REVIEW

Clinical Application of Antiangiogenic Therapy: Microvessel Density, What It Does and Doesn't Tell Us

Lynn Hlatky, Philip Hahnfeldt, Judah Folkman

A substantial number of clinical trials using antiangiogenic therapies are ongoing worldwide. How to achieve the maximum benefit from these therapies and how to monitor patient response are of paramount concern to investigators. There are currently no markers of the net angiogenic activity of a tumor available to aid investigators in the design of antiangiogenic treatment schemes. It stands to reason that quantification of various aspects of tumor vasculature might provide an indication of angiogenic activity. One often-quantified aspect of tumor vasculature is microvessel density. Studies over the last decade have demonstrated the value of using tumor microvessel density as a prognostic indicator for a wide range of cancers. In this context, measurement of microvessel density facilitates assessments of disease stage and the likelihood of recurrence and helps guide treatment decisions. Recently, however, it has been assumed by some investigators that measurements of microvessel density may also reveal the degree of angiogenic activity in a tumor. Based on this assumption, quantification of microvessel density is thought to constitute a surrogate marker for the efficacy of antiangiogenic agents as well as a means by which to assess which patients are good candidates for antiangiogenic therapy prior to treatment. Here we contend that, although microvessel density is a useful prognostic marker, it is not, by itself, an indicator of therapeutic efficacy, nor should it be used to guide the stratification of patients for therapeutic trials. In this review, we discuss the evidence for these assertions and what can and cannot be determined from measurements of microvessel density.

MICROVESSEL DENSITY IS A USEFUL PROGNOSTIC INDICATOR

As early as 1972, Brem and colleagues *(1)* saw the need to define a quantitative measure of tumor angiogenesis and proposed a grading system for human brain tumors that relied on the analysis of endothelial cell characteristics in conjunction with vessel density measurements. With the advent of specific antibodies to detect endothelial cells, quantitative studies of tumor vascularization intensified. In the early 1990s, Weidner et al. *(2–4)* showed that measurement of microvessel density within isolated regions of high vessel concentration (i.e., hotspots) was a prognostic indicator for human breast and prostate carcinomas. In this capacity, microvessel density measurements aid in assessing the stage of disease; the likelihood of metastasis, recurrence, or survival; and the planning of treatment course *(5,6)*. Since those early studies, hundreds of reports have examined the prognostic value of microvessel density in breast, prostate, and other cancers. Most of these studies report positive correlations between microvessel density and tumor recurrence, although some report no or even negative correlations between these end-

points. Some of the discrepancies may be explained by the fact that details of the methodology used to assay microvessel density can influence its value as a prognostic indicator. For example, the choice of the antibody (e.g., CD34, CD31, von Willebrand factor, or CD105) used to detect endothelial cells of the tumor microvasculature *(7–9)* has been reported to influence the study outcome, as does whether microvessel density is assessed at the periphery or center of the tumor *(10)*. Despite these technical issues, microvessel density as measured by the hotspot method of Weidner *(2–4)* is a valuable prognostic indicator for a wide range of tumor types. The accumulated data indicating the prognostic value of microvessel density in breast cancer was recently reviewed by Gasparini *(11)*. Earlier, Gasparini and Harris *(12)* reviewed published reports through 1999 on the prognostic value of microvessel density for a variety of solid tumors. We have summarized their compiled results and have reviewed the current literature for malignant melanoma *(13–19)*, non-small-cell lung *(7,8,20–34)*, genitourinary *(4,9,35–51)*, esophageal *(52–54)*, and gastrointestinal cancers *(55–65)*. These data are presented in Tables 1–4. Evidence is accumulating that measurements of microvessel density may also have predictive value for hematologic cancers *(66–71)*. Compiled in Table 5 are the results of studies to date that have examined the prognostic value of microvessel density measurements in the bone marrow of patients with hematologic malignancies *(67,68,71)*.

MICROVESSEL DENSITY MAY NOT BE AN INDICATOR OF ANTIANGIOGENIC TREATMENT EFFICACY

Despite its importance as a prognostic indicator in untreated tumors, microvessel density has not been shown to be a valid measure to guide or evaluate antiangiogenic treatment. The presumptions that the degree of tumor microvessel density is equivalent to the degree of tumor angiogenic activity, and that the quantification of microvessel density will provide the much needed dictates for antiangiogenic therapy, have led to two serious misconceptions: 1) that one can use the level of microvessel density of the untreated tumor to decide whether a patient will respond to antiangiogenic therapy and, thus, which patients

Table 1. Prognostic value of microvessel density measurements in malignant melanoma

Study (Ref. No.)	No. of patients	Prognostic value (survival or time to recurrence)
Srivastava et al. *(13)*	20	Yes
Fallowfield and Cook *(14)*	64	Yes
Carnochan et al. *(15)*	107	No
Busam et al. *(16)*	120	No
Graham et al. *(17)*	37	Yes
Vlaykova et al. *(18)*	31	Yes
Makitie et al. *(19)*	134	Yes

Table 2. Prognostic value of microvessel density measurements in non-small-cell lung cancers

