Published in final edited form as:

Semin Thromb Hemost. 2010 April ; 36(3): 321–331. doi:10.1055/s-0030-1253454.

Heterogeneity of the Tumor Vasculature

Janice A. Nagy, Ph.D.¹, Sung-Hee Chang, Ph.D.^{1,2}, Shou-Ching Shih, Ph.D.¹, Ann M. Dvorak, M.D.¹, and Harold F. Dvorak, M.D.¹

¹Center for Vascular Biology Research, Beth Israel Deaconess Medical Center and the Departments of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School

²Department of Pathology and Laboratory Medicine, Weil Cornell Medical School, New York, New York

Abstract

The blood vessels supplying tumors are strikingly heterogeneous and differ from their normal counterparts with respect to organization, structure, and function. Six distinctly different tumor vessel types have been identified, and much has been learned about the steps and mechanisms by which they form. Four of the six vessel types (mother vessels, capillaries, glomeruloid microvascular proliferations, and vascular malformations) develop from preexisting normal venules and capillaries by angiogenesis. The two remaining vessel types (feeder arteries and draining veins) develop from arterio-venogenesis, a parallel, poorly understood process that involves the remodeling of preexisting arteries and veins. All six of these tumor vessel types can be induced to form sequentially in normal mouse tissues by an adenoviral vector expressing vascular endothelial growth factor (VEGF)-A¹⁶⁴. Current antiangiogenic cancer therapies directed at VEGF-A or its receptors have been of only limited benefit to cancer patients, perhaps because they target only the endothelial cells of the tumor blood vessel subset that requires exogenous VEGF-A for maintenance. A goal of future work is to identify therapeutic targets on tumor blood vessel endothelial cells that have lost this requirement.

Keywords

Angiogenesis; arterio-venogenesis; tumors; vascular endothelial growth factor; VEGF-A

Normal tissues require an extensive vascular network for supply of nutrients and clearance of waste products. Not surprisingly, tumors have similar requirements. Some tumors satisfy these needs, in whole or in part, by coopting the normal vasculature.^{1,2} Cooption is dramatically illustrated in some forms of a highly malignant brain tumor, glioblastoma multiforme, in which tumor cells form cuffs that envelop normal brain blood vessels.³ For the most part, however, tumors must generate new blood vessels if they are to grow beyond minimal size.⁴ This article considers the structural and functional properties of the new blood vessels that tumors induce, reviews what is known about the mechanisms by which they form, and evaluates the potential of tumor blood vessels as therapeutic targets.

Copyright © 2010 by Thieme Medical Publishers, Inc.

Address for correspondence and reprint requests: Harold F. Dvorak, M.D., Department of Pathology, Beth Israel Deaconess Medical Center, 330 Brookline Avenue, RN227c, Boston, MA 02215 (hdvorak@bidmc.harvard.edu).

Under-Recognized Significance of Endothelial Heterogeneity: Hemostasis, Thrombosis, and Beyond; Guest Editors, William Aird, M.D., and Hau C. Kwaan, M.D., Ph.D.

Semin Thromb Hemost 2010;36:321–331. Copyright © 2010 by Thieme Medical Publishers, Inc., 333 Seventh Avenue, New York, NY 10001, USA. Tel: +1(212) 584-4662.

PROPERTIES OF TUMOR BLOOD VESSELS

The normal vasculature is arranged in a hierarchy of evenly spaced, well-differentiated arteries, arterioles, capillaries, venules, and veins. In contrast, the tumor vasculature is composed of a chaotic mixture of abnormal, hierarchically disorganized vessels that differ from those of normal tissues with respect to organization, structure, and function.⁵ Unlike normal blood vessels, those supplying tumors commonly follow a serpentine course, branch irregularly, and, on average, are larger than their normal counterparts; this results in an altered surface area to volume ratio that impairs tissue nutrition. As a result, and also because of arteriovenous shunts, nutrients are not taken up efficiently by tumors, as is demonstrated by the higher than normal oxygen content of the venous blood draining tumors. Poor clearance of carbon dioxide and other metabolites, coupled with high tumor cell glycolytic activity, results in a tumor microenvironment that is acidic, as compared with normal tissues (pH ~7.2 versus pH ~7.4).⁶ Not unexpectedly, this combination of events leads to zones of metabolic insufficiency, ischemia, and necrosis. As a consequence of ischemia, the transcription factor HIF-1 is stabilized, an event with important consequences for new blood vessel formation.⁷ Another long-recognized property of tumor blood vessels is that they are hyperpermeable to plasma and plasma proteins, although hyperpermeability is confined to only a subset of tumor blood vessels (Table 1).⁸⁻¹⁰ Vascular hyperpermeability leads to local edema and extravascular clotting of plasma. As a consequence, a cross-linked fibrin gel is deposited; this fibrin gel serves as a provisional matrix for cell migration, regulates endothelial cell gene expression, and stimulates angiogenesis and subsequent stroma formation.^{10,11} Also, clotting converts plasma to serum, a fluid not normally present in tissues that extensively alters the gene expression pattern of fibroblasts¹² and likely of endothelial cells as well.

Although it has been recognized for many years that tumor blood vessels are heterogeneous and not of a single type, few attempts have been made to define their individual properties. Warren, writing in the late 1970s, classified tumor blood vessels into eight distinct types, not all of which were found in any one tumor.⁵ He concluded that the main difference between normal and tumor blood vessels was that, in the latter, capillaries and veins became tortuous and “dilated.” The notion of tumor vessel “dilation” has unfortunately persisted widely in the literature, but it is actually a misnomer that inaccurately describes the mechanism by which tumor vessels increase in size. Vascular dilation is correctly understood as the vessel enlargement that results from the relaxation of vascular smooth muscle cells, as, for example, in response to nitric oxide or sympathetic innervation. In contrast, the greatly enlarged vessels found in tumors do not result from smooth muscle cell relaxation but rather from the extensive remodeling of preexisting smaller blood vessels.

