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Commentary

Intratumor Microvessel Density as a Prognostic Factor in Cancer

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In this issue, Hollingsworth et al[1] present evidence that increasing intratumor microvessel density in the areas of most intense neovascularization is associated with decreasing overall and disease-free survival in patients with advanced stage ovarian cancer. Moreover, using a Cox proportional hazards model, they showed that intratumor microvessel density may be a better predictor of disease-free survival than stage, grade, and tumor type, whereas stage was the best predictor of overall survival. The authors conclude that analysis of tumor neovascularization in advanced stage ovarian cancer may be a useful prognostic marker.

Clearly, for a tumor to grow, the tumor cells must not only proliferate, but benign host tissue, especially new blood vessels, must also form around the tumor cells. In 1971, Folkman proposed that tumor growth is dependent on angiogenesis.[2] Furthermore, he suggested that tumor cells and blood vessels composed a highly integrated ecosystem, that endothelial cells could be switched from a resting state to one of rapid growth by a diffusible signal from tumor cells or associated inflammatory cells, and that antiangiogenesis could be an effective anticancer therapy. Indeed, now there is considerable indirect and direct evidence to show that tumor growth is angiogenesis dependent, that tumor cells can produce diffusible angiogenic regulatory molecules, and that angiogenesis antagonists can slow or prevent tumor growth.

The indirect evidence is that tumors, both *in vitro* and *in vivo*, that lack access to blood vessels will grow only until passive diffusion can no longer provide adequate nutrients or allow waste products to exit into the adjacent medium.[3–5] At equilibrium, these avascular spheroids reach sizes of only 4 mm *in vitro*[6] and up to 2 mm *in vivo*.[7,8] Additional growth and metastases do not occur unless the spheroids become vascularized.[7–17] Other indirect evidence is that, in breast carcinoma, intratumor endothelial cells proliferate 45 times faster than endothelial cells in adjacent benign stroma,[18] and the rate of tumor progression is associated with increased intratumor microvessel density, a morphological measure of tumor angiogenesis.[19,20]

Direct evidence that tumor growth is angiogenesis dependent has been presented in several studies wherein different methods of specifically inhibiting angiogenesis (which are not cytostatic to tumor cells *in vitro*) clearly inhibited tumor growth *in vivo*.[21–30] For example, a synthetic analogue of fumagillin, a naturally secreted antibiotic of *Aspergillus fumigatus fresenius*, inhibits endothelial proliferation *in vitro* and tumor-induced angiogenesis *in vivo*,[24] and this angioinhibin (also known as AGM-1470 or TNP-470) will suppress tumor growth with few side effects. Indeed, this drug, as well as other angiogenesis inhibitors (ie, bryostatin, thalidomide, platelet factor 4, interferon-α, carboxyaminotriazole, metalloproteinase inhibitor (BB94), and D-gluco-D-galactan sulfate (DS4152)), are now in various phases of clinical trials as chemotherapeutic agents for a variety of malignant solid tumors, leukemias, and infantile hemangiomas.[22,23] Also, Kim et al[26] have shown that inhibition of vascular endothelial growth factor (VEGF)-induced angiogenesis suppresses tumor growth *in vivo*. These investigators injected human rhabdomyosarcoma, glioblastoma multiforme, or leiomyosarcoma cell lines into nude mice and found that treatment of these mice with a monoclonal antibody specific for VEGF inhibited the growth of the tumors and reduced tumor vessel density but had no effect on the growth rate of the

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tumor cells *in vitro*. Likewise, Millauer et al[28] showed that tumor growth is markedly suppressed by the introduction of defective VEGF receptors into tumor endothelial cells. They showed that tumor angiogenesis and tumor growth are inhibited *in vivo* by infecting tumor cells with a retrovirus vector encoding a dominant-negative, nonfunctional mutant of the VEGF receptor (flk-1). Most recently, Brooks et al[29] reported that a single intravascular injection of antagonists of the $\alpha^v\beta^3$ integrin (ie, either a cyclic peptide antagonist or monoclonal antibody) disrupts ongoing angiogenesis on the chick chorioallantoic membrane. This leads to the rapid regression of histologically distinct human tumors transplanted onto the chorioallantoic membrane. Also, induction of angiogenesis by a tumor or cytokine promotes vascular cell entry into the cell cycle and expression of $\alpha^v\beta^3$ integrin. After angiogenesis is initiated, antagonists of this integrin induce apoptosis (programmed cell death) of the proliferative angiogenic vascular cells, leaving preexisting quiescent blood vessels unaffected.