Study (Ref. No.)	No. of patients	Prognostic value (survival or time to recurrence)*
Macchiarini et al. *(20)*	87	Yes
Yamazaki et al. *(21)*	42	Yes
Fontanini et al. *(22)*	253	Yes
Giatromanolaki et al. *(23)*	107	Yes
Angeletti et al. *(24)*	96	Yes
Apolinario et al. *(25)*	116	Yes
Dazzi et al. *(26)*	76	Yes
Han et al. *(27)*	85	Yes
Cox et al. *(28)*	181	Yes
O'Byrne et al. *(29)*	223	Yes
Marrogi et al. *(30)*	106	No
Yano et al. *(7)*	108	Yes (adenocarcinoma by CD34) No (adenocarcinoma by vWF†)
Aikawa et al. *(31)*	112	Yes (squamous carcinoma)
Ushijima et al. *(10)*	255	Yes (tumor periphery)
Tanaka et al. *(8)*	236	Yes (CD105) No (CD34)
Masuya et al. *(32)*	104	Yes
Fontanini et al. *(33)*	470	Yes (stage II)
Fontanini et al. *(34)*	73	Yes (stage II)

*Qualifiers to prognostic value appear in parentheses.
†vWF = von Willebrand factor.

Table 3. Prognostic value of microvessel density measurements in genitourinary cancers

Study (Ref. No.)	No. of patients	Prognostic value (survival or time to recurrence)*
Testicular germinal cell tumor		
Olivarez et al. *(35)*	65	Yes
Jones et al. *(36)*	51	No
Cervical cancer		
West et al. *(37)*	107	Yes
Prostate cancer		
Wakui et al. *(38)*	101	Yes
Fregene et al. *(39)*	34	Yes
Weidner et al. *(4)*	74	Yes
Vesalainen et al. *(40)*	88	Yes
Brawer et al. *(41)*	37	Yes
Silberman et al. *(42)*	109	Yes
Barth et al. *(43)*	41	Yes
Rogatsch et al. *(44)*	46	Yes
Halvorsen et al. *(45)*	104	Yes
Rubin et al. *(46)*	60	No (PSA)†
de la Taille et al. *(9)*	102	Yes (PSA by CD34) No (PSA by CD31)
Bladder cancer		
Dickenson et al. *(47)*	45	Yes
Bochner et al. *(48)*	164	Yes
Grossfeld et al. *(49)*	163	Yes
Korkolopoulou et al. *(50)*	80	Yes (T2)
Reiher et al. *(51)*	84	No

*Qualifiers to prognostic value appear in parentheses.
†PSA = prostate-specific antigen.

to include in clinical trials; and 2) that the efficacy of the anti-angiogenic agent will necessarily be reflected by a decreasing microvessel density during the course of antiangiogenic treatment. Here we discuss these and other apparent misconceptions concerning microvessel density in the context of antiangiogenic therapy. The first five misconceptions concern the interpretation of microvessel density in untreated tumors, and the last two concern treated tumors.

1) Microvessel Density Is Not a Measure of the Angiogenic Dependence of a Tumor. To a Large Degree, It Reflects the Metabolic Burden of the Supported Tumor Cells.

Microvessel density varies widely with tumor type. The degree of variability in microvessel density among tumor types is often misconstrued to mean that some tumors depend on angiogenesis whereas others do not. All tumor types, even those with low microvessel densities, depend on a therapeutically targetable angiogenic process. This angiogenic dependence is based on the inviolable demand of a growing tumor for sufficient levels of nutrients and oxygen exchange *(72)*. These metabolic requirements are in addition to any dependence of the tumor cells on paracrine factors provided by the endothelial cells *(73,74)*. Even leukemia, considered to be a 'liquid tumor,' has now been shown to induce angiogenesis in the bone marrow to support its growth *(75)*. Evidence of the importance of angiogenesis in hematologic malignancies, in general, is rapidly accruing. Both myeloid and lymphoid disorders may be accompanied by an increase in the microvessel density of the bone marrow *(66–71,75)*.

Contrary to common belief, microvessel density does not reflect the angiogenic activity or angiogenic dependence of a tumor. Microvessel density is a measure of the number of vessels per high-power (microscope) field and, as such, reflects intercapillary distance. Intercapillary distances are determined at the local level by the net balance between angiogenic factors that stimulate and those that inhibit vessel growth in each microregion, as well as by nonangiogenic factors, such as the oxygen and nutrient consumption rates of the tumor cells. Oxygen and nutrient consumption limit how far away from the vasculature tumor cells can remain viable and, thus, the number of tumor cells that can squeeze between capillaries before some become necrotic (Fig. 1). The metabolic needs of cancer cells vary with the tissue of origin and change with tumor progression. Thus, the number of tumor cells that can be supported by a vessel varies, influencing, in turn, the vascular density of the tumor. As was pointed out by Thomlinson and Gray *(76)*, one can think of the supported tumor cells as forming a viable cuff around a vessel, with cuff size being roughly indicative of the metabolic burden of the cancer cells. This is well illustrated in the Dunning rat model of prostate carcinoma, shown in Fig. 2, wherein tumor cells within approximately 110 μm of the vasculature form a viable cuff around a functional tumor vessel. Outside this radius of oxygen and nutrient support, tumor cells cannot survive and an abrupt shift to necrosis is observed.

