**Tumor Stroma, Tumor Blood Vessels, and Antiangiogenesis Therapy**

Harold F. Dvorak, MD

**Abstract:** Solid tumors generally require a vascularized connective tissue stroma if they are to grow beyond minimal size. They generate that stroma in part by secreting vascular endothelial growth factor (VEGF), a potent vascular permeability and angiogenic factor. Increased vascular permeability leads to deposition of a provisional fibrin stroma, which supports tumor, connective tissue, and inflammatory cell migration and plays an active role in the formation of mature vascularized stroma. Vascular endothelial growth factor–induced tumor blood vessels are heterogeneous, of at least 6 distinct types, and develop linearly over time. They include both angiogenic (mother vessels, glomeruloid microvascular proliferations, vascular malformations, capillaries) and arteriovenous (feeding arteries, draining veins) blood vessels. Attacking the tumor vasculature with drugs that target VEGF or its receptors (VEGFR) has come into vogue but has been less effective than had been hoped for. One reason for this is that anti-VEGF/VEGFR therapy attacks only a subset of tumor blood vessels, the earliest to form. New targets on late-forming blood vessels such as feeding arteries would be useful in helping antivascular cancer therapy fulfill its promise.

**Key Words:** Angiogenesis, arteriovenogenesis, antiangiogenesis, fibrin, tumors, vascular permeability, VEGF, VPF, wounds


Tumors have a structure that is broadly similar to that of normal tissues. They are composed of parenchyma, that is, the tumor cells themselves, and a supporting stroma. Like normal tissues, tumor cells require nutrients, must clear waste products, and, in the case of solid tumors, have need of structural scaffolding. With few exceptions, tumors must acquire stroma if they are to grow beyond minimal size. However, tumors vary markedly in the quantity and quality of the stroma they induce. At one extreme are desmoplastic tumors, such as many carcinomas of the breast, stomach, and pancreas, in which stroma may account for 90% or more of the tumor mass, with malignant cells being only a minor component. At the other extreme are tumors such as medullary and lobular carcinomas of the breast and many lymphomas that induce minimal stroma. Differences in tumor stromal content may also be qualitative. For example, some carcinomas of the breast provoke the deposition of abundant elastic tissue and collagen, whereas others, such as medullary carcinomas of the breast and colon, induce an extensive lymphocytic infiltrate. The amount of tumor stroma is unrelated to the degree of malignancy. Tumors with widely varying degrees of malignancy can possess large or small amounts of stroma. Even within a single tumor, there may be substantial variations in stromal composition from one area to another. Such stromal heterogeneity should not be surprising in view of the now well-documented heterogeneity of tumor cells within individual tumors.

Although less obvious, “liquid” tumors also have stroma. Leukemic cells circulating in the blood have an ideal stroma, the plasma itself, which provides nutrients and clears waste products with an efficiency that is equivalent or superior to that of any normal tissues. Ascites tumors growing in suspension in the peritoneum or other body cavities are also liquid tumors. Carcinomas of the ovary, for example, can induce the accumulation of many liters of plasma protein-rich exudate because of the hyperpermeability of the angiogenic blood vessels lining the peritoneal cavity. The stroma of ascites tumors is inefficient because the laws of diffusion limit the exchange of nutrients and waste products across large fluid volumes.

**COMPOSITION OF TUMOR STROMA**

The connective tissue stroma of solid tumors is composed of a mixture of normal and abnormal elements. Among these are normal proteins such as types I and III collagen, proteoglycans, and glycosaminoglycans such as hyaluronan, but also products not usually found in normal adult tissues; these include proteins such as tenascin and fetal fibronectin, increased interstitial fluid (edema), and fibrin. Tumor stroma also includes metabolically activated fibroblasts that are responsible for the synthesis of the aforementioned structural elements. Other cellular components include mast cells and blood and bone marrow–derived lymphocytes and myeloid cells. Finally, tumor stroma includes newly formed blood vessels and sometimes abnormally enlarged, poorly functional lymphatics. Stroma generation commonly precedes tumor invasion, as in situ carcinomas of the cervix and breast.