Obviously, tumor neovascularization promotes growth because the new vessels allow exchange of nutrients, oxygen, and waste products by a crowded cell population for which simple diffusion of these substances across its outer surfaces is no longer adequate. It is also becoming apparent that, in addition to this perfusion effect, endothelial cells may release important paracrine growth factors for tumor cells (eg, basic fibroblast growth factor (bFGF), insulin growth factor-2, platelet-derived growth factor, and colony-stimulating factors).[30–33] Also, the invasive chemotactic behavior of endothelial cells at the tips of growing capillaries is facilitated by their secretion of collagenases, urokinases, and plasminogen activator.[34,35] These degradative enzymes likely facilitate spread of tumor cells into and through the adjacent fibrin-gel matrix and connective tissue stroma. Indeed, elevated levels of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 in breast carcinomas have been shown to be independent predictors of poor prognosis. It is important that Fox et al[34] have shown a significant association of urokinase-type plasminogen activator and plasminogen activator inhibitor-1 with intratumor microvessel density. As a consequence, these investigators concluded that the poor prognosis in breast carcinomas associated with elevated urokinase-type plasminogen activator and plasminogen activator inhibitor-1 might be a result of an interaction between endothelial and tumor cells using the urokinase-type plasminogen activator enzyme system. Thus, the additive impact of the perfusion and paracrine tumor effects plus the endothelial cell-derived invasion-associated enzymes all likely contribute to a phase of rapid tumor growth and signal a switch to a potentially lethal angiogenic phenotype. These same effects likely contribute to a much higher metastatic potential by facilitating entry of tumor cells into the lymphaticvascular system.

The process of tumor neovascularization shares many features with normal wound healing[36] and is likely mediated by similar and specific angiogenic molecules that are released by the tumor cells and/or host immune cells (ie, macrophages) into the tumor stroma or are possibly mobilized from a bound inactive state within the tumor stroma.[6,22,37,38] Although the factor(s) and/or cell(s) causing tumor angiogenesis have yet to be determined, the current leading candidates include bFGF[39,40] and VEGF.[27] VEGF and vascular permeability factor (VPF) are the same substance and is often designated VPF/VEGF. VEGF is a permeability and selective endothelial cell growth factor and likely an important tumor angiogenic factor. Indeed, its permeability promoting effects on endothelial cells may be more important than its growth promoting effects. Brown et al[41,42] have shown in a variety of solid tumor types that immunohistochemical staining of tumor cells revealed high levels of VEGF protein and VEGF mRNA, which was accentuated in tumor cells close to areas of necrosis. In contrast, tumor endothelial cells express VEGF protein but not VEGF mRNA, yet the same endothelial cells expressed high levels of mRNA for the VEGF receptors flk-1 and kdr, indicating that the endothelial cell staining likely reflects binding of VEGF protein secreted by adjacent tumor cells. It is important that endothelial cells away from the tumor did not express these proteins or mRNAs. Moreover, VEGF has been shown to induce in endothelial cells expression of plasminogen activator, plasminogen activator inhibitor, interstitial collagenase, and procoagulant activity.[41] VEGF promotes extravasation of plasma fibrinogen, leading to fibrin deposition within the tumor matrix, which promotes the ingrowth of macrophages, fibroblasts, and endothelial cells.[43] Moreover, the work of Kim et al[26] and Millauer et al[28] strongly suggest VEGF is an important tumor angiogenic factor, yet VEGF may not act alone, and the work by Goto et al[44] show that VEGF and bFGF can act in a synergistic manner to cause angiogenesis. Also, various low molecular weight, nonpeptide angiogenic factors have been reported, but the actual role in vessel formation of these nonpeptide angiogenic factors remains incompletely studied. Nonetheless, nitric oxide and an arachidonic acid metabolite, 12(R)-hydroxyeicosatrienoic acid may prove important in regulating the angiogenic process.[6,37,45,46] Of interest, the proto-oncogenes

c-*myc*, c-*jun*, and c-*fos* were activated within endothelial cells when they had been exposed to 12(R)-hydroxyeicosatrienoic acid.[46]

