Heterogeneity of Angiogenesis and Blood Vessel Maturation in Human Tumors: Implications for Antiangiogenic Tumor Therapies

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ABSTRACT

Microvessel density (MVD) counting techniques have been widely used to assess the vasculature in tumors. MVD counts assess the presence of blood vessels but do not give an indication of the degree of angiogenesis and the functional status of the tumor neovascularity. To analyze angiogenesis and the functional status of the tumor vascular bed, we have quantitated endothelial cell proliferation and the recruitment of pericytes in human tumors (glioblastomas (n = 30), renal cell carcinomas (n = 22), colon carcinomas (n = 18), mammary carcinomas (n = 24), lung carcinomas (n = 15), and prostate carcinomas (n = 19)). These findings were compared to the physiological angiogenesis in the cyclic bovine ovarian corpus luteum. Tissue sections were examined applying double-labeling immunohistochemical techniques to detect proliferating endothelial cells and to colocalize endothelial cells and pericytes. The following parameters were quantitated: (a) MVD count; (b) proliferating capillary index (PCI); (c) proliferating tumor versus endothelial cell index; and (d) microvessel pericyte coverage index (MPI).

Based on endothelial cell proliferation, angiogenesis was found to be present in all tumors with characteristic and significant differences between the tumor types (glioblastomas, PCI = 9.6 ± 6.1%; renal cell carcinomas, PCI = 9.4 ± 5.2%; colon carcinomas, PCI = 7.8 ± 5.2%; mammary carcinomas, PCI = 5.0 ± 4.8%; lung carcinomas, PCI = 2.6 ± 2.5%; prostate carcinomas, PCI = 2.0 ± 1.4%). There was a considerable degree of heterogeneity in the intensity of angiogenesis within each tumor group, as indicated by large standard deviations. Even in the most angiogenic tumors, angiogenesis was found to be 4 to 20 times less intense as compared with the physiological angiogenesis in the growing ovarian corpus rubrum (PCI = 40.6 ± 6.2%). Varying degrees of pericyte recruitment to the tumor microvasculature were determined in the different tumor types (glioblastomas, MPI = 12.7 ± 7.9%; renal cell carcinomas, MPI = 17.9 ± 7.8%; colon carcinomas, MPI = 65.4 ± 10.5%; mammary carcinomas, MPI = 67.3 ± 14.2%; lung carcinomas, MPI = 40.8 ± 14.5%; prostate carcinomas, MPI = 29.6 ± 9.5%). The data demonstrate distinct quantitative variations in the intensity of angiogenesis in malignant human tumors. Furthermore, the varying degrees of pericyte recruitment indicate differences in the functional status of the tumor vasculature in different tumors that may reflect varying degrees of maturation of the tumor vascular bed.

INTRODUCTION

Tumor growth and metastatic dissemination are critically dependent on the tumor’s supply of blood vessels (1–3). The angiogenesis dependency of tumor growth has led to the development of antiangiogenic therapies that are conceptually extremely appealing for a number of reasons (4–6); (a) as an oncofetal mechanism that is mostly down-regulated in the healthy adult, targeting of angiogenesis should lead to minimal side effects even after prolonged treatment; (b) tumor-associated angiogenesis is a physiological host mechanism; consequently, its pharmacological inhibition should not lead to the development of resistance (7); (c) each tumor capillary potentially supplies hundreds of tumor cells, and the targeting of the tumor vasculature should thus lead to a potentiation of the antitumorogenic effect; and (d) in contrast to the interstitial location of tumor cells, direct contact between the vasculature and the circulation allows efficient access to therapeutic agents.

Despite the enormous efforts aimed at elucidating the molecular determinants of angiogenesis (8–10) and the intense search for natural and synthetic angiogenesis inhibitors (4, 6), surprisingly little is known about the nature of the vascular bed in human tumors. Almost all of the studies that have assessed endothelial cell turnover in tumors were performed in experimental animal models with rapidly growing tumors whose growth kinetics are vastly different from the growth kinetics of human tumors (11, 12). In fact, the few endothelial cell turnover studies that have been performed in human tumors do suggest that endothelial cell proliferation in these tumors is detectable, albeit at a relatively low rate (13–16). Average tumor endothelial cell proliferation indices of 0.15% have been reported for prostatic carcinomas (13). The endothelial cell labeling index in mammary carcinoma varies between 2.2% (14) and 2.7% (15), and a value as high as 9.9% has been reported for colorectal adenocarcinomas (16).