Tumor stromal cells are benign; when isolated and implanted into host animals, they are not able to by themselves to form tumors. However, stromal cells often possess properties that are associated with malignancy and can enhance tumor growth. Tumor-derived fibroblasts exhibit increased invasive capacity and loss of heterozygosity and express growth factors.

**FIBRIN: A MISSING LINK IN TUMOR STROMA GENERATION**

An association between malignancy and abnormal hemostasis dates back more than 150 years. Using migratory thrombophlebitis as a sign (later to become Trousseau sign), the French physician Armand Trousseau diagnosed himself with the gastric malignancy that took his life. Vascular thrombosis is a common cause of death in cancer patients. However, in addition to forming intravascular thrombi, fibrin is also observed outside blood vessels in the stroma of solid tumors. There, fibrin forms a water-retaining gel whose deposition transforms the antiangiogenic stroma of normal tissues into a provisional stroma that is strongly...
proangiogenic. Coupled with impaired lymphatic drainage, fibrin retards the clearance of edema fluid, contributing to the increased interstitial pressure characteristic of solid tumors. The extent of fibrin deposited is highly variable in different tumors, and, like the total amount of tumor stroma, no simple relationship has been found between the quantity of fibrin deposited and the degree of malignancy. However, the net amount of fibrin deposited, a balance between clotting and fibrinolysis, may predict the amount of mature stroma that will ultimately develop (Fig. 1).

Fibrin plays an active role in tumor stroma generation. By serving as a provisional stroma, fibrin provides tumors with initial structure. It also provides a promiscuous matrix that facilitates the attachment and migration of many different cell types because it interacts with integrins (e.g., α5β1, α1β3/β5, αMβ2) that are expressed by tumor cells, fibroblasts, and endothelial and inflammatory cells. This is important because mammalian cells do not like to swim in interstitial fluid; they require a solid matrix on which to attach and migrate. Fibrin also causes stromal cells to express and sequester growth factors, thus preventing their degradation. Furthermore, fibrin has biologically active breakdown products that are proangiogenic. Finally, fibrin acts in ways that are not yet fully understood to promote angiogenesis and the generation of the vascularized connective tissue that replaces it.

Vascular hyperpermeability leads not only to the extravasation of procoagulant proteins, but also to the leakage of plasma proteins such as plasminogen that lead to fibrinolysis. Tumor-secreted plasminogen activators cleave extravasated plasminogen to generate the fibrinolytic protease, plasmin. As a consequence, the fibrin gel deposited by tumors is degraded and is replaced over time by the ingrowth of fibroblasts and new blood vessels; these give rise to a loose and highly vascular connective tissue that resembles the “granulation tissue” of healing wounds. As blood vessels regress and new collagen is laid down, this granulation-like tissue may be transformed into poorly vascularized, dense collagenous connective tissue, a process termed desmoplasia. Desmoplastic tumors often include older, centrally placed regions in which tumor cells are encased in dense collagen, as well as peripheral zones in which tumor cells actively invade the surrounding host tissue and initiate new rounds of stroma formation.

**Generation of Extravascular Fibrin**

As may be inferred from the preceding, 2 conditions must be fulfilled for fibrin deposition in tissues. First, local blood vessels must become hyperpermeable to allow fibrinogen and other necessary plasma clotting proteins to extravasate. Second, the clotting system must be activated in the extravascular space. With regard to the latter, tumor cells, such as platelets, express tissue factor and a surface for prothrombinase assembly (Fig. 1). In addition, many tumors shed microparticles (exosomes) that also express tissue factor and can initiate clotting.

