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Aberrant vascular architecture in tumors and its importance in drug-based therapies

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The vasculature is not simply a collection of hollow tubes through which blood flows – it is an organ in its own right, consisting of several cell types organized in a specific manner, and it mediates many unique biological and physiological functions such as selective filtration and angiogenesis. In tumors, blood vessels have structural and functional abnormalities that, to date, have generally hindered conventional therapies: the malformed networks make it difficult to deliver drugs uniformly to all cancer cells. Furthermore, flow in these vessels is chaotic, with intermittent stagnation followed by high-flow or even flow reversal in isolated segments. Cancer cells in stagnated or low-flow regions will receive suboptimal drug levels during chemotherapy. However, as more is learned about the formation of tumor vasculature and how it differs from that in normal tissue, more effective and directed therapies to fight cancer might be developed.

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▼ Terms such as 'abnormal,' 'malformed' and 'aberrant' are used to describe various aspects of tumor vasculature. However, much of what is labeled 'abnormal' with regard to tumor vessels is 'normal' in the context of physiological angiogenesis. In normal angiogenic responses, perivascular cells lose their association with the endothelium, endothelial junctions become leaky and the basement membrane is modified. What is abnormal in tumors is the dysregulation of angiogenesis: it does not follow the organized sequence that produces normal vessels in development, wound healing or the female reproductive cycle. Instead of considering these aspects of tumor vessels abnormal, perhaps it is better to envision solid tumors as 'wounds that never heal' [1], with the corollary that they are primitive wounds that are not able to make proper vessels.

Tumor blood vessels are poor conduits for drug delivery

Over the past century, we have begun to understand how solid tumors acquire their blood supply and how the resulting pathological vasculature functions. Much of this work has shown why traditional therapies are not effective [2,3]. For example, tumor vessels are 'leaky' because of unusually large gaps between adjacent endothelial cells, an abnormal basement membrane and extensive fenestration [4–6]. Studies have shown that high levels of vascular endothelial growth factor (VEGF) produced by tumor cells contribute to the high permeability of tumor vessels [5,7–10], and functional and structural analyses have assigned the range of effective pore size for the endothelial gaps to 200–2000 μm [6,11,12].

One would expect the high permeability and the large pore size of tumor vessels to facilitate the delivery of systemically injected drugs to tumor cells. However, the interstitial fluid pressure within solid tumors is high and roughly equivalent to the intravascular pressure [13]. This limits fluid convection out of the vessels to the cancer cells. In normal tissue, plasma proteins and nutrients travel from the blood vessels through the extravascular space to the lymphatics, and the geometry of the system has evolved to provide adequate nutrients to and extraction of waste products from all cells. In tumors, the disorganized branching structure [14] and lack of a functional lymphatic system [15] result in pockets of cancer cells that are not accessible by blood-borne drugs. It is thought that cancer cells in these regions of compromised nutrient delivery survive because they are

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genetically protected against apoptosis. Furthermore, oxygen and nutrients, which are generally much smaller than the injected molecular therapeutics and particles, might be able to diffuse into these isolated regions more easily. Drugs that do manage to extravasate from the vessels probably shunt through preferential channels between cancer cells. The drugs are then reabsorbed by nearby blood vessels or enter the lymphatics at the tumor periphery, bypassing many of the tumor cells. To make things worse, blood flow in individual segments is highly variable, often stopping or reversing temporarily.

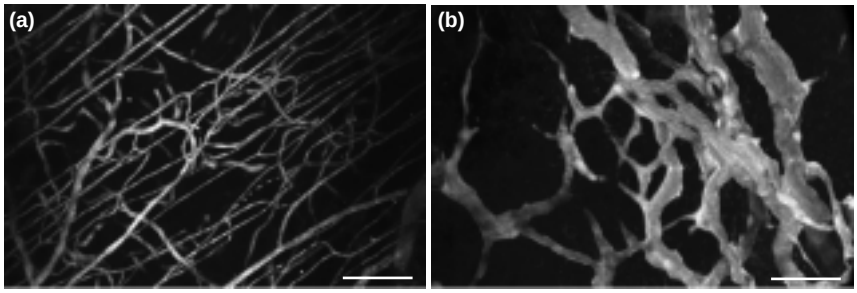

Figure 1. The striking difference between normal vasculature in the skin **(a)** and a tumor network in an LS174T human adenocarcinoma grown subcutaneously in a severe combined immunodeficiency (SCID) mouse **(b)**. Note that some regions lack vessels, making drug delivery difficult. Also, vessel diameters are not constant, resulting in chaotic flow patterns. Reproduced, with permission, from [26]. Scale bars = 50 mm.