As early as 1972, Brem et al. (17) proposed a microscopic angiogenesis grading system to assess the angiogenic status of the tumor vasculature. Based on the analysis of the vascular density, the number of endothelial cell nuclei, and the cytological properties of tumor-associated endothelial cells, an angiogenesis score was determined and used to establish an angiogenic rank order of different human brain tumors (17). In recent years, the vascular bed of human tumors has been characterized extensively by performing MVD counting studies (18, 19). These studies have revealed that high MVD counts within vascular hot spots of tumors correspond with a poor prognosis for the patient. MVD studies using panendothelial cell markers reflect the vascular status of a tissue, i.e., the presence of blood vessels. However, they do not give an indication of the angiogenic status of a tissue vascular bed, i.e., the rate of ongoing angiogenesis and the functional status of tumor neovascularity. To more realistically assess the angiogenic status of the vasculature within human tumors, the present study was aimed at functionally analyzing the properties of the tumor vascular bed. Based on the analysis of tumor endothelial cell proliferation and pericyte recruitment, angiogenesis and the functional status of the tumor microvascular bed were quantitated in six different types of malignant human tumors. These findings were compared with the angiogenesis kinetics in the cyclic ovarian corpus luteum, one of the few organ sites in the adult with significant physiological angiogenesis.

4 The abbreviations used are: MVD, microvessel density; PCI, proliferating capillary index; PTE, proliferating tumor versus endothelial cell; MPI, microvessel pericyte coverage index; α-SMA, α-smooth muscle actin; vWF, von Willebrand factor; PCNA, proliferating cell nuclear antigen; BS-I, Bandeiraea simplicifolia I; AEC, ethanol carbazole; HPI, high power field.

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Materials and Methods

Tissue Samples. Tissue specimens of formalin-fixed paraffin-embedded human tumors were retrieved from the pathology archive. All tumors (1–4 blocks/tumor) were histopathologically evaluated by two investigators independently of the original pathology report. Tumor-free tissue adjacent to the tumor as well as tumor-free specimen from necropsies served as normal control tissue. Specimens of bovine ovaries were retrieved from an established bank of tissues that has been extensively analyzed for its angiogenesis status (20, 21).

Staining of Proliferating Endothelial Cells. A double-labeling immunohistochemical technique was used to simultaneously stain nuclei of proliferating cells and endothelial cell. Deyparaffinized and rehydrated sections (4 μm) were microwaved, endogenous peroxidase was blocked, and sections were incubated with an antibody to PCNA (clone PC10; 1:100 dilution; final concentration, 3.9 μg/ml; Dako, Hamburg, Germany; 60 min, room temperature) or Ki67 (clone MIB1; 1:10 dilution; final concentration, 20 μg/ml; Dianova, Hamburg, Germany; 60 min, room temperature). A biotinylated secondary antibody, streptavidin alkaline phosphatase complex, and nitroblue tetrazolium as a substrate (Zymed, South San Francisco, CA) were used to visualize binding of the first antibody. Single-color-stained tissue sections were incubated with double-staining enhancer (Zymed) for 30 min, and then endothelial cells were stained for CD34 expression (human tissues; clone QBEnd/10; 1:25 dilution; Novocastra, Newcastle, United Kingdom; 2 h, room temperature; secondary antibody, Zymed) or binding of the lectin BS-I (bovine tissues; biotinylated BS-I; 10 μg/ml; Sigma, Deisenhofen, Germany; 37°C, 2 h) using streptavidin-peroxidase as enzyme and AEC as chromogen (Zymed, South San Francisco, CA).