Tumor blood vessels are stimulated to form by growth factors and cytokines that are secreted primarily by tumor cells, as well as by stromal cells present in the tumor microenvironment. Although many endothelial cell mitogens have been identified, it is now generally agreed that vascular permeability factor/vascular endothelial growth factor (VPF/VEGF, VEGF-A), and particularly its 164 (mouse; VEGF-A¹⁶⁴)/165 (human; VEGF-A¹⁶⁵) isoform (VEGF-A¹⁶⁴), is the most important of these. VEGF-A, and its endothelial cell receptors, are overexpressed in nearly all malignant tumors,¹⁰ and often in premalignant tumors prior to invasion.^{13,14} VEGF-A expression can be induced in tumor cells by a variety of mechanisms (e.g., activation of oncogenes, loss of tumor suppressor genes, hormones, other cytokines, etc.), and, importantly, by the transcription factor HIF-1, a gene product that is, as we already noted, activated by the ischemia commonly found in tumors.⁷ Further evidence for the importance of VEGF-A comes from the demonstration that anti-VEGF-A antibodies, as well as drugs that target VEGF-A or its receptors (VEGFR), can strongly inhibit tumor growth in mice, although they are generally less effective in cancer

patients.¹⁵⁻¹⁷ We have identified six structurally and functionally distinct blood vessel types in human cancers, and surrogates of each of these can be induced in mice with an adenoviral vector engineered to express VEGF-A¹⁶⁴ (Ad-VEGF-A¹⁶⁴) (Table 1, Fig. 1).^{8,10,18,19}

INDUCTION OF TUMOR SURROGATE BLOOD VESSELS WITH Ad-VEGF-A¹⁶⁴

Ad-VEGF-A¹⁶⁴ has served as a useful tool for investigating the steps and mechanisms by which tumor blood vessels form. The only restriction is that studies must be performed in immunosuppressed animals (e.g., nude or SCID mice) because adenoviral vectors are highly immunogenic and generate a strong inflammatory response that overwhelms the neovascular response. In immunosuppressed mice, Ad-VEGF-A¹⁶⁴ induces the formation of each of the six types of blood vessels recognized in human cancers, although it does not exactly replicate the tumor vascular mix. In tumors, VEGF-A is produced continuously at high concentrations over long periods of time, thereby generating a vasculature that at any one time is composed of a mixture of many different vessel types. Some of these are newly formed, whereas others have been present for a long time. Adenoviral vectors, in contrast, are not integrated into cellular DNA, and their encoded proteins are expressed for only a short time. Therefore, Ad-VEGF-A¹⁶⁴ generates in mouse tissues only a short-lived pulse of VEGF-A¹⁶⁴ protein that at the doses of virus used is similar initially to those that are constantly maintained in tumors (Fig. 2). However, in contrast to tumors, the level of VEGF-A¹⁶⁴ plunges rapidly and by a month has fallen to near control levels (Fig. 2). The result is a single, self-limited burst of new blood vessel formation that proceeds in a highly reproducible sequence. As a result, vessels of each type develop in large numbers at characteristic times after Ad-VEGF-A¹⁶⁴ injection and can be collected in fairly pure form for molecular analysis. A further consequence of the self-limited VEGF-A¹⁶⁴ expression induced by Ad-VEGF-A¹⁶⁴ is that it has allowed investigation of the effects that cessation of exogenous VEGF-A¹⁶⁴ expression has on each of the different types of surrogate tumor blood vessels. As will be seen, some of the new blood vessels induced by Ad-VEGF-A¹⁶⁴ require continuous VEGF-A¹⁶⁴ expression for their maintenance and undergo apoptosis when VEGF-A levels fall below threshold levels.²⁰ Others, however, once induced by VEGF-A¹⁶⁴, persist indefinitely in the absence of exogenous VEGF-A¹⁶⁴ and therefore have lost their dependency on exogenous VEGF-A¹⁶⁴. This finding has obvious consequences for tumor therapies that target VEGF-A or its receptors. Ad-VEGF-A¹⁶⁴ also induces the formation of abnormal “giant” lymphatic vessels that also acquire VEGF-A¹⁶⁴ independency and persist indefinitely after exogenous VEGF-A¹⁶⁴ levels have fallen to baseline levels.^{21,22} The extent to which new lymphatics are induced in tumors and their importance for tumor metastasis is a subject of considerable current interest²³⁻²⁶ but is beyond the scope of this review.

Ad-VEGF-A¹⁶⁴ induces the different types of tumor vessel surrogates by two parallel processes, angiogenesis and arterio-venogenesis (Table 1, Figs. 1-5).

ANGIOGENESIS

Angiogenesis refers to the generation of new blood vessels from preexisting microvessels. Tumor angiogenesis is thought to involve the unbalanced overexpression of a small set of growth factors, particularly VEGF-A^{164/5}.^{10,19,27} In growing tumors and in response to Ad-VEGF-A¹⁶⁴, mother vessels (MV) are the first new vessel type to form. MV are hyperpermeable blood vessels (Fig. 2B) and subsequently evolve into one of three different types of “daughter” vessels: capillaries, glomeruloid microvascular proliferations (GMP), and vascular malformations (VM) (Fig. 3).