**DISCOVERY OF VASCULAR PERMEABILITY FACTOR/VASCULAR ENDOTHELIAL GROWTH FACTOR**

The finding of fibrin in the stroma of freshly transplanted tumors was my most exciting moment in science, because it led to the discovery of vascular permeability factor, subsequently renamed vascular endothelial growth factor (VEGF). An older literature (reviewed by Brown et al.) indicated that some animal tumors exhibited increased vascular permeability to intravenously injected radiolabeled proteins, but the mechanisms involved had not been investigated. I set out to find the vascular permeabilizing activity that was responsible. Initially, I hypothesized that tumors secreted a factor that caused the degranulation of mast cells or basophils, whose permeability-enhancing activity was some 50,000 times that of histamine on a molar basis. Instead, vascular permeability factor/VEGF acted directly and selectively on venular endothelium to increase permeability to circulating macromolecules such as plasma proteins. Donald Senger and I purified VEGF to homogeneity, using the Miles permeability assay as a metric. Vascular endothelial growth factor turned out to be a dimeric, N-glycosylated protein whose permeabilizing-enhancing activity was some 50,000 times that of histamine on a molar basis. We demonstrated that VEGF was expressed by most malignant animal and human tumors and prepared a blocking antibody against it that prevented fluid accumulation in an animal ascites tumor model.

**FIBRIN DEPOSITION AND STROMA GENERATION IN TUMORS, WOUNDS, AND CHRONIC INFLAMMATION**

The discovery of VEGF clarified the steps by which tumors initiate stroma formation (Fig. 1). The increased vascular...
permeability induced by VEGF leads to deposition of extravascular fibrin and its eventual removal by vascularized connective tissue. This very same sequence of events takes place in the physiological process of corpus luteum formation (a form of wound healing) and in other examples of wound healing such as skin wounds and myocardial infarcts where VEGF comes to be overexpressed. In short, tumors have preempted and subverted a fundamental host mechanism, the wound healing response, as the means to acquire the stroma they need to grow and spread. In some sense then, “tumors are wounds that do not heal.”

The analogy between tumor stroma generation and wound healing can be taken one step further. The clotting that leads to fibrin deposition in tumors and wounds also results in the conversion of blood plasma to serum. Tissues are normally bathed in a protein-poor plasma filtrate. In pathological states, however, increased vascular permeability and extravascular clotting change the interstitial fluid environment to protein-rich serum. Serum contains proinflammatory components that likely modify and regulate tumor growth and wound healing. Powerful evidence in support of this concept comes from a study by Chang et al., who cultured fibroblasts in either 0.1% serum (as close an approximation to plasma as is possible in tissue culture) or the usual 10% serum. The authors found that a set of 677 genes were differentially expressed under these conditions; furthermore, they found that the gene pattern expressed by fibroblasts cultured in 10% serum was also found in malignant tumors where it correlated with a worse prognosis. These results provided additional evidence for an association between tumor stroma generation and wound healing.

Of course, there are also important differences between tumors and wounds. Platelets, which play critical roles in wound healing, seem not to participate to any great extent in the stroma formation of solid tumors, although they do contribute to the predilection of cancer patients to vascular thrombosis. However, tumor cells can subsume many platelet functions, for example, initiation of clotting and growth factor expression. Tumors also differ from healing wounds in another important respect. At wound sites, overexpression of VEGF-A and consequent vascular hyperpermeability are limited to a few days, that is, until newly formed blood vessels relieve hypoxia and turn VEGF off. In contrast, VEGF-A expression and therefore continued vascular hyperpermeability and angiogenesis persist indefinitely in tumors. Factors in addition to hypoxia and low pH stimulate VEGF expression in tumor cells. These include growth factors (e.g., epidermal growth factor, basic fibroblast growth factor), certain hormones (e.g., thyroglobulin), oncoproteins (e.g., src, ras), and tumor suppressor genes (e.g., von Hippel-Lindau protein) (reviewed by Dvorak).

**TUMOR ANGIOGENESIS**

Although others had previously proposed similar ideas, much credit is due the late Judah Folkman for bringing together and championing the importance of angiogenesis in tumor biology and therapy. In a highly cited 1971 *New England Journal of Medicine* article, Folkman proposed 3 basic postulates: (1) tumors must induce new blood vessels to grow beyond minimal size, and they do so by secreting the same TAF, that is, VEGF. Subsequent work has better characterized tumor blood vessels and determined that most are in fact structurally abnormal (Table 1, Fig. 2). Finally, therapy targeting VEGF and its receptors has found a place in treating human cancer. Unfortunately, however, anti-VEGF/VEGFR receptor therapy has not been the cure-all that was hoped for. Given the as yet unfulfilled promise of antiangiogenesis therapy, it is useful to inquire into the nature of tumor blood vessels in order to understand why anti-VEGF/VEGFR receptor therapy does not work better.

