VEGFR-2 were initially successful in preventing new blood vessel formation but were largely ineffective when administered 2 weeks later (27).

In an effort to explain our results mechanistically, we immunostained Ad-VEGF-A\textsuperscript{164}–induced blood vessels for VEGFR-2. Whereas early susceptible vessels (mother vessels, GMP) expressed high levels of VEGFR-2, late, rapamycin-, and VEGF Trap–resistant vessels (vascular malformations, feeder arteries, draining veins) expressed extremely low and sometimes undetectable levels of this receptor as determined by immunohistochemistry (23), and by reverse transcriptase–PCR (H.F. Dvorak, unpublished data). Thus, a possible explanation for the insensitivity of vascular malformations, feeder arteries, and draining veins to VEGF Trap is that their lining endothelial cells have lost dependence on exogenous VEGF-A, and that any remaining requirement for VEGF-A as a survival factor had been met by the pericytes or smooth muscle cells that closely enveloped them (28).

Our findings with rapamycin and VEGF-Trap on Ad-VEGF-A\textsuperscript{164}–induced tumor surrogate blood vessels are likely to be relevant to anti-VEGF/VEGFR therapy for mouse tumors and human cancer. Personal review of many different tumors and of the published photomicrographs of others indicate that vessels with the structure and functional properties of mother vessels predominate during early stages of the growth of most transplantable mouse tumors and tumor xenografts and also in such spontaneously arising tumors as the Rip-Tag2 model. Additional evidence for this conclusion comes from the finding that anti-VEGF/VEGFR drugs potently reverse tumor vascular hyperpermeability (7, 23, 29, 30). In the pathologic angiogenesis induced by tumors or Ad-VEGF-A\textsuperscript{164}, mother vessels (and to a lesser extent GMP) are the only hyperpermeable blood vessels that have thus far been identified in tumors (13, 14). The same reasoning suggests that the “vascular normalization” following anti–VEGF-A/VEGFR treatment results, at least in part, from the pruning of hyperpermeable mother vessels. Left behind are relatively unaffected pericyte- and smooth muscle–coated vascular malformations, feeder arteries, and draining veins, that is, vessels that by MRI and histology seem relatively normal in that they are not hyperpermeable and are smooth muscle cell coated.

Looking Back and Moving Forward

The work reviewed here invites some conclusions but also raises new questions. On the plus side, Folkman’s postulates have been affirmed. Targeting VEGF and its receptors offers benefit to patients with at least some types of cancer and provides proof-of-principle that attacking the vasculature is a valid approach to cancer therapy. At present, however, these benefits are limited and may have untoward consequences; just as inadequately treated bacterial infections can result in mutations that lead to antibiotic resistance, so residual tumor cells, rendered hypoxic but not killed by anti-VEGF/VEGFR therapy, can reprogram their genomes to express increased amounts of VEGF-A as well as other growth factors that can overwhelm or bypass the VEGF/VEGFR axis and stimulate new blood vessel growth. To mix metaphors, winding a bear can make him very angry!

A further conclusion is that tumor blood vessels are heterogeneous and that some are susceptible, whereas others are highly resistant, to anti-VEGF/VEGFR therapy. Resistant surrogate vessels such as vascular malformations, feeder arteries, and draining veins develop gradually over time after Ad-VEGF-A\textsuperscript{164} administration; they are relatively large vessels, coated by 1 or more layers of smooth muscle cells, and are lined by endothelial cells that express very low levels of VEGFR-2. Late-appearing vessels of this type are likely to predominate in human cancers, which are often present for months to years before they are discovered.

The mechanisms by which tumor blood vessels are actually destroyed by anti-VEGF/VEGFR therapy require further investigation (18). An obvious possibility is that vessels lined by endothelial cells expressing high levels of VEGFR-2 and coated with few pericytes require substantial amounts of VEGF-A for survival and, therefore, undergo apoptosis when the VEGF-A/VEGFR axis is disrupted. However, VEGF-R expression levels and pericytes may not provide the entire explanation for resistance to anti-VEGF/VEGFR therapy. Pericyte-coated capillaries are not always spared by anti-VEGF/VEGFR therapy (5), and other mechanisms can be envisioned for their destruction (18). In some cases, blood vessel pruning could result from tumor cell killing; for example, dying tumor cells could release cytokines or toxic products that damage blood vessels. A possibility that has received little attention is that anti–VEGF-A/VEGFR therapy could not only act on angiogenic capillaries but also act upstream on the larger feeder arteries that supply the tumor microcirculation. The VEGF-A–VEGFR-2–eNOS–NO pathway has an important
role in maintaining normal arterial tone, and, when interrupted, could cause constriction of the feeder arteries supplying tumors, leading to reduced downstream blood flow and necrosis of both tumor blood vessels and the tumor cells they supply. Consistent with this possibility is the finding that patients receiving anti-VEGF-A/VEGFR therapy commonly develop systemic hypertension (18). It would be ironic if treating this hypertension, as is commonly done, actually interfered with therapeutic benefit.

Finally, going forward, it is important to think beyond VEGF and its receptors as the sole vascular targets. We need to identify new molecular targets on the endothelial cells lining the large tumor blood vessels that supply and drain the tumor microvasculature (vascular malformations, feeder arteries, and draining veins) and that have proved resistant to anti-VEGF/VEGFR therapy. Attacking these large vessels would cut and draining veins) and that have proved resistant to anti-VEGF therapy. Eventually, those large vessels would cut off the blood supply to the entire tumor and, therefore, have a larger effect than pruning the innumerable VEGF-A–sensitive microvessels. As any plumber knows, shutting off the water supply at its source makes more sense than trying to turn off every faucet in the house. Evidence for the potential of this concept comes from studies in which photodynamic occlusion of feeder arteries and draining veins led to eradication of mouse ear tumors (31) and from findings that uterine artery embolization or myolysis can ablate uterine fibroids in patients (32). Progress is being made in this regard, and a number of tumor vessel markers worthy of further investigation have been identified (33, 34). We anticipate bright days ahead for targeting the vasculature as a mode of cancer therapy.

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