These aspects of tumor vasculature make uniform delivery of conventional chemotherapeutics difficult. Improving the situation would require coercing the tumor vasculature into a more-normal branching pattern and also encouraging a functional lymphatic network to form inside the tumor [16]. However, this goal requires a fundamental understanding of network formation, pruning and maturation in normal physiology, and how these processes go awry in tumors. Because we are still far from achieving this goal, therapies relying on drug delivery to the cancer cells will suffer from these limitations for years to come.

In addition to drug delivery problems, cancer cells have the tendency to mutate during therapy, becoming resistant to the therapeutic agent. These considerations have led to the popularity of anti-angiogenic and anti-vascular therapies [16–23], which target the genetically stable blood vessels directly, avoiding the delivery and drug resistance problems faced by traditional chemotherapeutics. Ultimately, the differences between tumor and normal vessels might be the key to targeting tumor vasculature specifically.

Strictly speaking, anti-vascular therapy attacks any blood vessel, whereas anti-angiogenic therapy only prevents new vessel recruitment. However, this distinction has become somewhat blurred because many anti-angiogenic therapies seem to affect even the pre-existing networks. This has been attributed to the vascular maintenance function provided by angiogenic growth factors such as VEGF, which are apparently necessary to sustain mature blood vessels [16,20,21].

Tumor vessel networks and vascular wall structure are malformed

Figure 1 illustrates the striking difference in the overall architecture of normal and tumor vessel networks. Tumor networks are tortuous and do not follow the regular structural hierarchy seen in normal tissue [14]. The haphazard branching patterns and larger, less-regular diameters of vessels in tumors contribute to the non-uniform perfusion of cancer cells. Tumor blood flow is also chaotic, with high flow rates in some vessel segments and stagnation in others [24], and these patterns can change in hours or minutes [25]. Flow stagnation or a lack of vasculature in certain vessel segments during systemic treatments such as chemotherapy renders certain regions of the tumor inaccessible to drugs.

These network malformations arise from the poorly regulated, unchecked angiogenesis that occurs in tumors. The best known form of vessel recruitment is capillary sprouting, in which endothelial cells in a pre-existing vessel proliferate and migrate to form additional vascular segments. However, tumors can also recruit new blood vessels by intussusceptive microvascular growth (IMG), a mechanism by which existing vessels are split into smaller looping segments, resulting in a mesh-like network that fills the tissue [27] (Box 1). We have shown that the process of IMG is dysregulated in tumors, and that this can contribute to network abnormalities [28,29]. Interestingly, perivascular cells are thought to play a crucial role in IMG by directing the network formation through matrix modification [27], and might also be responsible for burrowing ahead of endothelial sprouts in sprouting angiogenesis [30]. As more information is gathered about the relationship between the perivascular and endothelial cells participating in angiogenesis and their interactions with the extracellular matrix, additional targets for anti-angiogenic therapy should become apparent.

Irregularities in tumor vessel architecture at a finer scale have also been described. The biology and organization of

Box 1. Bridging and intussuceptive microvascular growth

Intussusceptive microvascular growth (IMG) occurs, in its simplest form, by (1) dilation of pre-existing vessel, (2) insertion of specialized matrix elements by perivascular cells to form an interstitial tissue structure (ITS) near the vessel wall oriented perpendicular to the blood flow, (3) attachment of endothelial cells to the ITS and migration around them, (4) fusion of the two endothelial processes at the side of the ITS distal from the vessel, (5) opening of the lumen at the point of attachment and (6) growth of the interstitial tissue structure and endothelium to expand the resulting loop [28,29], (Fig. I). Intricate processes such as this are dysregulated in tumors, resulting in blind-ending, non-perfused segments and non-uniform perfusion.