Most of the reported angiogenic factors have been shown to stimulate vessel growth, but inactivation of a suppressor gene resulting in loss of an angiogenic suppressor substance may allow for tumor angiogenesis. In fact, it is likely that the switch to the angiogenic phenotype and the intensity of active angiogenesis are the net effect of both stimulatory and inhibitory factors. For example, Zajchowski et al[47] have shown that somatic hybrid cells, produced by fusion of MCF-7 human breast carcinoma cells with normal immortalized human mammary epithelial cells did not form tumors in nude mice. The hybrids had the ability to increase the expression of the angiogenesis inhibitor thrombospondin, suggesting that angiogenic capability contributes to tumorigenicity in human breast carcinoma. Also, Dameron et al[48] showed that the switch to the angiogenic phenotype by fibroblasts cultured from Li-Fraumeni patients coincided with loss of the wild-type allele of the p53 tumor suppressor gene and to be the result of reduced expression of thrombospondin-1. Finally, O'Reilly et al[49] recently reported that a novel angiogenesis inhibitor, angiostatin, is released by the primary tumor mass of a Lewis lung carcinoma. When the primary tumor is present, metastatic tumor growth is suppressed by this angiostatin, but, after primary tumor removal, the metastases neovascularize and grow. The angiostatin activity co-purifies with a 38-kd plasminogen fragment. This mechanism may explain one form of dormancy, but some metastatic deposits appear to remain dormant despite the fact that the primary tumor had been previously removed. Maybe the latter deposits switch to a more angiogenic phenotype and then begin to grow.[50] Other reported endogenous negative regulators of endothelial proliferation include platelet factor 4, tissue inhibitors of metalloproteinases, a 16-kd fragment of prolactin, bFGF soluble receptor, and transforming growth factor-β.[22]

To metastasize, a tumor cell must successfully negotiate a series of obstacles, as well as present and respond to several growth factors or cytokines. In most primary tumors, less than 1 cell in 1000 or 10,000 has all of these abilities.[51–53] Tumor cells must gain access to the vasculature from the primary tumor, survive the circulation, escape immune surveillance, localize in the microvasculature of the target organ, escape from (or grow from within) the vasculature into the target organ, and induce tumor angiogenesis.[51,52] Tumor growth and spread are further amplified geometrically when the newly established metastasis sheds additional tumor cells to form even more metastases by following the same cascade of events.[52] If the metastasis is already highly angiogenic, then its daughter metastases (clones) are likely to be highly angiogenic as well.

Angiogenesis is necessary at the beginning of this journey because, without it, tumor cells are only rarely shed into the circulation.[54–56] Greater numbers of tumor vessels increase the opportunity for tumor cells to enter the circulation. Liotta et al,[55,56] using a transplantable mouse fibrosarcoma model, showed that the number of tumor cells shed into the bloodstream increased from 1.4×10^3 cells per 24 hours on day 5 after tumor implantation to 1.5×10^5 cells per 24 hours on day 15, and this increase correlated very closely with increasing intratumor microvessel density, especially when those intratumor microvessels were more than 30 μg in diameter. Also, these studies revealed that the establishment of lung metastases is directly related to the number of cells shed into the circulation.[6,55,56] These data strongly suggest that intratumor microvessel density might correlate with aggressive tumor behavior.