**TUMOR BLOOD VESSELS AND THE STEPS BY WHICH THEY FORM**

The pathological angiogenesis induced by tumors, such as that of healing wounds, is a much cruder process than the finely tuned angiogenesis of normal development and results from the unbalanced secretion of a small subset of growth factors, particularly VEGF. Nonetheless, tumor blood vessels are remarkably heterogeneous. We have recently classified them into 6 distinct types (Table 1, Fig. 2); all but tumor capillaries are highly abnormal in structure. Furthermore, tumor blood vessels arise not only by angiogenesis (from preexisting small vessels, i.e., capillaries and venules) but also by arteriovenogenesis (from preexisting normal arteries and veins). Vascularly generally represents only a small fraction of the tumor mass and, at any one time, includes a mixture of blood vessel types, making their formation difficult to study. Therefore, we developed a reductionist model, based on the generally accepted belief that tumors induce new blood vessels by overexpressing VEGF. We used an adenovirus-expressing murine VEGF-A, the most common VEGF-A isoform, to deliver VEGF locally to the tissues of immunocompetent nude mice, and were able to...
induce large numbers of each of the 6 types of tumor blood vessels in the absence of tumor cells.\textsuperscript{10,11,26,59,63} Because adenoviral vectors are not integrated into the genome, their encoded proteins are expressed for only a limited period. As a result, VEGF levels fall exponentially to ineffectual levels within a few days. Therefore, in contrast to tumors in which VEGF levels remain elevated indefinitely, Ad–VEGF-A\textsubscript{164} delivers a single, self-limited pulse of VEGF. This has allowed us to elucidate some of the steps and mechanisms by which VEGF induces new blood vessel formation and furthermore to isolate surrogate forms of the different tumor blood vessel types in nearly pure form for molecular characterization.\textsuperscript{64,65}

Mother vessels (MVs) are the first new angiogenic vessel type to form, both in tumors\textsuperscript{66} and in response to Ad–VEGF-A\textsubscript{164} (Fig. 1, Table 1). Mother vessels are large vascular channels lined only by greatly thinned endothelial cells and are the principal blood vessel subset that is hyperpermeable in tumors.\textsuperscript{67} Mother vessels derive from preexisting normal venules and capillaries by a 3-step process that involves proteolytic degradation of basement membranes, pericyte detachment, and 4- to 5-fold vessel enlargement. Basement membrane degradation is a crucial first step because basement membranes are rigid structures that limit vessel expansion\textsuperscript{68} and provide a foothold for pericytes. In mouse tumor models, and following injection of Ad–VEGF-A\textsubscript{164}, basement membrane degradation is accomplished by increased local expression of cathespins proteases, along with a parallel decreased expression of cysteine protease inhibitors, the natural cathespin inhibitors.\textsuperscript{69} Lacking basement membrane and pericyte support, intravascular hydrostatic pressure drives vascular expansion, causing previously cuboidal venular endothelial cells to undergo extensive thinning. This endothelial cell thinning requires a significant increase in plasma membrane that is accommodated by transfer of vesiculovacuolar organelle membranes to the cell surface. Vesiculovacuolar organelles are an extensive plexus of interconnected cytoplasmic vesicles and vacuoles whose membranes collectively constitute a surface area several times that of the venular plasma membrane.\textsuperscript{70} Vesiculovacuolar organelles also have a role in the vascular hyperpermeability induced by VEGF and other vascular permeabilizing agents\textsuperscript{63} (Fig. 1).

Lacking basement membrane and pericyte support, MVs are unstable and differentiate into several types of daughter vessels (Figs. 2 and 3; Table 1). The first of these are glomeruloid microvascular proliferations (GMPs), structures that resemble renal glomeruli (hence the name). Glomeruloid microvascular proliferations result from MV collapse, accompanied by accumulation of pericytes and macrophages and extensive synthesis of new basement membrane.\textsuperscript{59,71,72} They are found in many human cancers but are particularly abundant in glioblastoma multiforme.\textsuperscript{73} Over time, GMPs devolve into normal-appearing capillaries (Fig. 3).