Mother Vessels

The term *mother vessel* was coined by Paku and Paweletz to describe the first type of new blood vessel induced by experimental tumors.²⁸ MV (Table 1, Fig. 1A) are also the first new blood vessel type to form in response to Ad-VEGF-A¹⁶⁴, as well as in healing wounds, and, most probably, in chronic inflammation.^{18,29-31} MV begin to develop from preexisting venules (and capillaries) within hours after injection of Ad-VEGF-A¹⁶⁴ or tumor cells into mice. Initially, there is little vascular cell division, but, by 3 to 5 days, $\geq 90\%$ of MV endothelial cells incorporated ³H thymidine-positive (unpublished data). This is a substantially higher level of cell division than has been found in tumor blood vessels. For example, Denekamp and Hobson reported a range of vascular labeling of 3.6 to 32.3% in studies of 131 different tumors.³² The explanation for this difference probably lies in the fact that tumor blood vessels are heterogeneous and that only some, predominantly MV and GMP, exhibit active cell division; MV and GMP generally account for only a small fraction of the blood vessels found in tumors that have been growing for a long time.

The mechanisms by which MV form has recently been elucidated and involves a three-step process of vascular basement membrane (VBM) degradation, pericyte detachment, and vascular expansion.

Vascular Basement Membrane Degradation and Pericyte Detachment

VBM degradation is the essential first step in MV formation. VBM are noncompliant (nonelastic) structures that limit microvessel expansion to $\sim 30\%$.³³ In contrast, MV acquire a cross-sectional area that is three- to fivefold greater than that of the venules and capillaries from which they arise. It follows, therefore, that VBM must be degraded to allow the vascular expansion characteristic of MV formation. Recent data have shown that two essential VBM components, collagen IV and laminin $\beta 1$ chain, are degraded in developing MV in both tumors and at Ad-VEGF-A¹⁶⁴ injection sites (Figs. 4A and B).³⁴ To our surprise, matrix metalloproteases 2 and 9, proteases that have frequently been implicated in tumor angiogenesis, were apparently not involved in this process. Instead, cathepsin (B > S > L) expression and activity increased substantially in parallel with MV formation (Fig. 4C). GB123, a fluorescent probe that binds tightly to the active sites of cysteine proteases, allowed sites of cysteine protease activity to be localized precisely to the pericytes of developing MV (Fig. 4D), indicating that pericytes were the source of the cathepsin proteases that digested VBM. Additional evidence pointing to the importance of cathepsins was the finding that Ad-VEGF-A¹⁶⁴-induced angiogenesis was strikingly reduced in cathepsin B-null mice.³⁴

Cathepsin activity does not proceed unopposed and in normal tissues is held in balance by a family of small (11 to 13 kDa) proteins called cysteine protease inhibitors (CPI). CPI include stefin A (cystatin A) and cystatins B and C. All three are high affinity competitive inhibitors of cysteine proteases that are expressed in normal blood vessels and have been implicated in tumor progression.³⁵ Expression of all three CPIs was strikingly reduced in developing MV in Ad-VEGF-A¹⁶⁴-injected sites (Fig. 4E). Increased cathepsin B and decreased cystatins were also found in developing tumor MV (Fig. 4F).³⁴

Taken together, these data indicate that VEGF-A¹⁶⁴ initiates MV formation by upsetting the local cathepsin/CPI balance. Increased pericyte cathepsins, relieved of CPI inhibition, degrade VBM, causing pericytes to disassociate and leaving behind developing MV that are composed almost entirely of endothelial cells.

Vascular Enlargement

Vascular enlargement is the final step in MV formation and results in a three- to fivefold increase in vascular lumen cross-sectional area (Fig. 5). Presumably this increase in size is driven by centripetal vascular pressure on vessels that have lost the constraints normally imposed by VBM and pericytes. To accommodate this increase in luminal size, MV endothelial cells thin and expand to cover a greatly enlarged surface area. This endothelial cell thinning and expansion requires increased plasma membrane. Although some new membrane may derive from new synthesis, a significant amount comes from the transfer to the cell surface of membrane that is normally stored internally in venular endothelial cells in the form of vesiculo-vacuolar organelles (VVOs) (Fig. 5). VVOs are “bunch of grapelike” clusters of interconnected, uncoated vesicles and vacuoles whose membrane content in normal venular endothelium is equivalent to more than twice that of the endothelial cell plasma membrane (Fig. 5).^{8,36,37} VVOs were originally discovered as a pathway for transcellular extravasation of plasma from tumor vessels and normal venules rendered permeable by VEGF-A and other vascular permeability factors.³⁶⁻³⁹ They have now been found to have a second function,^{8,19} that of contributing their membrane to the cell surface to provide the additional plasma membrane required to cover an enlarged surface area.

Daughter Vessels

MV are transient structures that do not persist long before evolving into one of three different types of “daughter” vessels: capillaries, GMP, and VM (Figs. 1 and 3).^{8,10,18-20} Daughter vessels begin to form ~7 days after Ad-VEGF-A¹⁶⁴ injection by mechanisms that are at present understood only at the descriptive level; further, the mechanisms by which individual MV choose to evolve into each of the three different daughter vessel types is not understood at any level.

Capillaries

One mechanism by which mother vessels evolve into capillaries (Table 1, Fig. 1B) involves intraluminal bridging, a process that was originally discovered in tumor vessels⁴⁰ and was subsequently found to occur in exercised skeletal muscle⁴¹ and in healing wounds (myocardial infarcts³⁰). Endothelial cells project cytoplasmic processes into and across MV lumens, forming transluminal bridges that divide blood flow into multiple smaller channels (Fig. 1B). This process differs from intussusception in that the intraluminal projections are composed entirely of endothelial cell processes and do not include surrounding cells or stroma. The mechanisms by which these endothelial cell projections proceed to divide MV into capillaries are unknown. The capillaries that form in this manner, as well as those that result from the devolution of GMP, are apparently normal in structure and function and persist for a considerable time after tissue VEGF-A¹⁶⁴ levels fall toward normal.