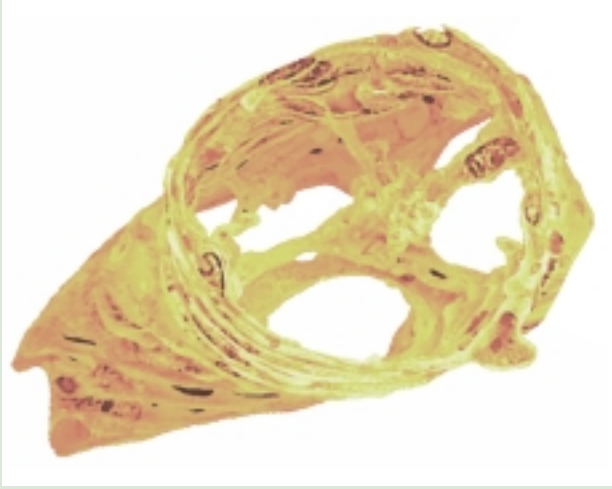

Figure I. 'Bridging', a form of intussusceptive microvascular growth (IMG) seen in a venule near the edge of a healing wound. Endothelial cells span the lumen, eventually dividing this vessel into four independent segments.

the endothelial cells, the basement membrane and the supporting mural cells are highly abnormal. Electron microscopy studies have helped define the differences between normal and tumor vessels, reporting abnormally fenestrated endothelia with large gaps between the cells and unusual intracellular organelles. Extravascular erythrocytes and microthrombi are also commonly observed [6,12,31–33].

An intriguing, yet controversial, aspect of tumor vasculature, originally reported in 1965 by Warren and co-workers and recently revisited, is the concept that cancer cells can participate in angiogenesis independent of endothelial cells. By performing intravital microscopy in the hamster cheek pouch in conjunction with ultrastructural studies, Warren concluded that plasma and blood cells travel through channels lined by cancer cells in melanomas [32]. This was later supported by Hammersen and co-workers, who further proposed that melanoma cells participate actively in the neovascularization. These researchers claimed that cancer or mesenchymal cells can promote angiogenesis by incorporation into capillary sprouts or by integration into the lining of larger vessels [34]. Recent studies of the matrix arrangement suggested that aggressive melanomas can form blood vessels without the involvement of stromal or endothelial cells, and that these channels co-localize with periodic acid Schiff (PAS)-positive structures. These researchers concluded that the melanoma cells could adopt an endothelial phenotype, expressing endothelial adhesion molecules [35].

This so-called 'vasculogenic mimicry' stimulated several commentaries and independent investigations that questioned the nature and functionality of the channels and the generality of the observation [36–39]. For example, the examination of similar melanomas by electron microscopy revealed that all blood-carrying vessels are lined by endothelium [38]. Although the formation and function of these PAS-positive structures are still under investigation, it is probable that they represent preferential channels for fluid transport that compensate for the lack of an intratumoral lymphatic network. It remains to be seen what blood elements are carried in such channels, their prevalence in various tumor types, and how stromal or cancer cells contribute to their formation. Potentially, elements in fluid channels lined by cancer cells could be a new target in tumors that depend on them.

A related study quantified the cellular composition of blood vessel walls in human adenocarcinomas. Using green fluorescent protein (GFP)-expressing cancer cells, a cocktail of antibodies targeted to endothelial markers and intravenous injection of fluorescent lectins, tumor cells, endothelial cells and perfused vessels were unambiguously identified using confocal microscopy (Fig. 2) [40]. About 15% of the vessels examined (4% of the vascular surface area) lacked immunoreactivity to common endothelial antibodies. However, instead of actively participating in angiogenesis, it appears that these cancer cells find their way to the lumen by incidental loss of the endothelial lining. Endothelial cells in mosaic regions are probably adversely affected by the tumor microenvironment and the frail or missing basement membrane, as opposed to vasculogenic mimicry in which cancer cells assume the role of endothelial cells; this apparently leads to phenotypic changes in the endothelium, apoptosis, loss of adhesion and shedding. The kinetics, mechanisms and the fate of mosaic blood vessels (i.e. whether they are eventually repaired or shut down) are still under investigation. These studies might elucidate

ways to target the basement membrane to induce endothelial shedding and/or destruction of fragile tumor vessels [17,41].

A potential cause for the leakiness and cellular disorganization of tumor vessels lies in the abnormal arrangement of the basement membrane and perivascular cells [42–44]. Normally, pericytes are in close contact with endothelial cells sharing the same basement membrane, and produce soluble factors that help fortify the vessel wall and control the barrier of the endothelial junctions. In tumors, studies have shown that pericyte association with the endothelium is less frequent and less intimate. For example, pericyte recruitment to tumor vessels can vary from 67.3% (lung carcinoma) to 12.7% (glioblastoma) [45], and the lack of pericyte recruitment by T241 fibrosarcomas and KRIB osteosarcomas is thought to result from the limited pool of mural cells available to the tumor [46]. Using immunostaining of desmin and α-smooth muscle actin, Morikawa and colleagues studied the involvement of pericytes with vessels in RIP-Tag2 tumors, MCa-IV breast carcinomas and Lewis lung carcinomas. These researchers found that >97% of vessels had associated pericytes, but that these pericytes were only loosely associated with endothelial cells [30]. Similar observations were made using intravital two-photon microscopy; many host stromal cells accumulated at the periphery of implanted tumors, and some migrated into the tumor. Although these cells eventually lined up along the blood vessels, resembling pericytes, they did not adopt the close apposition seen in normal, quiescent vessels [26].