Quantification of MVDs, PCI, and MPI. Sections were assessed for uniformity of staining at low power (%100), and individual microvessel counts were then performed in on a ×400 field. To express MVD counts microscope-independent, counts were transformed and expressed as the number of microvessels/mm² (1 HPF = 0.0681 mm²). Density counts of CD34, BS-I, or vWF-stained microvessels were performed independently by three investigators, as described previously (18, 20). At least five independent microscopic fields per tissue section were analyzed by two independent investigators to count PCNA-positive tumor cells and endothelial cells. Tumor cell proliferation and endothelial cell proliferation were quantitated in vascular hot spots that were identified by screening for the areas with highest vessel density at low magnification. A PCI was determined by calculating the ratio of the number of microvessels with proliferating endothelial cells/the total number of microvessels. A MPI was correspondingly established by quantitating the percentage of microvessels that colocalized endothelial cell staining (CD34 or BS-I) and pericyte staining (α-SMA). For MPI quantitation, at least five independent microscopic fields per section were independently analyzed by two investigators.

Statistical Analysis. Results were analyzed for statistical significance by an ANOVA and the Mann-Whitney U test. Two-sided statistical calculations were performed using the Statistica 5.1 program (StatSoft, Tulsa, OK) on an IBM-compatible personal computer.

Results

Endothelial Cell Proliferation in Human Tumors. Angiogenesis and pericyte recruitment were assessed in six different types of human tumors that were all histologically diagnosed as malignant tumors, namely, glioblastomas, renal cell carcinomas, colon carcinomas, mammary carcinomas, lung carcinomas, and prostate carcinomas (Table 1). To quantitate angiogenesis in these tumors, we used a double-labeling immunohistochemical technique, simultaneously staining endothelial cells for the expression of CD34 and detecting proliferating cells by using the proliferation markers PCNA and Ki67. This approach facilitated the detection of the relatively few proliferating endothelial cells among the numerous tumor cells (Fig. 1). Counting the CD34-positive microvessels in tumor vascular hot spots revealed uniformly high MVDs in the different tumor types, with glioblastomas and renal cell carcinomas having the highest average MVDs (Fig. 2). Nevertheless, it is noteworthy that MVD counts in all tumors except for prostate carcinomas were lower than the MVD counts for the corresponding normal tissues (brain tissue versus glioblastomas, 128%; kidney tissue versus renal cell carcinomas, 141%; colon tissue versus colon carcinomas, 103%; mammary tissue versus mammary carcinomas, 135%; lung tissue versus lung carcinomas, 348%; prostate tissue versus prostate carcinomas, 68%).

Both PCNA and Ki67 proved to be useful in assessing endothelial cell and tumor cell proliferation. On average, PCNA staining yielded 1.31 ± 0.23 (mean ± SD) times higher values than staining with Ki67, confirming previously reported differences between PCNA and Ki67 (22, 23). When quantitating a PCI reflecting the percentage of capillaries with PCNA-positive endothelial cell nuclei within vascular hot spots, significant differences were detected between the different tumor types (Fig. 2B). Glioblastomas (mean ± SD, 9.6 ± 6.1%; median, 8.6%) and renal cell carcinomas (mean ± SD, 9.4 ± 5.2%; median, 8.3%) had significantly higher PCIs than mammary carcinomas (mean ± SD, 5.0 ± 4.8%; median, 3.4%), lung carcinomas (mean ± SD, 2.6 ± 2.5%; median, 2.3%), prostate carcinomas (mean ± SD, 2.0 ± 1.4%; median, 1.9%; P < 0.005). Colon carcinomas had intermediate PCI values of 7.8 ± 5.2% (mean ± SD; median, 6.6%). In contrast, PCI values of all corresponding normal tissues were at the detection limit, with only an occasional PCNAlabeled endothelial cell being detectable (median of all tissues, 0%). The organs of the female reproductive system represent the only organ system with significant physiological angiogenesis (20, 24). We consequently determined PCI values in the cylvic ovarian corpus luteum. The highest PCI values were determined in the growing corpus rubrum (mean ± SD, 40.6 ± 11.2%; median, 37.8%; Fig. 2B). PCI values during corpus luteum angiogenesis were significantly higher than the PCI values of all analyzed tumor groups (P < 0.005).