Cuff size tends to vary inversely with tumor metabolic demand. Tumors that have high rates of oxygen or nutrient con-

Table 4. Prognostic value of microvessel density measurements in esophageal and gastrointestinal tumors

Study (Ref. No.)	No. of patients	Prognostic value (survival or time to recurrence)*
Esophageal cancer		
Tanigawa et al. (52)	43	Yes
Shih et al. (53)	117	Yes
Hironaka et al. (54)	73	Yes†
		(for T[2-4]M[0] after CRT)‡
Gastric cancer		
Maeda et al. (55)	124	Yes
Tanigawa et al. (56)	181	Yes
Sanz-Ortega et al. (57)	34	Yes
Colorectal cancer		
Saclarides et al. (58)	48	Yes
Tomisaki et al. (59)	175	Yes
Takebayashi et al. (60)	166	Yes
Bossi et al. (61)	178	No
Cianchi et al. (62)	84	No
Pietra et al. (63)	119	No (for N[0])
Sternfeld et al. (64)	146	Yes (after recurrence)
Lindmark et al. (65)	212	Yes†

*Qualifiers to prognostic value appear in parentheses.
†High vascularity associated with good prognosis.
‡CRT = chemotherapy plus radiation therapy.

Table 5. Prognostic value of microvessel density measurements in bone marrow for hematologic malignancies

Study (Ref. No.)	No. of patients	Prognostic value (survival or time to recurrence)
Sezer et al. (67)	44	Yes
Munshi and Wilson (68)	122	Yes
Rajkumar and Kyle (71)	74	Yes

sumption, such as glioblastomas, have small cuffs only two to three cells wide and have a high vascular density. By contrast, tumors of low metabolic demand, such as chondrosarcomas, have relatively large cuff sizes, with many cell layers supported and a relatively low vascular density. In normal tissues, cuff size also varies with metabolic demand. For example, in the liver, a tissue that has a high oxygen consumption rate, cuff size is one cell layer thick, and every hepatocyte is adjacent to an endothelial cell. In human tumors, the oxygen consumption rate is often substantially lower than that of the corresponding normal tissue (77). It is therefore consistent, albeit counterintuitive, that certain human tumor types, despite their often considerable angiogenic activity, can exhibit lower microvessel densities than the corresponding normal tissues. For example, the microvessel densities for human lung, mammary, renal cell, and colon carcinomas are lower than those of their normal tissue counterparts (78). In addition, the microvessel density for glioblastoma is less than that for brain tissue (78), and pituitary adenomas are less vascular than the normal pituitary gland (79). [Prostate tumors and germ cell tumors of the testis are two notable exceptions to this trend—these tumors show higher microvessel densities than their normal tissue counterparts (36,78,80)]. Thus, the fact that increases in microvessel density do not necessarily coincide with the onset of angiogenesis, a hallmark of neoplastic transformation (81), underscores the fact that microvessel density is neither a measure of tumor angiogenic activity nor a measure of tumor angiogenic dependence.

2) The Measurement of Microvessel Density Is Not Predictive of Tumor Response Under Antiangiogenic Treatment and Therefore Is Not Useful for Stratifying Patients for Clinical Trials. Low Microvessel Density Does Not Portend a Poor Response to Antiangiogenic Therapy.

In recent years there has been a trend toward individualizing tumor treatment by using predictive assays to select patients who are the most likely to respond to specific cancer therapies (82–84). Predictive assays are used to evaluate to what degree an individual patient's tumor exhibits the specific attributes [e.g., the level of tumor hypoxia (84)] that a specialized cancer therapy (e.g., neutron radiotherapy) targets. It is a common misconception that antiangiogenic treatment can be applied only to cancers that have high microvessel densities. This thinking promotes the further misconceptions that the measurement of microvessel density is the relevant predictive assay for antiangiogenic therapy and that the level of microvascular density within untreated tumors forecasts the efficacy of tumor response to antiangiogenic treatment. Thus, it is widely assumed that tumors with high microvessel densities are good candidates for clinical trials of antiangiogenic therapies, whereas tumors that typically have low microvessel densities (e.g., astrocytomas) are thought to be poor candidates for such clinical trials (85). However, experimental evidence does not support this method of patient stratification, but rather shows that both poorly vascularized and highly vascularized tumors can respond to antiangiogenic therapy. For example, bladder tumors that had less vascularity were found to be treatable by lower doses of an antiangiogenic agent than were those that had more vascularity (86). Because virtually all tumors are angiogenesis-dependent (72,87), low microvessel counts within tumors are not sufficient criteria to exclude patients from treatment with angiogenesis inhibitors.

3) Tumor Microvessel Density May Not Vary in Accordance With the Tissue or Blood Levels of any Single Proangiogenic Factor.

Many studies have investigated the relationship between microvessel density and the levels of single angiogenic factors such as vascular endothelial growth factor (VEGF) (27,32). It is now clear that individual tumors can make a wide variety of proangiogenic factors, and the relative expressions of these factors can change over time. For example, when breast cancers were screened for their expression of a panel of angiogenic factors, the majority originally expressed only a single positive factor, either VEGF or acidic fibroblast growth factor (aFGF), but evolved over time to express up to six angiogenic stimulators (88). The net angiogenic influence of the tumor microenvironment should be thought of as the sum of the positive and negative regulators of angiogenesis that arise from both the tumor cells (89) and host tissues (90). Human breast cancer fibroblasts, for example, exhibit high levels of VEGF (90). Because it is the net effect of total angiogenic stimulation and inhibition that determines the level of neovascularization, the levels of single angiogenic factors are not, as a general rule, strictly associated with measures of tumor vascularization. This principle has been borne out by investigations showing no correlation between tumor microvessel density measurements and levels of VEGF and aFGF or basic fibroblast growth factor (bFGF) (91–94). Such findings need to be considered when proposing antiangiogenic cancer therapies that seek to block a single proangiogenic signal.