Glomeruloid Microvascular Proliferations

GMP, also referred to as glomeruloid bodies (Table 1, Fig. 1C), are poorly organized vascular structures that are so named because of their macroscopic resemblance to renal glomeruli.^{18,20} They have long been known to be present in a variety of human tumors, particularly glioblastoma multiforme, but also in cancers of the breast where their presence has been associated with an unfavorable prognosis.^{42,43} As far as is known, all of the human tumors in which GMP are found express VEGF-A; further, tumors such as glioblastoma multiforme that make unusually large amounts of VEGF-A are among those that most commonly induce GMP. Like MV, GMP are permeable to plasma and plasma proteins.

In response to Ad-VEGF-A¹⁶⁴, nascent GMP first appear as focal accumulations of large poorly differentiated CD31- and VEGFR-2-positive cells in the endothelial lining of

MV.^{18,20} The source of these cells, whether from local MV endothelium or from circulating progenitor cells, is not known. Whatever their source, these cells proliferate rapidly, extending inwardly into MV lumens, and also outwardly into the surrounding extravascular matrix. They thus encroach upon and compress the MV from which they arose, eventually dividing single large MV lumens into multiple, much smaller channels. Initially, the great majority of cells comprising GMP continue to express endothelial cell markers. Subsequently, however, cells expressing pericyte and macrophage markers and ultrastructural characteristics also appear.²⁰ An additional prominent feature is deposition of abundant abnormal, multilayered VBM. Like MV, GMP require the continued presence of exogenous VEGF-A¹⁶⁴ for their maintenance. As adenoviral vector-derived VEGF-A¹⁶⁴ expression declines (Fig. 2), GMP undergo apoptosis or devolve into normal-appearing capillaries.²⁰

Vascular Malformations

VM, the third type of daughter vessel (Table 1, Fig. 1D), form from MV by acquiring a stabilizing coat of smooth muscle cells.¹⁸ The source of these smooth muscle cells is unknown, although reattachment of previously detached pericytes could offer a partial explanation. VM are readily distinguished from normal arteries and veins by their inappropriately large size (for their location) and by their thinner and often asymmetrical muscle coat. Vessels of this description closely resemble certain of the benign vascular malformations that occur in brain and other tissues,⁴⁴ suggesting a mechanism by which such malformations form. As their structure implies, VM are not permeable to circulating macromolecules. Also unlike MV and GMP, VM persist indefinitely, long after adenoviral vector-induced VEGF-A¹⁶⁴ expression has ceased. Thus VM have attained independence from exogenous VEGF-A¹⁶⁴, although it is quite possible that they are maintained by VEGF-A secreted locally by the smooth muscle cells that closely envelop them. This independence from high levels of exogenous VEGF-A has important implications because tumor VM would not be expected to be susceptible to therapies that target VEGF-A.

ARTERIO-VENOGENESIS: THE FORMATION OF FEEDER ARTERIES AND DRAINING VEINS

As described earlier, tumors and tissue sites injected with Ad-VEGF-A¹⁶⁴ form a localized, highly vascularized zone of angiogenesis. However, immediately external to zones of angiogenesis are found a relatively small number of greatly enlarged, radially extending feeder arteries (FA) and draining veins (DV) (Table 1, Figs. 1E and 6). These FA and DV supply and drain angiogenic sites, whether induced by tumors or by Ad-VEGF-A (Fig. 6).^{22,27,45,46} Arteriovenogenesis proceeds in parallel with angiogenesis, but almost nothing is known about the mechanisms involved. FA and DV would seem to offer an attractive therapeutic target, if only based on plumbing principles. Compared with angiogenic blood vessels, they are relatively few in number and are upstream and downstream, respectively, of the tumor mass and its associated angiogenic blood vessels. Therefore, their ablation would be expected to cut off the tumor blood supply more efficiently than strategies aimed at angiogenic blood vessels within the tumor proper. FA and DV have long been appreciated by ophthalmologists, who have observed them as vessels that supply and drain retinal tumors.⁴⁷⁻⁴⁹ However, FA and DV are by no means exclusive to the eye and have been found in all tumors that have been appropriately examined.

VASCULAR MIMICRY

In some tumors, particularly ocular melanomas, spaces filled with red blood cells are found that are lined by tumor cells rather than endothelial cells.^{50,51} Such spaces have been

referred to as “vascular mimicry” (Table 1, Fig. 1F). The tumor cells lining such spaces are reported to have acquired some of the molecular properties of endothelial cells.⁵² That red blood cell-filled spaces exist in certain tumors is not in question, but their significance is much debated.⁵³ The issue hangs on whether these spaces are part of a functional vascular network or whether they simply reflect sites of hemorrhage into tumors from leaky or damaged but otherwise conventional blood vessels.

THE POTENTIAL OF TUMOR VESSEL ENDOTHELIAL CELLS AS THERAPEUTIC TARGETS

The concept of antiangiogenesis as an approach to tumor therapy has a long history and was brilliantly formulated by the late Judah Folkman.⁴ Hopes for this approach were encouraged by the success that anti-VEGF-A antibodies, as well as drugs targeting VEGF-A or its receptors (VEGFR), had on inhibiting the growth of many rodent tumors.^{54,55} However, anti-VEGF-A/VEGFR therapy has proved to be less successful in cancer patients.¹⁵⁻¹⁷ There are many possible reasons for this; for example, cancer patients are older and sicker than the young healthy mice used in laboratory experiments, humans as a species may be more susceptible to side effects of antiangiogenic drugs than rodents, tumor vessels may undergo “normalization” in response to therapy,⁵⁶ and so on. However, another possibility is suggested by the finding that only a subset of Ad-VEGF-A¹⁶⁴-induced surrogate tumor blood vessels (MV, GMP) require VEGF-A for their maintenance, whereas others (VM, FA, DV, and likely capillaries) have acquired VEGF-A independence. Moreover, these VEGF-A independent vessels are long lasting and are likely to become the predominant vessel type in tumors that have been growing for any length of time. Human tumors, of course, develop over a period of many months or years prior to diagnosis and treatment. Therefore, the subset of vessels in human tumors that depends on VEGF-A and is therefore vulnerable to anti-VEGF-A/VEGFR therapy may be considerably smaller than in rodent tumors that are treated shortly after transplant.