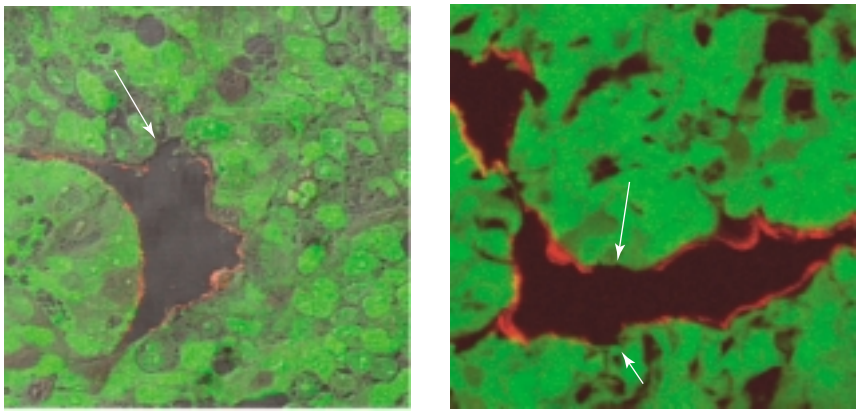

Figure 2. 'Mosaic' blood vessels in human tumor xenografts in severe combined immunodeficiency (SCID) mice. Immunostaining of endothelium (red) reveals regions lacking endothelial markers (arrows). The human adenocarcinoma cells express GFP constitutively (green) allowing unambiguous identification of the cancer cells [40].

Reports such as these suggest that stromal cells in tumors might play a crucial role in orchestrating angiogenesis and maintaining or protecting blood vessels. The outcome of anti-angiogenic treatment has been shown to depend on the stage of vessel maturation, determined mainly by pericyte investment [21]. Treatment with recombinant interleukin-12 resulted in the disappearance of small, non-fortified vessels but not the larger, pericyte-associated vessels [21]. Thus, these perivascular and stromal cells, which previously lurked in anonymity, are now squarely in the cross-hairs of researchers developing new therapies [21,26,30,44,47,48].

Targeting tumor endothelium

A key objective of cancer research has been to identify tumor-specific antigens, either on the cancer cells or endothelium, that would allow direct targeting of tumors without affecting normal tissue. Unfortunately, in the case of cancer cells, many factors have thwarted these efforts, including the genetic instability of cancer cells and the differential expression of surface antigens in cancers of different origin. However, endothelial cells do not, in theory, have these problems. Recently, researchers using phage-display methodology showed that endothelial cells in different organs (including tumors) express distinct surface markers [49]. This has given rise to the possibility of finding the vascular 'address' of tumor endothelium and targeting it with cytotoxic drugs [50], gene vectors or thrombogenic agents [18,41]. Assuming that appropriate, uniformly expressed targets can be found, this anti-vascular approach has the potential for specifically attacking tumors by shutting down their blood supply acutely [17]. This contrasts with anti-angiogenic therapies that block VEGF activity, for example, and gradually prune the network.

Tumors and the (dys-) regulation of angiogenesis

In normal tissue, there is an exquisite control system that directs endothelial cells, under various conditions, to: (1) open intercellular junctions, allowing plasma extravasation; (2) proliferate and migrate, providing new vasculature; and (3) modulate the expression of leukocyte adhesion receptors, encouraging immune surveillance. These do not necessarily all occur in individual physiological responses in normal tissue. For example, in inflammation, the recruitment of leukocytes and plasma leakage is more important than neo-vascularization. However, in the normal female reproductive cycle, angiogenesis can occur

without the need for enhanced leukocyte invasion. In wound healing, all three might take place.