When comparing PCI values of individual tumors, a large degree of variation was seen (Fig. 3A). PCI values in glioblastomas, renal cell carcinomas, and colon carcinomas varied over a wide range. Some tumors were found to have extremely high PCI values (>20%), whereas others had PCI values that were not higher than those of the groups with low PCI values (mammary, lung, and prostate carcinomas). With few exceptions, PCI values in these tumors were consistently low.

To assess the relative angiogenesis-inducing capacity of different types of tumor cells in relation to their own proliferative capacity, we determined an index of the overall ratio of proliferating tumor cells: proliferating endothelial cells (PTE index; Fig. 2C). Glioblastomas

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<th>Table 1 Summary of Tumors</th>
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<td>Histology/grading</td>
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<td>---------------------------</td>
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<tr>
<td>Glioblastomas</td>
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<tr>
<td>All grade IV astrocytomas</td>
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<tr>
<td>Renal cell carcinomas</td>
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<tr>
<td>4 grade I and 18 grade II tumors</td>
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<tr>
<td>Colon carcinomas</td>
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<tr>
<td>15 grade II and 3 grade III tumors</td>
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<td>Mammary carcinomas</td>
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<td>19 grade II and 5 grade III tumors</td>
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<td>Lung carcinomas</td>
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<td>11 grade II and 4 grade III tumors</td>
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<tr>
<td>Prostate carcinomas</td>
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<td>3 grade I, 15 grade II, and 1 grade III tumors</td>
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and renal cell carcinomas had PTE indices around 10, colon and mammary carcinomas had PTE indices between 20 and 30, and lung and prostate carcinomas had PTE indices between 40 and 70.

**Pericyte Coverage of the Neovasculature in Human Tumors.** To quantitatively assess the functional status of the tumor neovasculature, we applied a double-labeling immunohistochemical technique to simultaneously stain endothelial cells for CD34 expression and mural cells (pericytes/smooth muscle cells) with an antibody to α-SMA (21). Association of α-SMA with capillary endothelial CD34 expression was interpreted to reflect pericyte staining, whereas α-SMA association with CD34 expression in arteries and veins was interpreted to reflect smooth muscle cell staining (Fig. 4). A MPI was quantitated that reflects the percentage of capillaries associated with α-SMA-positive pericytes. MPIs were determined for all tumor types. Glioblastomas and renal cell carcinomas were identified as the tumor types with the lowest MPI values (glioblastomas, mean ± SD = 12.7 ± 7.9% and median = 9.7%; renal cell carcinomas, mean ± SD = 17.9 ± 7.8% and median = 17.6%; Figs. 2D and 3B). Mammary carcinomas had the highest MPI values (mean ± SD, 67.3 ± 14.2%; median, 70.4%). Similarly, colon carcinomas also had relatively high MPI values (mean ± SD, 65.4 ± 10.5%; median, 67.7%). Lastly, lung and prostate carcinomas had intermediate MPI values of 40.8 ± 14.5% (mean ± SD; median, 40.6%) and 29.6 ± 9.5% (mean ± SD; median, 29.3%), respectively.

The pericyte coverage data in the different types of malignant tumors were compared with the status of the neovasculature during cyclic ovarian angiogenesis. Angiogenesis in the cyclic ovary is a physiological event with a coordinated program of sprouting angiogenesis and mural cell recruitment. This is reflected by a MPI of 60.2% even in the angiogenic corpus rubrum (Refs. 21; Figs. 2D and 4G). The MPI of the mature midstage corpus luteum (62.4%) characterizes an overall immature neovasculature that rapidly undergoes regression at the onset of luteolysis.

**DISCUSSION**

Antiangiogenic targeting of the neovasculature within tumors is considered one of the most promising strategies in the search for novel antineoplastic therapies (5, 6). The concept that tumor vessels can be selectively targeted without affecting the quiescent organ vasculature is based on the fact that the molecular phenotype of immature, angiogenic blood vessels is distinctly different from that of resting blood vessels. A number of molecular determinants of angiogenic endothelial cells have been identified in recent years (4) and are being extensively explored for their suitability to target angiogenic blood vessels. Correspondingly, numerous animal studies have shown that
the activated, angiogenic neovasculature can be selectively targeted without affecting the normal organ vasculature (7, 25–27).