Fig. 1. Microvessel density reflects intercapillary distance. As this schematic of a tumor section with two vessels illustrates, intercapillary distance (indicated by the **yellow arrow**), and thus microvessel density, is influenced by both angiogenic and nonangiogenic factors. Microvessel density is therefore not a pure measure of angiogenic activity. FGF = fibroblast growth factor; VEGF = vascular endothelial growth factor; TGF-β = transforming growth factor-β; O_2 = oxygen.
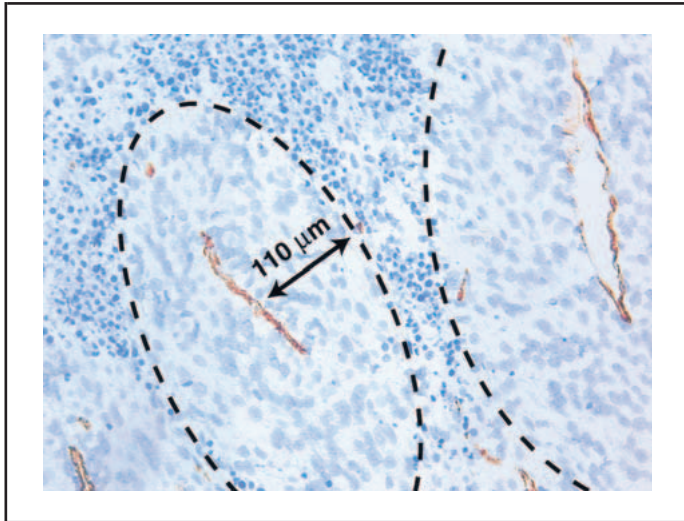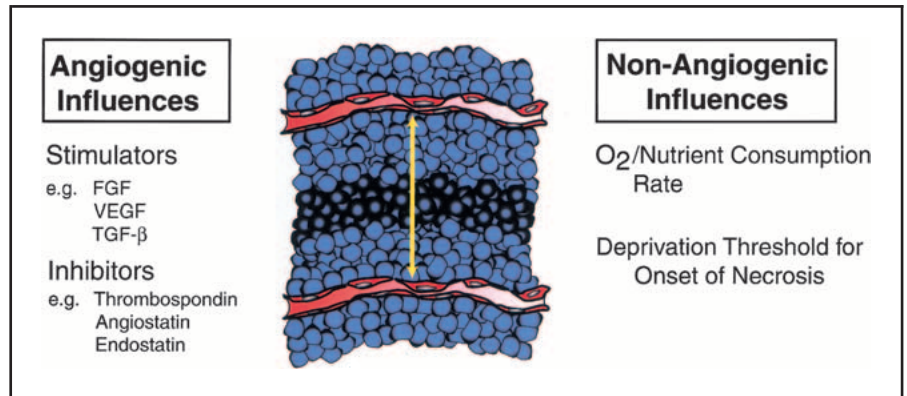
Fig. 2. Supported tumor cells forming cuffs (areas of viable tumor cells surrounding functional vessels—indicated by **black dashed ovals**) are shown for a Dunning rat prostate carcinoma xenograft. Cuff size is roughly indicative of the metabolic burden of the carcinoma cells. Tumor cells within approximately 110 μm of the vasculature are viable; beyond this radius of oxygen and nutrient support, an abrupt shift to necrosis is observed. Section was stained with hematoxylin for DNA, highlighting areas of necrosis, and with an antibody to CD31, showing the endothelium.

Recent work from the Kerbel group *(95)* showed that, when the proangiogenic factor VEGF is targeted by antiangiogenic therapy, tumors that express p53 respond more rapidly than tumors that do not. They interpreted the reduced response in the absence of p53 as resistance. More likely, p53-null status does not confer resistance. Rather, p53-null status is proangiogenic *(96)* through increased expressions of VEGF, interleukin 8 (IL-8), and bFGF, the suppression of which requires a suitably inclusive antiangiogenic counterattack.

4) A Minimum Vessel Density Is Determined by Tumor Cell Metabolic Demand, but Vessel Density Can Exceed the Metabolic Requirements of a Tumor.