New, proliferating capillaries have fragmented basement membranes and are leaky, making them more accessible to tumor cells than mature vessels.[57] Furthermore, the invasive chemotactic behavior of endothelial cells at the tips of growing capillaries is facilitated by the secretion of collagenases, urokinases, and plasminogen activator.[35] These degradative enzymes may facilitate the escape of tumor cells into the tumor neovasculature. Indeed, the invading capillaries may actively participate in the metastatic process by engulfing and thus facilitating the entry of tumor cells into vascular spaces, allowing systemic spread. Indeed, Sugino et al[58] observed, in a naturally occurring mouse mammary carcinoma model (C3H/He mice), that intravasating tumor cells and tumor emboli within blood vessel lumina retained their nested architecture within a continuous basement membrane and were also invested by an endothelial cell layer. These investigators believed that the findings indicated a passive mechanism of tumor cell intravasation, distinct from invasive properties of tumor cells, in which endothelial cells in sinusoidal vessels can envelope tumor cell nests, which then become detached into the blood. Arrest of such encapsulated emboli in pulmonary arterioles downstream could form new metastatic tumor foci.

Also, supporting the role of angiogenesis in the metastatic process is the observation that India ink injected into the rabbit cornea will stay at the injection site indefinitely as a tattoo, unless neovascularization is induced in the cornea.[59,60] As new capillaries approach the ink spot, the ink fragments and reappears

in the ipsilateral lymph nodes. Tumor cells can invade adjacent lymphatics that form concomitantly with blood capillaries or, hypothetically, they can pass from the blood stream into the lymphatics via lymphaticovenous junctions.[6,61] Also, tumor angiogenesis may facilitate this process by increasing tumor volume, thus enhancing tumor cell-lymphatic contact at the growing edge of the tumor.

Brem et al[62] were among the first to suggest that the intensity of intratumor angiogenesis may correlate with tumor grade and aggressiveness. For this purpose, they devised a microscopic angiogenesis grading system based on an index incorporating vascular density, endothelial cell hyperplasia, and endothelial cytology, yet the first clear-cut evidence that tumor angiogenesis in a human solid tumor could predict the probability of metastasis was reported for cutaneous melanoma. Srivastava et al[63] studied the vascularity of 20 intermediate thickness skin melanomas (0.76- to 4.0-mm levels of invasion). Vessels were highlighted with *Ulex europaeus*-1 agglutinin conjugated with peroxidase, and the stained histological sections were analyzed with a semi-automatic image analysis system. The 10 cases that developed metastases had a vascular area at the tumor base that was more than twice that seen in the 10 cases without metastases ($P = 0.025$). Age, sex, Breslow's tumor thickness, and Clark's level of invasion were similar in the two groups.

In 1990, my colleagues and I asked whether the extent of angiogenesis (ie, measured by intratumor microvessel density) in human breast carcinoma correlated with metastasis. We also sought to discover whether this intratumor microvessel density could be quantitated on a biopsy specimen rapidly and reproducibly so as to be useful in predicting the prognosis or the potential for metastasis at the time of diagnosis. If so, such information might prove valuable in selecting subsets of breast carcinoma patients for aggressive adjuvant therapies. For this to be true, it is important that a spectrum of intratumor microvessel densities exist within the spectrum of invasive breast carcinomas. Indeed, when the microvessel counts in a number of invasive breast carcinomas are sorted in ascending order on a log scale, the spectrum of low to high microvessel densities becomes apparent. The densities are an evenly distributed continuum, extending from approximately 10 to 200 microvessels per 0.74 mm^2 (×200) field.

My colleagues and I examined primary tumor specimens from 49 randomly selected patients with invasive breast carcinoma (26 node-positive and 23 node-negative); four of the node-negative patients subsequently developed distant metastases and died from cancer.[19] Microvessel endothelial cells were stained for factor VIII-related antigen/von Willebrand's factor (F8RA/vWF) by a standard immunoperoxidase technique in which tissue sections are treated with trypsin before application of the anti-F8RA/vWF. Hematoxylin-and-eosin-stained sections of breast tumor were used to choose one generous, paraffin-embedded tissue block representative of the invasive carcinoma. One 5 μm-thick section was stained for F8RA/vWF.