In fact, the limitations of anti-VEGF/VEGFR strategies should have come as no surprise. Bergers et al found that mouse tumors responded effectively to anti-VEGF-A/VEGFR treatment at early intervals after transplant, whereas tumors that had been growing for some time responded poorly to such treatment.⁵⁷ We recently demonstrated that therapy with rapamycin also targets tumor blood vessels differentially. Rapamycin prevented the formation of both MV and GMP in response to Ad-VEGF-A¹⁶⁴, but VM, once formed, were not affected.⁵⁸

CONCLUSION

Together the findings detailed here indicate that if therapies targeting tumor blood vessels are to be more successful, they will have to attack not only MV and VM but also later angiogenic vessels (capillaries, VM), as well as the FA and DV that result from arteriovenogenesis. To do so will require that new targets be identified on these several vessel types. An extensive search for new tumor vessel endothelial cell antigens has already begun, making use of several techniques, including direct isolation of such antigens from human tumors.⁵⁹ We have taken another approach, isolating tumor surrogate blood vessels at late times from tissue sites injected with Ad-VEGF-A¹⁶⁴. This approach has allowed us to identify several genes that are highly overexpressed on late tumor blood vessels. One of these is transmembrane -4-L-six-family -1 (TM4SF1), a tetraspanin-like protein that is highly overexpressed in the endothelial cells of VM found in Ad-VEGF-A¹⁶⁴ injected sites and also in a variety of common human cancers.⁶⁰ Knockdown of this protein caused cultured endothelial cells to undergo senescence, and, in vivo, prevented MV differentiation, making TM4SF1 a potentially attractive therapeutic target. We expect this general approach

to identify additional potential target gene products on late tumor and tumor-surrogate blood vessels.

Acknowledgments

This work was supported by supported in part by U.S. Public Health Service grants HL-64402 and P01 CA92644, by S.D.G., and by a contract from the National Foundation for Cancer Research. Portions of this work have been published previously in modified form as indicated in the text.

REFERENCES

1. Holash J, Maisonpierre PC, Compton D, et al. Vessel cooption, regression, and growth in tumors mediated by angiopoietins and VEGF. *Science*. 1999; 284(5422):1994–1998. [PubMed: 10373119]
2. Leenders WP, Küsters B, de Waal RM. Vessel co-option: how tumors obtain blood supply in the absence of sprouting angiogenesis. *Endothelium*. 2002; 9(2):83–87. [PubMed: 12200959]
3. Du R, Lu KV, Petritsch C, et al. HIF1 α induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. *Cancer Cell*. 2008; 13(3): 206–220. [PubMed: 18328425]
4. Folkman J. Tumor angiogenesis: therapeutic implications. *N Engl J Med*. 1971; 285(21):1182–1186. [PubMed: 4938153]
5. Warren, B. The vascular morphology of tumors. In: Peterson, H-I., editor. *Tumor Blood Circulation: Angiogenesis, Vascular Morphology and Blood Flow of Experimental and Human Tumors*. CRC Press; Boca Raton, FL: 1979. p. 1-47.
6. Stubbs M, McSheehy PM, Griffiths JR, Bashford CL. Causes and consequences of tumour acidity and implications for treatment. *Mol Med Today*. 2000; 6(1):15–19. [PubMed: 10637570]
7. Semenza GL. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. *Trends Mol Med*. 2001; 7(8):345–350. [PubMed: 11516994]
8. Nagy JA, Feng D, Vasile E, et al. Permeability properties of tumor surrogate blood vessels induced by VEGF-A. *Lab Invest*. 2006; 86(8):767–780. [PubMed: 16732297]
9. Dvorak, H. Tumor blood vessels. In: Aird, W., editor. *The Endothelium: A Comprehensive Reference*. Cambridge University Press; Cambridge, United Kingdom: 2007.
10. Dvorak HF. Rous-Whipple Award Lecture. How tumors make bad blood vessels and stroma. *Am J Pathol*. 2003; 162(6):1747–1757. [PubMed: 12759232]
11. Dvorak HF. Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. *N Engl J Med*. 1986; 315(26):1650–1659. [PubMed: 3537791]
12. Chang HY, Sneddon JB, Alizadeh AA, et al. Gene expression signature of fibroblast serum response predicts human cancer progression: similarities between tumors and wounds. *PLoS Biol*. 2004; 2(2):E7. [PubMed: 14737219]
13. Brown LF, Berse B, Jackman RW, et al. Expression of vascular permeability factor (vascular endothelial growth factor) and its receptors in breast cancer. *Hum Pathol*. 1995; 26(1):86–91. [PubMed: 7821921]
14. Guidi AJ, Abu-Jawdeh G, Berse B, et al. Vascular permeability factor (vascular endothelial growth factor) expression and angiogenesis in cervical neoplasia. *J Natl Cancer Inst*. 1995; 87(16):1237–1245. [PubMed: 7563170]
15. Ellis LM, Rosen L, Gordon MS. Overview of anti-VEGF therapy and angiogenesis. Part 1: Angiogenesis inhibition in solid tumor malignancies. *Clin Adv Hematol Oncol*. 2006; 4(suppl):1–12. [PubMed: 16562372]
16. Hurwitz H, Fehrenbacher L, Novotny W, et al. Bevacizumab plus irinotecan, fluorouracil, and leucovorin for metastatic colorectal cancer. *N Engl J Med*. 2004; 350(23):2335–2342. [PubMed: 15175435]
17. Jain RK, Duda DG, Clark JW, Loeffler JS. Lessons from phase III clinical trials on anti-VEGF therapy for cancer. *Nat Clin Pract Oncol*. 2006; 3(1):24–40. [PubMed: 16407877]