Although normally functioning tissues rarely overvascularize, tumors can engage in angiogenic activity beyond that dictated by their metabolic needs, thus leading to overvascularization. As previously discussed, a major factor contributing to vessel density is metabolic demand. Metabolic demand places a lower limit on the density of vessels within the tissue required to maintain tissue viability. If vascular density should fall below

the required minimum, the flux of oxygen and glucose to the cells will be inadequate, triggering just enough tumor cell death to restore the minimal vascular density. Attesting to this process, regions of necrotic tumor cells adjacent to, and resulting from, oxygen or nutrient shortages are seen in many human tumors *(97)* (*see* Fig. 2). In normal tissues, by contrast, the level of microvascular density fairly accurately reflects the metabolic demands of the cells. This is because evolutionary pressures have forced a tight and efficient coupling between vascular supply and metabolic need. In the genetically unstable tumor, the close coupling between vascular density and oxygen or nutrient consumption may be loosened. In tumors, angiogenic factor expression often becomes uncoupled from normal regulatory controls and, consequently, some angiogenic factors are constitutively expressed at high levels. A prime example is the dissociation of VEGF expression from its regulation by oxygen concentration; in normal tissues, VEGF expression is increased and VEGF mRNA is stabilized under conditions of hypoxia or ischemia *(90,98,99),* whereas in tumor cells, VEGF is often constitutively expressed at high levels regardless of the ambient oxygen tension *(37,100,101)*. Notable examples of the decoupling of oxygen tension from VEGF expression occur in p53-null tumors *(102)* and in renal cell carcinomas from individuals that carry a mutation in the von Hippel-Lindau (VHL) gene *(103)*. Both p53 and VHL genes are associated with the degradation of hypoxia-inducible factor-1α, an upstream regulator of VEGF.

5) Rapid Tumor Growth Does Not Imply High Vascular Density. The Microvessel Density of a Tumor Need Not Be Higher, and Is Often Lower, Than That of its Corresponding Normal Tissue, Which Is Experiencing no Net Growth.

Whereas microvessel density frequently increases during tumor progression to accommodate an increased metabolic demand or a decoupling of net angiogenic stimulator expression with metabolic need, microvessel density levels do not reflect growth rate. In fact, Eberhard et al. *(78)* have shown that for five of the six human cancers examined (prostate carcinoma being the exception), the microvessel density of the tumor was lower than that of the corresponding nongrowing normal tissue. In lung carcinoma, for example, microvessel density was found to be only 29% that of normal lung tissue. In glioblastoma, an exceptionally highly vascularized tumor, microvessel density was found to be 78% that of normal brain tissue. This apparent

paradox can be partially explained by the lower oxygen consumption rate of tumor cells (77). In addition, tumor cells are known to tolerate oxygen deprivation and to be resistant to apoptosis under hypoxic conditions (104). Because tumor cells can remain viable at lower oxygen concentrations, they can exist at greater distances from the vasculature than can their normal-cell counterparts. Both the lowered oxygen consumption of tumor cells and their tolerance of hypoxic conditions promote increased intercapillary distance in tumors relative to their normal tissue counterparts.

Although the amount of total tumor vascularization (an extrinsic variable applicable to the tumor as a whole) must increase rapidly in fast-growing tumors to support a rapidly increasing tumor mass, the density of the vessels (an intrinsic variable that is locally defined) need not be high. In addition, the efficacy of antiangiogenic therapy would not be expected *a priori* to vary with the growth rate of the tumor. The lack of growth rate dependence for antiangiogenic effect stands in marked contrast to what is seen under standard chemotherapy. Chemotherapy targets proliferating cells directly and thus exerts a more demonstrable effect for fast-growing tumors than it does for slow-growing tumors.

6) The Efficacy of Antiangiogenic Agents Cannot Be Simply Visualized by Alterations in Microvessel Density During Treatment.

There is an urgent need to assess the clinical activity of the numerous antiangiogenic agents that are now in patient trials (105,106). Because antiangiogenic therapy suppresses tumor cell growth indirectly by inhibiting endothelial cell growth, reductions in tumor sizes or growth rates under antiangiogenic therapy are likely to occur over longer time frames than they would when standard chemotherapy is used (89). It follows that rapid tumor shrinkage, which is the classic end point scored for in clinical trials of chemotherapeutic agents, cannot be used to reliably discriminate between a null and a positive response to antiangiogenic agents. Instead, an appropriate measure of vascular inhibition is needed to assess the efficacy of an antiangiogenic agent at an early stage in a trial (i.e., before tumor growth inhibition is observed).
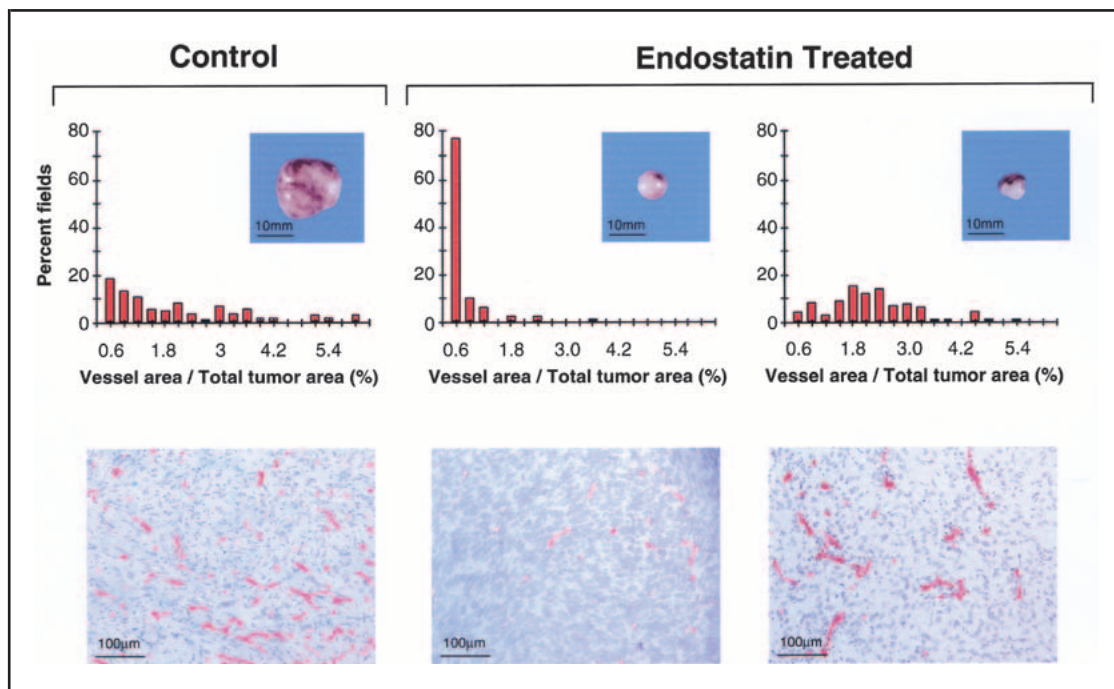
A common, albeit erroneous, impression is that measurements of microvessel density made during antiangiogenic therapy may be used to evaluate the therapeutic response to antiangiogenic agents. Numerous clinical protocols for antiangiogenic therapy include the periodic taking of biopsies to evaluate tumor microvessel density (107). However, the efficacy of an antiangiogenic agent cannot be evaluated by measuring the changes in microvessel density that the agent induces. This is because changes in microvessel density do not independently measure vascular inhibition, but rather, reflect the changing ratio of the vascular component of the tumor to its tumor-cell component. Under antiangiogenic therapy, capillary inhibition or elimination occurs first, followed by tumor-cell elimination, and both influence microvessel density. The tightness of the coupling of these two tumor components determines how much the vascular density fluctuates. Consequently, microvessel density is a time-dependent measure that varies in a nonmonotonic manner during antiangiogenic treatment—first decreasing in response to capillary inhibition or elimination, then decreasing more slowly