Intratumor microvessel density was assessed by light microscopic analysis for areas of the tumor that contained the most capillaries and small venules (microvessels). Finding these neovascular hot spots is critical to accurately assess a particular tumor's angiogenic potential. This is to be expected as there is considerable evidence that, like tumor proliferation rate, tumor angiogenesis is heterogeneous within tumors.[19,33,39,63,64] The technique for identifying neovascular hot spots is very similar to that for finding mitotic hot spots for assessing mitotic figure content and is subject to the same kinds of inter- and intraobserver variability. In our study, sclerotic, hypocellular areas within tumors and immediately adjacent to benign breast tissue were not considered in intratumor microvessel density determinations. Only tumors that produced a high quality and distinct microvessel immunoperoxidase staining pattern with low background staining were included in this or subsequent studies. This is very important, because the quality of immunoperoxidase staining can vary considerably between laboratories and, before measuring intratumor microvessel density, high quality immunoperoxidase staining must be consistently achieved.

Areas of highest neovascularization were found by scanning the tumor sections at low power (×40 and ×100 total magnification) and selecting those areas of invasive carcinoma with the greatest density of distinct F8RA/vWF-staining microvessels. These highly neovascular areas could occur anywhere within the tumor but most frequently appeared at the tumor margins. After the area of highest neovascularization was identified, individual microvessel counts were made on a ×200 field (×20 objective and ×10 ocular, Olympus BH-2 microscope, 0.74 mm^2 per field with the field size measured with an ocular micrometer). Any highlighted endothelial cell or endothelial cell cluster clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was considered a single, countable microvessel. Even those distinct clusters of brown-staining endothelial cells, which might be from the same microvessel snaking its way in and out of the section, were considered distinct and countable as separate microvessels. Vessel lu-

mens, although usually present, were not necessary for a structure to be defined as a microvessel, and red cells were not used to define a vessel lumen. Results were expressed as the highest number of microvessels in any single ×200 field. An average of multiple fields was not performed.

Invasive breast carcinomas from patients with metastases (either lymph nodal or distant site) had a mean microvessel count of 101 per ×200 field (SD = 49.3; range, 16 to 220). For those carcinomas from patients without metastases, the corresponding value was 45 per ×200 field (SD = 21.1; range, 15 to 100; $P = 0.003$). Also, we plotted the percentage of patients with metastatic disease in whom a vessel count was carried out within progressive 33-vessel increments. The plot showed that the incidence of metastatic disease increased with the number of microvessels, reaching 100% for patients having invasive carcinomas with >100 microvessels per ×200 field.

Subsequently, a number of other studies performed on different patient data bases by different investigators at different medical centers have observed this same association of increasing intratumor microvessel density with measures of tumor aggressiveness such as greater incidence of metastases and/or decreased patient survival. In many of these studies, intratumor microvessel density was found to have independent prognostic significance when compared with traditional prognostic markers by multivariate analysis. This has been shown in studies of patients with carcinomas of breast,[20,65–74] lung,[75–77] prostate,[78–82] head and neck (squamous),[83–87] rectum,[88] testicles,[89] and bladder,[90] as well as in malignant melanoma,[91–96] soft tissue tumors,[97] central nervous system tumors,[98] and multiple myeloma.[99] Now, Hollingsworth et al[1] have documented this association in ovarian carcinoma.

Many pathology laboratories have the reagents and technology available to perform the standard immunohistochemical assays for assessing intratumor microvessel density. However, it is very important that previously published protocols for measuring it be followed carefully.[19,20] Furthermore, considerable experience at the senior staff pathologist level is needed for assessing intratumor microvessel density, not only to supervise the immunostaining of endothelial cells but also for selecting representative invasive tumor and for localizing the neovascular hot spot. Counting microvessels has been shown to be reproducible,[19,67,80] especially after a period of training.[100] Brawer et al[81] compared manual intratumor microvessel determinations with those determined by automated counting (ie, Optimas Image Analysis) and found a very tight correlation ($r^2 = 0.98$, $P < 0.001$). Finally, accurate staging and adequate patient follow-up are needed to determine those patients who have metastases or will experience recurrent tumor, and proper technique must be supplemented with unbiased case selection and proper statistical analysis of data.