Although many of the cytokines and growth factors that contribute to these distinct endothelial responses have been identified, and some clues regarding their regulation are known, it is not clear how they cooperate and interact to produce the range of endothelial responses seen in normal physiology. It is therefore difficult to understand the dysregulation that leads to the primitive vasculature of tumors. For example, it is known that VEGF is induced by hypoxia and can cause vessel leakiness as well as endothelial proliferation and migration. It also upregulates endothelial adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1). However, basic fibroblast growth factor (bFGF), which is also involved in angiogenesis, can downregulate these same adhesion molecules. Thus, some angiogenic factors can facilitate lymphocyte recognition of angiogenic vessels, whereas others provide these vessels with a mechanism that protects them from cytotoxic lymphocytes [51].

The kinetics of expression of various growth factors might also be crucial for normal vessel development, but impaired in tumors. Placenta growth factor (PlGF), a member of the VEGF family, does not directly affect vascular permeability. However, when PIGF is administered to endothelial cells 2–4 h before the addition of VEGF, it greatly increases the transport of water across the endothelial barrier [52]. Experiments such as this highlight the need to consider the cooperation amongst the various molecules involved in angiogenesis to achieve a complete understanding of the process. However, designing the appropriate spatio-temporal experiments at the necessary small-length scales is difficult, especially *in vivo*.

Until the roles of each growth factor in angiogenesis are understood and rational, tailored therapies against specific tumors based on stage, origin and molecular profile can be devised, a reasonable strategy is to administer a cocktail of drugs aimed at blocking as many angiogenic molecules as possible. This approach is supported by studies showing that Herceptin®, a monoclonal antibody against the cell-surface receptor human epidermal growth factor receptor-2 (HER2), induces regression of the vasculature in an experimental human breast tumor by modulating the effects of multiple pro- and anti-angiogenic factors [53].

Conclusions

The areas where abnormal tumor vasculature might be vulnerable to future therapies are listed in Table 1 and Fig. 3, and can be summarized as follows:

Anti-vascular therapies

By targeting the basement membrane or tumor endothelium via specific antigens, it might be possible to specifically shut down the blood supply to pre-existing and new vessels, thereby starving the tumor. Cancer cells exposed to blood flow in mosaic vessels provide an additional target for this type of therapy. Many strategies could be applied to stop blood flow, including the induction of thrombosis, destruction of the endothelium, or disruption of the endothelial–basement membrane association. Other antivascular approaches take advantage of differences in the cytoskeletal structure of 'immature' endothelial cells in recently formed vessels. For example, combretastatin has shown promise in selectively attacking these young endothelial cells,which use tubulin, but not actin, to asemble the endothelial lining [54–56].

Anti-angiogenic therapies

Arguably the most active area of research, anti-angiogenesis is aimed at blocking the action of angiogenic growth factors such as VEGF, bFGF and epidermal growth factor (EGF) [19,23,53,57–59] or interfering with endothelial migration [60–65]. Although originally devised to stop new blood vessel recruitment by tumors, many of these therapies also cause pre-existing vasculature to regress by interfering with vascular maintenance function. Several endogenous inhibitors of angiogenesis, such as angiostatin and endostatin [23,66] and naturally occurring flavonoids [67,68], are also under investigation for this purpose. Other researchers are trying to block endothelial migration to prevent angiogenesis, with the adhesion molecules $\alpha(v)$ integrins being the most promising targets. Endothelial precursor cells have also received much attention as potential participants in vasculogenesis and angiogenesis, and could be valid targets [69,70]. Finally, the contribution of stromal cells to angiogenesis has recently been identified, and future efforts should focus on blocking their migration, matrix interactions and production of growth factors [26,30].

Preferential fluid channels and improved drug delivery

Although still poorly understood, preferential channels for fluid transport surrounded by tumor cells could represent specific targets for localizing drugs in tumors, or could be blocked to starve the tumor. Conversely, improving drug delivery to cancer cells by normalizing the tumor vasculature [16] and lymphatic system could improve treatments based on conventional cytotoxic chemotherapeutics or radiation therapy (by improving tissue oxygenation).

Although anti-angiogenic therapy is still a promising approach, it has met with initial clinical difficulties and might not provide an immediate solution [23].