18. Pettersson A, Nagy JA, Brown LF, et al. Heterogeneity of the angiogenic response induced in different normal adult tissues by vascular permeability factor/vascular endothelial growth factor. *Lab Invest.* 2000; 80(1):99–115. [PubMed: 10653008]
19. Nagy JA, Dvorak HF, Dvorak AM. VEGF-A and the induction of pathological angiogenesis. *Annu Rev Pathol.* 2007; 2:251–275. [PubMed: 18039100]
20. Sundberg C, Nagy JA, Brown LF, et al. Glomeruloid microvascular proliferation follows adenoviral vascular permeability factor/vascular endothelial growth factor-164 gene delivery. *Am J Pathol.* 2001; 158(3):1145–1160. [PubMed: 11238063]
21. Nagy JA, Vasile E, Feng D, et al. Vascular permeability factor/vascular endothelial growth factor induces lymphangiogenesis as well as angiogenesis. *J Exp Med.* 2002; 196(11):1497–1506. [PubMed: 12461084]
22. Nagy JA, Vasile E, Feng D, et al. VEGF-A induces angiogenesis, arteriogenesis, lymphangiogenesis, and vascular malformations. *Cold Spring Harb Symp Quant Biol.* 2002; 67:227–237. [PubMed: 12858545]
23. Dadras SS, Detmar M. Angiogenesis and lymphangiogenesis of skin cancers. *Hematol Oncol Clin North Am.* 2004; 18(5):1059–1070. viii. [PubMed: 15474335]
24. He Y, Karpanen T, Alitalo K. Role of lymphangiogenic factors in tumor metastasis. *Biochim Biophys Acta.* 2004; 1654(1):3–12. [PubMed: 14984763]
25. Jain RK. Angiogenesis and lymphangiogenesis in tumors: insights from intravital microscopy. *Cold Spring Harb Symp Quant Biol.* 2002; 67:239–248. [PubMed: 12858546]
26. Wirzenius M, Tammela T, Uutela M, et al. Distinct vascular endothelial growth factor signals for lymphatic vessel enlargement and sprouting. *J Exp Med.* 2007; 204(6):1431–1440. [PubMed: 17535974]
27. Nagy JA, Chang SH, Dvorak AM, Dvorak HF. Why are tumour blood vessels abnormal and why is it important to know? *Br J Cancer.* 2009; 100(6):865–869. [PubMed: 19240721]
28. Paku S, Paweletz N. First steps of tumor-related angiogenesis. *Lab Invest.* 1991; 65(3):334–346. [PubMed: 1716330]
29. Brown LF, Yeo KT, Berse B, et al. Expression of vascular permeability factor (vascular endothelial growth factor) by epidermal keratinocytes during wound healing. *J Exp Med.* 1992; 176(5):1375–1379. [PubMed: 1402682]
30. Ren G, Michael LH, Entman ML, Frangogiannis NG. Morphological characteristics of the microvasculature in healing myocardial infarcts. *J Histochem Cytochem.* 2002; 50(1):71–79. [PubMed: 11748296]
31. Secomb TW, Konerding MA, West CA, Su M, Young AJ, Mentzer SJ. Microangiectasias: structural regulators of lymphocyte transmigration. *Proc Natl Acad Sci U S A.* 2003; 100(12):7231–7234. [PubMed: 12782790]
32. Denekamp J, Hobson B. Endothelial-cell proliferation in experimental tumours. *Br J Cancer.* 1982; 46(5):711–720. [PubMed: 7171453]
33. Swayne GT, Smaje LH, Bergel DH. Distensibility of single capillaries and venules in the rat and frog mesentery. *Int J Microcirc Clin Exp.* 1989; 8(1):25–42. [PubMed: 2785976]
34. Chang SH, Kanasaki K, Gocheva V, et al. VEGF-A induces angiogenesis by perturbing the cathepsin-cysteine protease inhibitor balance in venules, causing basement membrane degradation and mother vessel formation. *Cancer Res.* 2009; 69(10):4537–4544. [PubMed: 19435903]
35. Cox JL. Cystatins and cancer. *Front Biosci.* 2009; 14:463–474. [PubMed: 19273078]
36. Dvorak AM, Kohn S, Morgan ES, Fox P, Nagy JA, Dvorak HF. The vesiculo-vacuolar organelle (VVO): a distinct endothelial cell structure that provides a transcellular pathway for macromolecular extravasation. *J Leukoc Biol.* 1996; 59(1):100–115. [PubMed: 8558058]
37. Feng D, Nagy JA, Hipp J, Dvorak HF, Dvorak AM. Vesiculovacuolar organelles and the regulation of venule permeability to macromolecules by vascular permeability factor, histamine, and serotonin. *J Exp Med.* 1996; 183(5):1981–1986. [PubMed: 8642308]
38. Feng D, Nagy JA, Hipp J, Pyne K, Dvorak HF, Dvorak AM. Reinterpretation of endothelial cell gaps induced by vasoactive mediators in guinea-pig, mouse and rat: many are transcellular pores. *J Physiol.* 1997; 504(Pt 3):747–761. [PubMed: 9401980]