Mother vessels can also differentiate into vascular malformations (VMs), stable vessels that retain their large size by acquiring a coat of smooth muscle cells and perivascular collagen. Vascular malformations can be distinguished from feeding arteries (FAs) by their thinner and often asymmetric muscular coat and from draining veins (DVs) by their smaller lumens. Vascular malformations resemble the nonmalignant VMs that are found in skin, brain, and so on, perhaps suggesting a mechanism by which VMs form in those circumstances. As their structure implies, VMs are not hyperpermeable to plasma proteins. Also unlike MVs and GMPs, VMs persist indefinitely, long after adenoviral vector-induced VEGF-A\textsubscript{164} expression has ceased. Thus, VMs have attained independence from exogenous VEGF, although it is likely that their endothelial cells are supported by paracrine VEGF secretion from the smooth muscle cells that envelop them.

Feeding arteries and DVs are also present in all solid tumors thus far examined. Feeding arteries and DVs develop in parallel with angiogenesis, but it is unclear whether they form in response to the needs of angiogenic blood vessels or form independent of angiogenesis in direct response to VEGF. Once formed, FAs and

\begin{figure}[h]
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\caption{Abnormal blood vessels induced by tumors and Ad–VEGF-A\textsubscript{164}. A, Typical MV with detached pericytes and degraded basement membrane. B, Glomeruloid microvascular proliferation with 1 large and several small lumens. Republished from Pettersson et al.\textsuperscript{59} with permission. C, Vascular malformations. D, Feeding artery and DVs. Scale bars, 20 μm.}
\end{figure}
DVs, like VMs, persist indefinitely, even though the angiogenic vessels they had supplied had long since regressed.

### WHY DOES NOT ANTI-VEGF/VEGFR THERAPY WORK BETTER IN HUMAN CANCER?

At present, antiangiogenesis therapy is essentially anti-VEGF/VEGFR therapy and, although highly effective in treating many mouse cancers, has not as yet fulfilled its promise in the clinic. There are many possible reasons for the lack of greater success. One explanation is that cancer patients are often elderly and sick, whereas the mice used in experimental studies are generally young and healthy. Another is that Bv8 and IL-17–expressing T\textsubscript{H} stromal cells impede the response to treatment.\textsuperscript{74} Furthermore, because anti-VEGF/VEGFR therapy is only partially successful, it leaves behind a population of ischemic tumor and stromal cells that respond to hypoxia by up-regulating the synthesis not only of VEGF but also of other growth factors.\textsuperscript{75} These growth factors then stimulate tumor growth as well as new blood vessel formation and so counter antiangiogenic drug therapy. Of course, this criticism also applies to radiation and chemotherapy, which can also leave behind residual ischemic tumor cells. The elusive goal remains that of killing all tumor cells.

Three additional possibilities deserve consideration to explain the limited success of anti-VEGF/VEGFR therapy. One possibility is that some tumors live on the edge and are able to tolerate an inadequate blood supply. A second possibility is that some tumors do not need to induce new blood vessels because they co-opt preexisting normal blood vessels.\textsuperscript{76,78} We have found this to be the case in mouse tumors growing in the lung. Primary subcutaneous B16 melanomas induce an abundant, MV-rich vasculature; however, when metastatic to the lung, they grow to large size without generating new blood vessels (unpublished data). This is perhaps not surprising in that the lung provides an oxygen-rich environment with an abundant supply of capillaries capably designed for efficient gas and nutrient exchange.