These reasons may explain why some investigators have not found this association between intratumor microvessel density and prognosis in solid tumors. Using anti-F8RA/vWF to highlight microvessels, Van Hoef et al[101] reported on intratumor microvessel density in the carcinomas of 93 patients with node-negative disease. These authors found no correlation between relapse-free or overall survival and intratumor microvessel density. But the number of microvessels reported in their study appeared much higher than those obtained by Weidner et al,[19,20] even though the latter employed larger counting areas and the same immunostaining techniques. The mean and median (range) microvessel counts from the Weidner et al study[20] was 60 and 56 (range, 8 to 167), respectively, with a 0.74-mm^2 counting area. In contrast, Van Hoef et al[100] obtained higher ranges of 80 and 72 (range, 32 to 156), respectively, with a 0.476-mm^2 counting area, or 64% of the field used by Weidner. The microvessel densities obtained by the Van Hoef group are in a range greater than what would be expected by using anti-CD31 to highlight microvessels, and Horak et al[67] found anti-CD31 to be the most sensitive endothelial marker for highlighting intratumor microvessels. These discrepancies suggest methodological problems.

Hall et al[102] were unable to find a relationship between intratumor microvessel density and metastasis in breast carcinoma. They reported microvessel counts by using a 0.1256-mm^2 microscopic field, which is much smaller than the optimal 0.74-mm^2 field used in our studies. Significance of the intratumor microvessel density drops when the field size is smaller than 0.19 mm^2.[19] They also excluded as vessels single cells that stained for anti-F8RA/VWF, believing that a lumen was necessary for it to be classified as a vessel and that single cells were frequently not of vascular origin. This is a significant deviation from the Weidner et al[19,20] procedure for determining intratumor microvessel density. We have found that anti-F8RA/vWF immunostaining is very specific for endothelial cells. Also, Hall et al[102] studied 87 breast carcinoma patients, 50 of whom had only 1.5 years median follow-up and, of the 50 node-negative patients, only three (6%) developed axillary or distant recurrence. A 6% incidence of disease relapse is far less than the expected 20 to 30% rate expected for breast carcinoma patients with node-negative dis-

ease. Carnochan et al[103] and Leedy et al[104] failed to show a relationship between tumor-related microvessel density and outcome in patients with malignant melanoma and lymph nodal metastases and in patients with squamous carcinoma of the tongue, respectively. Also, Leedy et al[104] failed to show a relationship of p53 protein accumulation and lymph node status. Why these reports are contradictory to other reports is not clear; however, Leedy et al[104] noted that the tongue was already a highly vascular organ. They implied that tumor growth and spread may be facilitated by pre-existing vessels in highly vascular organs, and it may be that intratumor microvessel density will prove not as useful in predicting outcome in patients with tumors developing in such highly vascular organs such as the tongue, liver, skin, kidney, or gastrointestinal tract.

Thus far, no endothelial marker developed has been trouble free. When applied properly, anti-F8RA/vWF remains the most specific endothelial marker, providing very good contrast between microvessels and other tissue components. Unfortunately, anti-F8RA/vWF may not highlight all intratumor microvessels.[100] Although apparently more sensitive, CD31 strongly cross-reacts with plasma cells.[105] This complication can markedly obscure the microvessels in those tumors with a prominent plasma cellular inflammatory background. CD34 is an acceptable alternative and the most reproducible endothelial cell highlighter in many laboratories, but CD34 will highlight perivascular stromal cells and has been noted to stain a wide variety of stromal neoplasms.[106,107] Like antibodies to F8RA/vWF, antibodies to CD31, CD34, and PAL-E also do not immunostain all intratumor microvessels.[108]