39. Feng D, Nagy JA, Dvorak AM, Dvorak HF. Different pathways of macromolecule extravasation from hyperpermeable tumor vessels. *Microvasc Res.* 2000; 59(1):24–37. [PubMed: 10625568]
40. Nagy JA, Morgan ES, Herzberg KT, Manseau EJ, Dvorak AM, Dvorak HF. Pathogenesis of ascites tumor growth: angiogenesis, vascular remodeling, and stroma formation in the peritoneal lining. *Cancer Res.* 1995; 55(2):376–385. [PubMed: 7529135]
41. Egginton S, Zhou AL, Brown MD, Hudlická O. Unorthodox angiogenesis in skeletal muscle. *Cardiovasc Res.* 2001; 49(3):634–646. [PubMed: 11166277]
42. Goffin JR, Straume O, Chappuis PO, et al. Glomeruloid microvascular proliferation is associated with p53 expression, germline BRCA1 mutations and an adverse outcome following breast cancer. *Br J Cancer.* 2003; 89(6):1031–1034. [PubMed: 12966421]
43. Straume O, Chappuis PO, Salvesen HB, et al. Prognostic importance of glomeruloid microvascular proliferation indicates an aggressive angiogenic phenotype in human cancers. *Cancer Res.* 2002; 62(23):6808–6811. [PubMed: 12460889]
44. McKee, P. *Pathology of the Skin with Clinical Correlations.* Mosby International; London, United Kingdom: 1996.
45. Nagy JA, Dvorak AM, Dvorak HF. VEGF-A(164/165) and PlGF: roles in angiogenesis and arteriogenesis. *Trends Cardiovasc Med.* 2003; 13(5):169–175. [PubMed: 12837578]
46. Fu, Y.; Nagy, J.; Dvorak, A.; Dvorak, H. Tumor blood vessels: structure and function. In: Teicher, B.; Ellis, L., editors. *Cancer Drug Discovery and Development. Antiangiogenic Agents in Cancer Therapy.* Humana Press; Totowa, NJ: 2007. p. 205-224.
47. Baker JL. Retinal capillary hemangioma. *J Am Optom Assoc.* 1991; 62(10):776–779. [PubMed: 1813504]
48. Farah ME, Uno F, Höfling-Lima AL, Morales PH, Costa RA, Cardillo JA. Transretinal feeder vessel ligation in von Hippel-Lindau disease. *Eur J Ophthalmol.* 2001; 11(4):386–388. [PubMed: 11820314]
49. Goel A, Muzumdar D, Desai K, Chagla A. Retroorbital hemangiopericytoma and cavernous sinus schwannoma—case report. *Neurol Med Chir (Tokyo).* 2003; 43(1):47–50. [PubMed: 12568323]
50. Folberg R, Hendrix MJ, Maniotis AJ. Vasculogenic mimicry and tumor angiogenesis. *Am J Pathol.* 2000; 156(2):361–381. [PubMed: 10666364]
51. Hess AR, Seftor EA, Gruman LM, Kinch MS, Seftor RE, Hendrix MJ. VE-cadherin regulates EphA2 in aggressive melanoma cells through a novel signaling pathway: implications for vasculogenic mimicry. *Cancer Biol Ther.* 2006; 5(2):228–233. [PubMed: 16481735]
52. Lin AY, Ai Z, Lee SC, et al. Comparing vasculogenic mimicry with endothelial cell-lined vessels: techniques for 3D reconstruction and quantitative analysis of tissue components from archival paraffin blocks. *Appl Immunohistochem Mol Morphol.* 2007; 15(1):113–119. [PubMed: 17536318]
53. McDonald DM, Munn L, Jain RK. Vasculogenic mimicry: how convincing, how novel, and how significant? *Am J Pathol.* 2000; 156(2):383–388. [PubMed: 10666365]
54. Ferrara N. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: therapeutic implications. *Semin Oncol.* 2002; 29(6, suppl 16):10–14. [PubMed: 12516033]
55. Kim KJ, Li B, Winer J, et al. Inhibition of vascular endothelial growth factor-induced angiogenesis suppresses tumour growth in vivo. *Nature.* 1993; 362(6423):841–844. [PubMed: 7683111]
56. Jain RK. Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy. *Science.* 2005; 307(5706):58–62. [PubMed: 15637262]
57. Bergers G, Song S, Meyer-Morse N, Bergsland E, Hanahan D. Benefits of targeting both pericytes and endothelial cells in the tumor vasculature with kinase inhibitors. *J Clin Invest.* 2003; 111(9):1287–1295. [PubMed: 12727920]
58. Xue Q, Nagy JA, Manseau EJ, Phung TL, Dvorak HF, Benjamin LE. Rapamycin inhibition of the Akt/mTOR pathway blocks select stages of VEGF-A164-driven angiogenesis, in part by blocking S6Kinase. *Arterioscler Thromb Vasc Biol.* 2009; 29(8):1172–1178. [PubMed: 19443844]
59. Seaman S, Stevens J, Yang MY, Logsdon D, Graff-Cherry C, St Croix B. Genes that distinguish physiological and pathological angiogenesis. *Cancer Cell.* 2007; 11(6):539–554. [PubMed: 17560335]

60. Shih SC, Zukauskas A, Li D, et al. The L6 protein TM4SF1 is critical for endothelial cell function and tumor angiogenesis. *Cancer Res.* 2009; 69(8):3272–3277. [PubMed: 19351819]

Figure 1.

The six types of new blood vessels induced by many tumors and by Ad-vascular endothelial growth factor (VEGF)-A¹⁶⁴ in mouse tissues. (A) Typical mother vessel (MV). (B) MVs with intraluminal extension of cytoplasmic processes, dividing the lumen into multiple smaller spaces that will eventually split to form capillaries (arrows). (C) Immunohistochemistry of a typical glomeruloid microvascular proliferation stained with antibodies against CD31. (D) Vascular malformations (VM). (E) Feeder artery. (F) Vascular mimicry (Mim) and MV in a B16 melanoma. All but (C) are Giemsa-stained 1-μm Epon sections. (A–E) were taken at early to late intervals from Ad-VEGF-A¹⁶⁴ injection sites. Scale bars, 25 μm.

Figure 2.