A third possibility is that anti-VEGF/VEGFR therapy targets only some tumor blood vessels. As was noted above, tumor blood vessels are heterogeneous, of at least 6 distinct types. Furthermore, VEGF-induced blood vessels develop linearly over time, and “early” vessels (VMs, GMPs) evolve into “late” vessels (VMs, capillaries, FAs, DVs) (Fig. 3). In mice, anti-VEGF/VEGFR receptor therapy effectively prevents the growth of freshly transplanted tumors\textsuperscript{58} that are populated primarily by early blood vessels, that is, MV, and FA, and DV precursors.\textsuperscript{69,79,80} but is much less effective against tumors with an established vasculature rich in “late” blood vessels.\textsuperscript{81} Human cancers, of course, have grown for many months or years before detection and are therefore presumably supplied by many late blood vessels.\textsuperscript{9–11} Together, these findings suggest the hypothesis that early blood vessels may be VEGF-dependent, whereas late vessels, although initially induced by VEGF, have become VEGF-independent and therefore are no longer susceptible to anti-VEGF/VEGFR therapy.\textsuperscript{82,83} Consistent with this hypothesis, early formed MVs and GMPs highly express VEGFR-2 (the receptor through which VEGF triggers angiogenesis), whereas VMs, FAs, and DVs do not.\textsuperscript{82} Vessels expressing high levels of VEGFR2 might be expected to be more susceptible to anti-VEGF/VEGFR attack than vessels that express VEGFR2 at low levels.

To test this hypothesis more rigorously, we made use of our reductionist model, administering drugs at early and successively later times after injection of Ad–VEGF-A\textsuperscript{164} into the skin of nude mice. The results with rapamycin,\textsuperscript{84} a drug active downstream of the VEGF–VEGFR-2 pathway, and with aflibercept and other drugs targeting VEGF or its receptors, have been consistent.\textsuperscript{82,83} All of these drugs prevented the formation of MVs, the majority “early”-type blood vessel found in freshly transplanted mouse tumors; they also caused the regression of already formed MVs and GMPs when administered a few days later. However, their antivascular activities were progressively less effective when treatment was delayed further. None had a significant effect on VMs, FAs, or DVs. These findings, then, are consistent with the limited success that anti-VEGF/VEGFR therapy has against human tumors; such therapy may be expected to prevent further angiogenesis and prune early tumor vessels (MV, GMPs) but have little or no effect on vessels of the late type. They are also supportive of the work of Carmeliet and Jain\textsuperscript{7,59} and Goel and colleagues,\textsuperscript{85,86} who demonstrated that anti-VEGF therapy causes tumor blood vessel “normalization.” Jain and colleagues found that anti-VEGF/VEGFR therapy reduces the hyperpermeability characteristic of tumor blood vessels, along with the consequent resulting edema and interstitial pressure. This result would be expected if these drugs were primarily targeting MVs, the hyperpermeable tumor blood vessel subset.

### SUMMARY AND FUTURE PROSPECTS

As Folkman\textsuperscript{52} foresaw many years ago, most tumors require a vascularized stroma if they are to grow beyond minimal size.
The concept of treating tumors by attacking their blood supply is attractive, and anti-VEGF/VEGFR therapy is effective in treating many animal tumors. Used alone, anti-VEGFR therapy is useful in treating patients with renal cell carcinoma and glioblastoma multiforme; it also provides benefit in patients with other solid tumors when combined with chemotherapy. An important limitation of current antiangiogenesis therapy is that it is VEGF/VEGFR centric. Tumor and stromal cells make other angiogenic factors besides VEGF, and targeting them could be beneficial. Also, it would be desirable to find new molecular targets on tumor blood vessels. One recently described candidate is TM4SF1, a tetraspanin-like molecule that is overexpressed by tumor cells and selectively by tumor blood vessel endothelial cells.64,65

Finally, the tumor vasculature is heterogeneous, consisting of at least 6 distinct blood vessel types. Although all 6 types are induced by VEGF, many, including VMs, FAs, and DVs, have lost their dependency on VEGF and therefore are not susceptible to anti-VEGF/VEGFR therapy. Feeding arteries would be ideal targets because they are relatively few and provide the blood supply to all of the other 5 vessel types. By analogy, plumbers find it more efficient to shut off the water supply at its entry into the house, rather than turning off the faucets in each room. That this approach may have potential comes from studies using photodynamic therapy to occlude the FAs supplying mouse ear tumors87 and also from tumor artery embolization (myolysis), an approach that is being used to ablate uterine fibroids.88 These examples, of course, are special cases, and it remains to be determined whether FAs can be successfully targeted in other cancers.

REFERENCES