Essentially all of the antiangiogenic animal studies have used experimental models with a very high intensity of angiogenesis. These include rapidly growing tumor models such as the Lewis lung carcinoma (7, 25), the rabbit cornea assay with an implanted angiogenic factor (26), or the naturally occurring angiogenic processes in the female reproductive system (27). Contrasting these experimental models with a high intensity of angiogenesis, little is known about the degree of active angiogenesis and the functional status of the vasculature within human tumors. Vessel density counting studies have used panendothelial cell markers that facilitate the quantitation of the number of blood vessels within tumors (17, 18). These studies have demonstrated that high vessel densities in tumors correspond with poor prognosis. However, the use of panendothelial cell markers, such as CD31 or CD34, facilitates the assessment of the vascular status of a tumor but does not give an indication of the tumor’s angiogenic status. In fact, recent histomorphological studies indicate that some tumors may be vascularized without significant angiogenesis, probably by using the preexistent organ vasculature (28) or even by forming vascular channels on their own through a nonendothelial cell process designated as vascular mimicry (29). Recent studies have tried to circumvent this problem by using marker molecules that are up-regulated during angiogenesis, such as CD105 (30) and the integrin α-SMA-positive pericytes (see “Materials and Methods”). MPI indices of the different types of malignant human tumors were compared with the MPI values of the transient neovasculature during ovarian angiogenesis.

Fig. 2. Quantitative analysis of endothelial cell proliferation and pericyte recruitment during pathological tumor angiogenesis and physiological ovarian angiogenesis. All data are expressed as mean ± SE. A, colon carcinomas, mammary carcinomas, lung carcinomas, and prostate carcinomas have similarly high MVD counts. Glioblastomas and renal cell carcinomas have higher average MVD counts (P < 0.005). B, a PCI (Prol. Cap. Index) was quantitated assessing the percentage of microvessels with proliferating endothelial cells (see “Materials and Methods”). A ranked order of the intensity of angiogenesis in the different types of tumors was determined as follows: glioblastomas > renal cell carcinomas > colon carcinomas > mammary carcinomas > lung carcinomas > prostate carcinomas. PCI values in malignant human tumors were compared with the intensity of angiogenesis in the cyclic bovine corpus luteum (CR; early corpus rubrum (angiogenesis)), midstage corpus luteum (CL; maturation), and regressing corpus luteum (REG-CL; regression). C, a quantitative ratio of the total number of proliferating tumor cells in all tumors to the total number of endothelial cells (Prol. Tumor/EC Index) was determined for each type of tumor to establish a relative parameter that reflects the per tumor cell angiogenic capacity of the tumors. D, a MPI (Peric. Cov. Index) was quantitated assessing the percentage of microvessels that are associated with α-SMA-positive pericytes (see “Materials and Methods”). MPI indices of the different types of malignant human tumors were compared with the MPI values of the transient neovasculature during ovarian angiogenesis.

Fig. 3. Endothelial cell proliferation and pericyte recruitment in individual malignant human tumors. A, PCIs (Prol. Cap. Index) in glioblastomas, renal cell carcinomas, and colon carcinomas vary over a wide range. In contrast, with few exceptions, mammary carcinomas, lung carcinomas, and prostate carcinomas have uniformly low PCI values. B, MPIs (Peric. Cov. Index) in the different tumor types varied over a much smaller range than the PCIs.
thelial cell proliferation studies in human tumors have been reported (13–16). These studies describe tumor endothelial cell proliferation indices between 0.15% (13) and 9.9% (16). Much of the variation in the literature data may be attributed to differences in methodology. Thus, one of the goals of the present study was to standardize one technique and to comparatively apply this technique to different tumors. When assessing angiogenesis based on endothelial cell proliferation in different types of human tumors, we identified significant differences in the degree of active angiogenesis between different types of tumors as well as within one tumor type. Glioblastomas, renal cell carcinomas, and colon carcinomas were identified as the most angiogenic types of tumors. There was a high degree of variation among the individual tumors, indicative of a low rate of active angiogenesis in a subgroup of these tumors. In contrast to the intense angiogenesis in some types of tumors, lung carcinomas, prostate carcinomas, and most of the mammary carcinomas had relatively low PCI values (around 2%) that are still indicative of active angiogenesis in these tumors. Endothelial cell turnover in the corresponding normal tissues was below the detection limit, with only single PCNA- or Ki67-positive endothelial cells being detectable. A very careful analysis of endothelial cell turnover in normal tissue has identified a very low percentage of proliferating endothelial cells in normal tissues (12), which is still lower than the PCI values for prostate and lung carcinomas by a factor of 20. When comparing tumor PCI values to angiogenesis in the cyclic ovary, it became apparent that angiogenesis in human tumors is operative, albeit at a much lower rate than in the corpus luteum. Angiogenesis in the growing corpus luteum in the first few days after ovulation was found to be fourfold to twentyfold more intense than the angiogenesis in the different malignant human tumors (PCI > 40%), corresponding to previous reports on the high intensity of angiogenesis in the female reproductive system (33, 34).