or even increasing somewhat as tumor cells drop out. Finally, in those cases where the angiogenic inhibitor is cleared from the bloodstream following bolus dosing (89), vessel density may asymptotically approach the pretreatment microvessel density level if treatment does not alter the metabolic demand or the angiogenic factor production of the tumor cells. The time required to partially or fully restore microvessel density to its pretreatment level following the clearance of an administered bolus dose of an angiogenic inhibitor would depend on the tightness of the coupling between vessel dropout and tumor-cell dropout. This would vary for different tumor types. If the restoration time is less than the typical times between biopsies, one might expect that most measures of microvessel density that are made over the course of treatment would yield a value little changed from the pretreatment value.

Although decreases in vascular density certainly provide a rough indication of the activity of an antiangiogenic agent, the nonmonotonic nature of microvessel density under antiangiogenic therapy renders microvessel density unsuitable as a stand-alone measure to monitor antiangiogenic effect. The concept that measures of vascular density alone do not reflect the efficacy of angiogenic inhibitors is demonstrated in Fig. 3. An untreated tumor (control) and two tumors treated with the angiogenic inhibitor endostatin are shown. Despite the fact that the inhibitor substantially inhibited growth in both of the treated tumors, the post-treatment levels of vascularization of the two tumors vary substantially. Compared with the control tumor, vessel density was substantially lower in one treated tumor and slightly higher in the second. Thus, detection of a decrease in microvessel density during treatment with an antiangiogenic agent suggests that the agent is active. However, the absence of a drop in microvessel density does not indicate that the agent is ineffective.

The lack of a parallel relationship between tumor size and tumor vessel density points to the error in interpreting the lack of a decrease in microvessel density during therapy as a failure to inhibit vascularization. Measuring slight decreases, no change, or even increases in microvessel density is still consistent with vessel inhibition because microvessel density is a dynamically complex quantity that is influenced by the initial vascular suppression plus the consequent interaction between the vascular and tumor-cell compartments (Fig. 4). A seminal clinical study illustrates this point. The study showed that multiple myeloma that was resistant to high-dose chemotherapy regressed in response to treatment with a single antiangiogenic agent, thalidomide (107), even though not all tumor regressions were accompanied by a decrease in microvessel density. This finding has prompted some skepticism about whether thalidomide acts via antiangiogenic mechanisms to promote tumor regression (107,108). However, the observation of tumor regression without a corresponding decrease in microvessel density does not indicate that mechanisms other than antiangiogenesis play a causal role in treatment response. The tumor cell population may simply decrease in direct proportion to, and as a direct consequence of, the loss of its supporting vasculature. The tendency to read too much into the behavior of microvessel density over the course of treatment of multiple myeloma patients with thalidomide further highlights the need for the development of appropriate surrogate markers to accurately assess angiogenic suppression in clinical trials. In a recent study, Bertolini et al. (109) reported that decreases in microvessel density in multiple my-