Wang et al[109,110] have developed a monoclonal antibody (MAb E9) that was raised against proliferating, or activated, endothelial cells of human umbilical vein origin and grown in tissue culture. MAb E9 strongly reacted with endothelial cells of all tumors and fetal organs and in regenerating and/or inflamed tissues, but it only rarely and weakly immunostained endothelial cells of normal tissues. Unfortunately, MAb E9 immunoreacted only in frozen tissue sections, although they did not mention whether microwave antigen retrieval techniques applied to formalin-fixed, paraffin-embedded tissues were tried. Antibodies like MAb E9 may provide the most sensitive staining of intratumor microvessels and preferentially immunostain activated or proliferating endothelial cells such that the overall staining intensity may correlate best with the intensity of tumor angiogenesis and, hence, tumor aggressiveness. Automated (machine) immunostaining and application of computer-aided image analysis may help to standardize microvessel counts and help eliminate inter-observer and even intra-observer variables, such as inexperience and hot spot selection biases.[111] The latter approach may make determination of intratumor microvessel density a simple, reliable, and reproducible prognostic factor in a variety of solid tumors, not just in breast carcinoma.

Actually, measuring intratumor microvessel density may prove to be a relatively crude method for estimating a tumor's angiogenic capacity. Other methods may prove more reliable and reproducible, such as measuring levels of angiogenic molecules in serum or urine, or directly measuring angiogenic molecules or inhibitors from tumor extracts (ie, in a manner similar to hormone receptor assays). Indeed, using an immunoassay, Watanabe et al[112] and Nguyen et al[113] reported elevated levels of bFGF in the serum and urine of patients with a wide variety of solid tumors, including breast carcinoma. Higher levels were found in patients with metastatic disease *versus* those of localized disease. Moreover, Li et al[114] measured bFGF in the cerebral spinal fluids of children with various brain tumors and correlated increasing fluid levels with greater intratumor microvessel density and increased likelihood of recurrence.

The association between intratumor microvessel density and various measures of tumor aggressiveness could be explained in a number of ways. First, a highly angiogenic primary tumor with a high intratumor microvessel density is more likely to seed distant sites with highly angiogenic clones.[22,115] Second, solid tumors are composed of two discrete yet interdependent components (ie, the malignant cells and the stroma they induce), and measuring intratumor microvessel density could be a valid measure of the success that a particular tumor has in forming this very important stromal compartment. Also, the endothelial cells of this stromal component may be stimulating the growth of the tumor cells in a reverse paracrine fashion. If true, the more microvessels and, thus, the more endothelial cells, the greater this paracrine growth stimulation. Third, the density of the microvessel bed within a tumor is likely a direct measure of the size of the vascular window through which tumor cells pass to spread to distant body sites. The larger that window, the greater the number of circulating tumor cells from which a metastasis could develop. Finally, if it is true that endothelial cells play a very active role in the metastatic process and that tumor cells are actually more passive than previously thought, then intratumor microvessel density could be a measure of those endothelial-derived forces promoting metastases. I believe all of these factors are acting together

to encourage tumor growth and metastasis. Indeed, it is no surprise that intratumor microvessel density correlates with various measures of tumor aggressiveness.

In closing, it should be emphasized that tumor angiogenesis alone is not sufficient to cause metastases. Tumor cells must also proliferate, penetrate host tissues and vessels, survive within the vasculature, escape the host's immune system, and then begin growth at a new body site. The behavior of typical bronchial carcinoids illustrates this point; they are highly vascular tumors, yet they uncommonly metastasize. Also, it remains to be seen whether intratumor microvessel density as reviewed here or reported by Hollingsworth et al[1] will be universally reproducible and continue to hold up as a predictor of metastasis or patient outcome when utilized in a prospective manner by pathologists in many different centers. As tumor therapies become more effective in preventing tumor recurrence, the ability of a prognostic test to stratify patients into various prognostic categories becomes diminished. With a 100% cure rate, all prognostic tests for predicting tumor recurrence become meaningless. In any event, the well documented association of increasing intratumor microvessel density with various measures of tumor aggressiveness have increased our understanding about the critical role of angiogenesis in human tumor growth and metastasis.

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