(A) Expression levels of vascular endothelial growth factor (VEGF)-A protein at successive intervals after intradermal injection of Ad-VEGF-A¹⁶⁴. (B) Kinetics of the angiogenic and permeability responses at indicated days after Ad-VEGF-A¹⁶⁴ injection. Ears were photographed (–EB) and then were injected intravenously with Evans blue dye for photography 30 minutes later to assess vascular permeability (+EB). Note peak of blue dye staining at day 5 when MV predominate. (C) Neovascular sites in flank skin induced by Ad-VEGF-A¹⁶⁴. (A, B) Reprinted with permission in modified form from Nagy et al.⁸ d, day.

Figure 3. Schematic diagram of the angiogenic response to Ad-VEGF-A¹⁶⁴. Reprinted with permission from Nagy et al.¹⁹

Figure 4.

Vascular basement membrane degradation as the first step in mother vessel (MV) formation. (A) Confocal microscopy of ear whole mounts from normal control (Ctl.) mice and from mice whose ears had been injected 3 days earlier with Ad-vascular endothelial growth factor (VEGF)-A¹⁶⁴. Immediately prior to sacrifice, mice were injected intravenously with FITC-lectin (green). Ears were then immunostained with antibodies against laminin and visualized with Texas Red conjugated secondary antibodies. Note extensive loss of red laminin staining at 3 days in MV as compared with control venules. Scale bar, 50 μ m. (B) Immunoblot with an antibody against laminin $\beta 1$ chain performed on extracts of normal ears (time 0) and on ears harvested at 1 to 21 days following Ad-VEGF-A¹⁶⁴ injection. Note increasing low molecular weight laminin $\beta 1$ chain fragments (bracket) at 1 to 5 days. In contrast, on days 7 and 10 there is increased expression of intact laminin $\beta 1$ chain (arrow), as well as high molecular weight fragments, reflecting synthesis of new vascular basement membrane (VBM) in developing glomeruloid microvascular proliferations (GMP). (C) Increased expression of proteolytically active cathepsins B, S, and L during the course of MV formation in response to Ad-VEGF-A¹⁶⁴. NM, normal ear skin. Ad-Lac Z, control adenovirus injection. (D) Confocal microscopy of GB123 (red) and FITC-lectin (green) in normal mouse ears (NM) and in ears injected 3 days previously with Ad-VEGF-A¹⁶⁴. Only rare stromal cells stain for GB123 (red) in NM ears, whereas pericytes, but not endothelial cells (white arrows), stain intensely red in Ad-VEGF-A¹⁶⁴-injected ears. White asterisk indicates detaching GB123-positive pericytes. Blue color, DAPI staining. Scale bar, 20 μ m. (E) Immunohistochemical staining for cystatin C in control (0) and in mouse ears 3 days after Ad-VEGF-A¹⁶⁴ injection. Note substantial reduction in staining in MV as compared with control venules (V). Scale bar, 50 μ m. (F) Reciprocal change in immunohistochemical cathepsin B and cystatin C staining in MV generated 3 days after subcutaneous injection of 10⁶ TA3/St mammary carcinoma cells. MV exhibit reduced cysteine protease inhibitor (CPI) staining and increased cathepsin B staining as compared with control vessels (insets). Scale bar, 25 μ m. Reprinted in modified form from Chang et al³⁴ with permission.

Figure 5.

Transmission electron micrographs of (A) control ear venule and (B) a mother vessel 3 days after ear injection of Ad-vascular endothelial growth factor (VEGF)-A¹⁶⁴. (A) Typical normal venule lined by cuboidal endothelium. The cytoplasm contains prominent vesiculo-vacuolar organelles (VVOs; clear cytoplasmic vesicles) and is enveloped by a complete coating of pericytes (P). R, red blood cell. (B) Typical mother vessel (MV) is greatly enlarged sinusoid, characterized by extensive endothelial cell thinning; striking reduction in VVOs; prominent nuclei that project into the vascular lumen; mitotic figures (arrows); and decreased pericyte (P) coverage. The mother vessel lumen is packed with red blood cells, indicative of vascular leakage and resulting plasma extravasation. Inset. Reproduction of the normal venule depicted in (A) at the same magnification as the MV to illustrate differences in relative size of normal venules and mother vessels. OCUB processing. Reprinted with permission in modified form from Nagy et al.⁸ Scale bars: a, 1 μm; b, 5 μm.

Figure 6. Macroscopic image of a mouse ovarian tumor (T) 7 days after transplant into the subcutaneous of ^{Q1} a syngeneic C3Heb/FeJ mouse. Angiogenesis is confined to the region indicated by the white dotted line. Note arteriovenogenesis in the vessels radiating from the tumor mass (arrows). Scale bar, 0.5 mm.

Classification of Tumor Blood Vessels

Table 1

Vessel Type	Vessel Properties		
Angiogenesis			
MV	Large, thin-walled, hyperpermeable, lightly fenestrated pericyte-poor sinusoids that are engorged with red blood cells.	+	+
Capillaries	Formed from MV by a process that involves internal division.	–	–/?
Glomeruloid microvascular proliferations	Poorly organized vascular structures that macroscopically resemble renal glomeruli. They are composed of endothelial cells and pericytes with minimal vascular lumens and reduplicated basement membrane	+	+
Vascular malformations	MV that have acquired an often asymmetrical coat of smooth muscle cells and/or fibrous connective tissue. Resemble arteriovenous malformations found in other settings.	–	–
Arterio-venogenesis			
Feeder arteries, draining veins	Enlarged, often tortuous structures that are derived from preexisting arteries and veins. They extend radially from the tumor mass, supplying and draining the angiogenic vessels within.	–	–
Other			
Vascular mimicry	Blood-filled spaces lined by tumor cells that have acquired some endothelial cell properties and may contribute to tumor circulation.	?	?

VEGF, vascular endothelial growth factor; MV, mother vessel.