Fig. 3. Microvessel density may not coincide with antiangiogenic treatment response. Human liposarcoma xenografts were untreated (Control) or treated with the angiogenic inhibitor endostatin (Endostatin Treated). Tumor masses were measured with calipers using the formula length × width × width/2, and differences of mean masses (n = 6) were quantified using Student's *t* test. Shown are representative tumors (photo insets), vascularization within those tumors as detected by an antibody to CD34 (a transmembrane glycoprotein constitutively expressed on endothelial cells), and vascular density as quantified by digitized imaging. Compared with the control tumor, the endostatin-treated tumors displayed statistically significantly (*P*<.001) less tumor

growth, yielding a treated tumor to control tumor mass ratio (T/C) of 0.085. The post-treatment levels of vascularization of the two tumors shown varied considerably. Entire tumor sections were scored for vessel density by densitometric detection of CD34, which was assessed by imaging microscope fields at ×200 across the entire section (ignoring necrotic regions). Microvessel density dropped sharply in one treated tumor (**center histogram**) but increased slightly in the second treated tumor (**right histogram**). The histograms show the pro-

portion of microscope fields over the tumor sections that had the indicated ratios of vessel area/tumor area. A drop in microvessel density is reflected by a leftward shift of the histogram distribution relative to the control, because more fields show low ratios of vascularization relative to tumor area. A leftward shift of the distribution, and thus a drop in microvessel density, is seen for the endostatin-treated tumor in the middle but not for the endostatin-treated tumor on the right.

Fig. 4. Microvessel density in a shrinking tumor is determined by the combined effects of capillary dropout and tumor cell dropout. The time-dependent coupling of these two forms of cell dropout is reflected by the intercapillary distance and, in turn, by microvessel density. Thus, following antiangiogenic therapy a regressing tumor may exhibit decreased microvessel density, essentially unchanged levels of microvessel density, or even increased microvessel density.
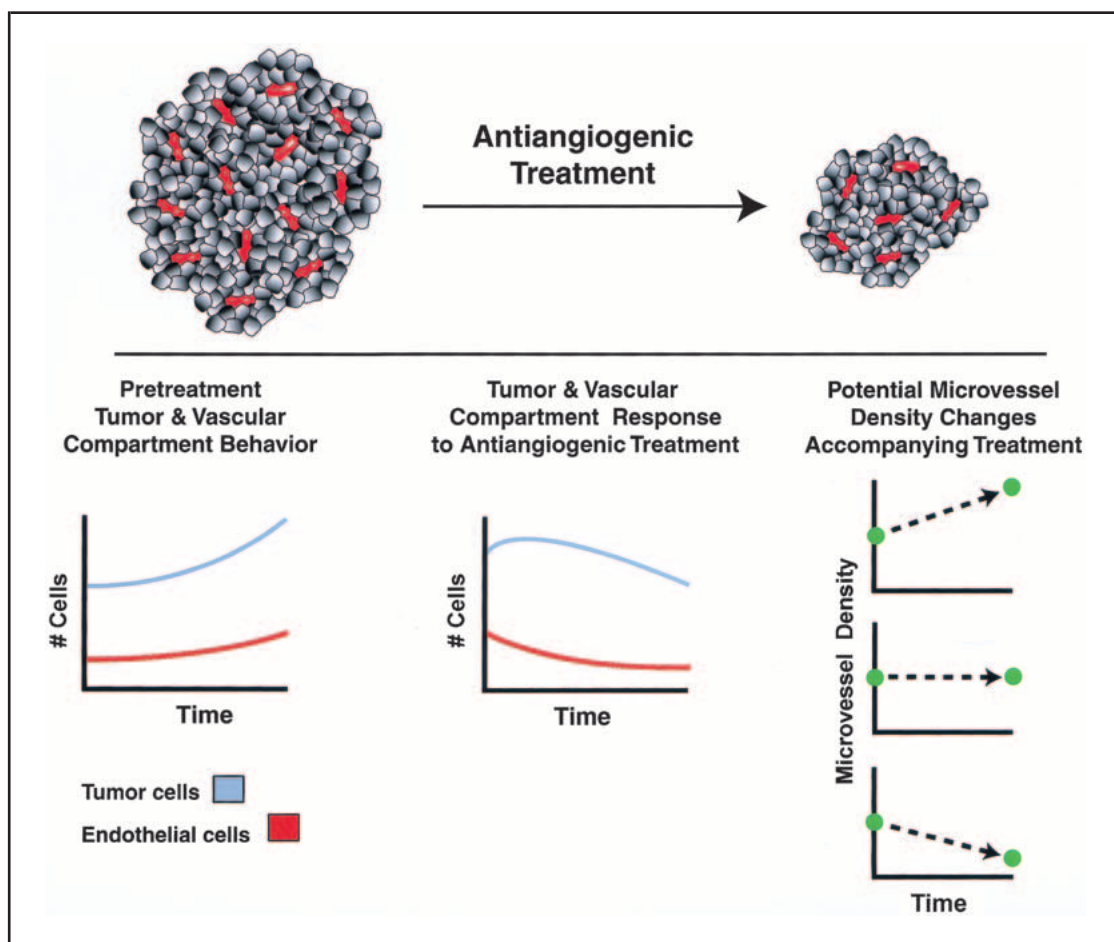
eloma patients who responded to thalidomide did not correlate with treatment response, although other measures of decreased angiogenic activity did correlate with treatment response in these same patients. Specifically, the levels of activated circulating endothelial cells derived from bone marrow decreased 10-fold after thalidomide treatment.