Table 1. Targeting tumor vessel abnormalities[a]

Approach	Process or structure	Target	Goal	Advantages	Problems	Refs
Anti-vascular	Basement membrane	Collagen IV, laminin, perlecan, fibronectin	Shut down tumor blood supply by inducing thrombosis or endothelial shedding	Relatively easy access because of leaky endothelium	Tumor-specific BM components yet to be characterized; non-specificity can lead to damage to normal tissue; could increase metastasis by disrupting endothelial barrier	[42–44]
	Mosaic vessels	Cancer cell-specific antigens	Kill tumor cells and/or shut down blood flow	Direct access for i.v. drugs	Only 15% of vessels might be affected; must first identify cancer cell-specific antigens.	[40]
	Endothelial cells	Tumor-specific endothelial surface molecules; tubulin in immature endothelial cells	Shut down tumor blood supply by inducing thrombosis; use vascular addresses to localize gene delivery vehicles in tumor	Direct access for i.v. drugs; potential for high level of specificity; rapid shut-down possible.	Therapy must be tailored to tumor type, site of growth to optimize treatment.	[17,19,41, 54–56]
Anti-angi-ogenic	Angiogenic growth factors	VEGF, PlGF, bFGF, Ang1, Ang2, Ephrins (or their receptors or Activation pathways)	Stop new blood vessel formation; destabilize existing vessels	Genetically stable target	Might be necessary to block more than one growth factor; mechanisms not yet well defined	[19,23,53, 57–59, 71–73]
	Endothelial migration	Integrins	Stop new blood vessel formation	Genetically stable target	Potentially no effect on pre-existing vessels	[60–65]
	Stromal contributions to angiogenesis and vessel fortification	Perivascular cells, fibroblasts, pericytes	Block the contribution of these cells to angiogenesis; destabilize fortified, mature blood vessels	Genetically stable target; attacks many aspects of angiogenesis (growth factor production, matrix production and modification)	Potentially will inhibit normal angiogenesis (e.g. wound healing) and might affect mature vessel physiology	[21,26, 30,44, 47,48]
Other	Preferential fluid channels	Cancer cells, matrix components	Interrupt nutrient supply	Easy access for i.v. drugs	Relative contribution of these channels is unknown; might be tumor- dependent	[74]
	Drug delivery to cancer cells	Blood and lymphatic vessels	Encourage normal physiology to improve drug delivery	All existing chemotherapeutics (and radiation treatment) could benefit	Difficult to sufficiently 'normalize' the blood and lymphatic networks in tumors	[15,16]

[a]Abbreviations: BM, basement membrane; bFGF, basic fibroblast growth factor; PlGF, placenta growth factor; VEGF, vascular endothelial growth factor.

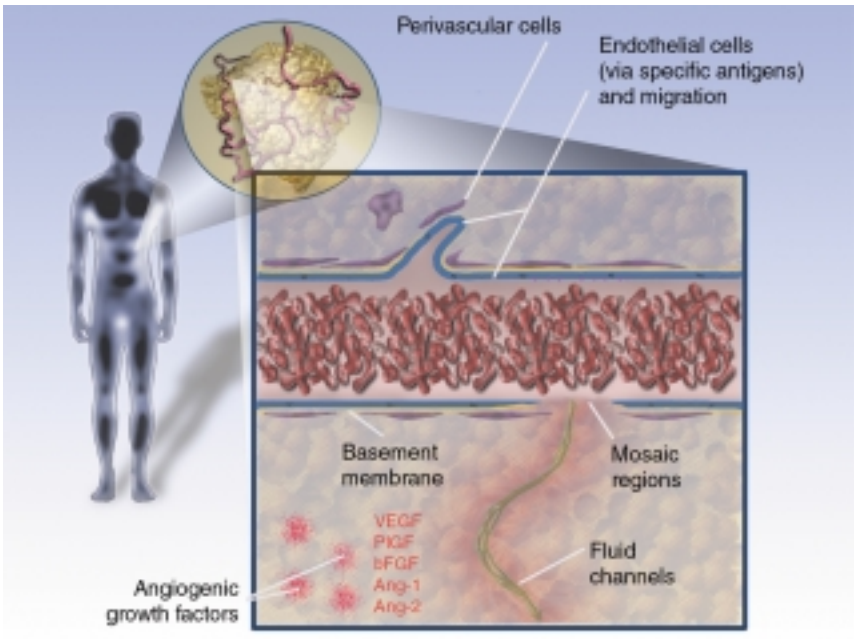

Figure 3. Potential points of attack: basement membrane, perivascular and/or stromal cells, tumor-lined fluid channels, mosaic vessels, angiogenic growth factors and endothelial 'addresses'.

Optimizing anti-angiogenic therapy requires a deeper understanding of the fundamentals of tumor angiogenesis, including growth factor and cellular cooperation, matrix involvement, endothelial biology and the influence of the cancer cells. Focusing on the abnormalities in tumor vasculature should further our understanding of anti-angiogenic treatment and point to new approaches for attacking these vessels.

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