In addition to assessing tumor endothelial cell proliferation, we quantitated the recruitment of mural cells (pericytes, smooth muscle cells) to the tumor neovasculature. The identification of the angiopoietins (35–37) and the phenotype of platelet-derived growth factor-β-deficient mice ( inability to recruit pericytes) (38, 39) has focused attention on the molecular mechanisms of blood vessel maturation mediated by the recruitment of pericytes. The mature phenotype of the quiescent organ vasculature in most organs is characterized by an extensive coverage with pericytes that appear to play a role in controlling the quiescent endothelial cell phenotype. Correspondingly, it has long been speculated that the tumor vasculature is characterized by a distinct maturation defect that is at least partially responsible for the irregular, tortuous, and leaky blood vessels found within tumors (40, 41). It was recently shown that androgen ablation therapy of prostate tumors leads to a down-regulation of vascular endothelial growth factor within the tumor, leading selectively to the regression of immature tumor microvessels that were not covered by pericytes (42). In line with these findings, we determined in the present study that only one-third of the vasculature within prostate carcinomas is covered by pericytes, despite the fact that prostate tumors were identified
as not very angiogenic tumors based on the assessment of endothelial cell proliferation.

The degree of pericyte recruitment to the neovasculature in the different tumor types varied significantly. The neovasculature in mammary and colon carcinomas had the highest rate of pericyte coverage, with as many as 70% of all microvessels being in contact with mural cells. In contrast, glioblastomas and renal cell carcinomas had pericyte coverage indices between 10% and 20%, indicating that most microvessels did not establish mural cell contact. These quantitative differences in mural cell recruitment could reflect varying degrees of vessel maturation of the tumor vascular bed. There is good evidence to suggest that pericyte coverage is a correct functional reflection of the degree of microvessel maturation (38, 39, 42, 43). However, it should be noted that pericyte coverage is not the only mechanism of vessel maturation, as indicated by the fact that the quiescent organ vasculature in some organs such as the lungs is not extensively covered by pericytes.

In summary, the present study has demonstrated that malignant human tumors are characterized by varying degrees of angiogenesis and pericyte recruitment. Furthermore, they indicate that the degree of angiogenesis in human tumors varies widely and may be very low in some types of tumors. Despite the fact that little is known about the mechanism of action of most angiogenesis inhibitors, the data suggest that the suitability of tumors for antiangiogenic therapies may differ between different tumor types and even within one type of tumor. Tumors with a low intensity of angiogenesis may not benefit much from antiangiogenic therapies that depend on the rate of endothelial cell proliferation. This stresses the importance of techniques such as those described in this study need to be implemented in clinical practice to assess the angiogenic status of a patient identifying those who will benefit most from antiangiogenic therapy. Furthermore, the data also indicate that the vasculature in most tumors is not very extensively covered by pericytes, which may reflect a functional immaturity of the tumor vascular bed. Not only may pericyte coverage of microvessels control vessel maturation, but it has also been shown to define a plasticity window for blood vessel remodeling (43). Thus, our data provide support for the concept that in addition to antiangiogenic therapies, angioregressive therapies could be developed that are capable of selectively inducing the regression of the immature tumor vasculature with an open plasticity window (42).

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