7) All Tumor Microvessels Are Not Functionally Equal. Inhibition of Ineffective Vessels Has Little Consequence in the Reduction of Tumor Growth, Further Emphasizing the Lack of a Simple Quantitative Relationship Between Alterations in Microvessel Density and Tumor Inhibition During Antiangiogenic Treatment.

All tumor vessels are not equal in their ability to provide oxygen and nutrients to the tumor cells they support. Vessels may be inefficient for several reasons. Tumor vessels can themselves be hypoxic and carry little oxygen, or they can have oscillating rather than directed blood flows and thus be ineffective at transporting oxygen and nutrients. Clearly, inhibiting hypoxic vasculature would shrink a tumor mass less than would inhibiting oxygen-rich vessels. In addition, the vascularization of a tumor may be greater than necessary to support its metabolic needs, thereby creating a state of overvascularization. Inhibiting redundant vasculature would likewise be expected to shrink a tumor mass less than would inhibiting the more effective vessels. In these cases, however, there is benefit to extending treatment. For example, in an effectively overvascularized p53-null tumor, there may be an initial period during antiangiogenic therapy when there is little tumor suppression because the excess vasculature is being targeted. As therapy continues and more critical vasculature becomes the target, a shift to more marked suppression would be observed. This appears to be what is happening in the study by Yu et al. *(95),* which looked at the response of p53-null colon carcinoma to antiangiogenic therapy using vinblastine and DC101, an antibody specific to VEGF receptor-2 expressed on endothelium.

The disconnect between vascular reduction and tumor response points to the lack of a simple quantitative relationship between therapeutic knockout of capillaries, reduction of microvessel density, and measurable tumor regression. In current theoretical treatments of therapeutic response to antiangiogenic agents, the inability to attribute tumor response to local vessel densities because of inhomogeneities in transport capacities is being dealt with by considering only the "effective vasculature" feeding the tumor. By focusing specifically on the functional vessels in a tumor, the underlying dynamics connecting vessel inhibition and tumor regression are likely to be clarified *(89).*

Looking Beyond Measurements of Microvessel Density

Switching the target of cancer treatment from the exceptionally heterogeneous tumor cell population *(110)* to the considerably more homogeneous tumor vasculature is a revolutionary therapeutic approach *(87).* In principle, this approach offers the means to treat cancers systemically by reaching both the primary tumor and its metastases *(111)* while circumventing, to large extent, three classic problems associated with chemotherapy: drug resistance *(87,112,113),* normal tissue toxicity *(112),* and limited access of drugs to target cells. A current challenge is to find appropriate characteristics of the vasculature that could

serve as clinical markers to guide antiangiogenic treatment and dosing. In this regard, there has recently been heightened interest in the role that microvessel density may play in indicating therapeutic responsiveness. Quantifying microvessel density has proven to be a valuable independent prognostic indicator in a wide variety of human cancers *(12)* and, as such, has become a useful tool for assessing treatment options. Arguably, the prognostic value of microvessel density points to the importance of the mechanisms that drive increased vascularization (such as a shift in the balance of angiogenic factors in favor of stimulation) in the biology of tumor progression. On the other hand, measures of microvessel density are not sufficient to reveal the functional or angiogenic status of tumor neovasculature. Microvessel density, accordingly, offers no indication as to which patients might best respond to antiangiogenic therapy. In addition, although decreases in microvessel density following antiangiogenic treatment can give an indication of the antivascular activity of a particular agent, microvessel density as a single end point fails to provide an adequate measure for resolving the vascular response to antiangiogenic agents.

Although microvessel density *per se* would not influence the responsiveness of a tumor to antiangiogenic treatment, the detailed composition of the tumor vessels [e.g., the fraction of proliferating endothelial cells they contain *(78)* or whether pericytes are present *(114)*] might well modify the action of a particular antiangiogenic agent. VEGF neutralization (e.g., with VEGF-specific antibodies) may not affect mature vessels but has been shown to lead to the regression of immature vessels that lack smooth muscle cells *(115).* Assays that quantify the maturation state of vessels might also provide some indication of the susceptibility of the existing vasculature in the tumor bed to specific antiangiogenic agents. These assays include those that measure the ratio of concentrations of the angiopoietins Ang-2 and Ang-1 *(116),* or the presence or absence of an epitope recognized by the monoclonal antibody LH39 that is expressed in the lamina lucida and is associated with vessel maturity *(117).* Currently, there is a flurry of investigation into the fundamental biology of angiogenesis *(118–121)* that will inevitably yield further insights into the effective use of antiangiogenic agents *(122).* With antiangiogenic therapy, the challenge comes not in restricting the patient pool, but in providing the proper guidelines for using combination therapies [e.g., those that use several antiangiogenic agents in combination or combined with radiation *(123,124)* or chemotherapy *(125,126)*]; delivering the agents [e.g., extended or continuous versus bolus dosing schemes *(89,125–129)*]; classifying the different types of antiangiogenic agents according to target *(115),* mode of action, or stage of cancer most amenable to antiangiogenic agent attack *(130)*; unveiling and exploiting the serendipitous antiangiogenic effects of classic chemotherapeutic agents *(125,126,131–136)*; and accurately assessing patient response, including the identification of appropriate surrogate end points. Recognizing the limitations of microvessel density as a surrogate end point will no doubt improve our resolve to explore supplementary assays for evaluating the efficacy of antiangiogenic agents.

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