Different Patterns of Angiogenesis in Sarcomas and Carcinomas

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INTRODUCTION

The growth and metastasis of solid tumors are dependent on their ability to initiate and sustain new capillary growth, i.e., angiogenesis (1). This hypothesis is supported by multiple animal experiments in which tumors implanted in chicks, rodents, and rabbits are restricted in growth during the avascular phase, but rapid growth and metastasis occur shortly after vascularization. In addition, angiogenesis inhibitors that are not cytostatic to tumor cells in vitro inhibit tumor growth and metastasis in the animals in vivo (2). Clinical observations in human cancer have added support to the hypothesis that tumors are angiogenesis dependent. The levels of various angiogenic factors in bodily fluids have been demonstrated to correlate with prognosis in cancer patients (3). Multiple agents have been developed to inhibit this phenomenon of tumor-induced angiogenesis (4). Many of these agents have completed Phase I and Phase II clinical trials and are presently in the midst of Phase III trials.

Another application of angiogenesis research, as determined by the amount of vascularity in the tumor specimen, was first shown to have prognostic significance in breast cancer by Weidner et al. (5) in 1991. The number of vessels in a tumor specimen correlates with the disease stage and can add prognostic prediction independent of other routinely used markers. Since 1991, an overwhelming majority of published reports have shown a significant correlation between the density of intratumoral microvessels of invasive breast carcinoma and the incidence of metastases and/or patient survival. Similar associations have now been reported for patients with gastrointestinal carcinoma, melanoma, prostate carcinoma, testicular carcinoma, ovarian carcinoma, bladder carcinoma, central nervous system tumors, multiple myeloma, non-small cell lung carcinoma, and squamous carcinoma of the head and neck (6).

Although vascular density has been prognostic in carcinomas, two recent reports showed that microvessel counts in sarcomas do not correlate with patient outcome (7, 8). We therefore set out to investigate whether microvessel count correlates with clinical outcome in our patient population with soft tissue sarcomas.

MATERIALS AND METHODS

Patient Population. From the group of patients with soft tissue sarcomas treated at the University of California, Los Angeles Medical Center between 1984 and 1995, we chose 25 cases in which we had access to tissue prior to any treatment modality including surgery, radiation, and chemotherapy. There were 19 patients with leiomyosarcoma and six with malignant fibrous histiocytoma. Median follow-up was 35 months, with a range from one month to 12 years. One patient was lost to follow-up. Metastases included disease in remote organs, such as lung or liver. Disease-specific survival reflected death from tumor-related causes. Information was obtained from review of the patients’ medical records and verbal contact with the patients’ primary physicians.
Immunohistochemistry. The specimens were obtained from archival paraffin-embedded tissues of the above 25 patients as well as from selected cases of breast carcinoma. Sections (5 μm) were cut, and H&E preparations of each specimen were performed to confirm the presence of nonnecrotic tumor. Immunostaining was performed with an avidin-biotin-peroxidase complex technique (9). The slides were sequentially incubated at room temperature as follows: (a) in 0.3% H$_2$O$_2$ to block the endogenous peroxidase activity; (b) in 5% serum to block nonspecific antibody binding; (c) with the specific primary antibody overnight; (d) with the secondary antibody; (e) with avidin and biotinylated horseradish peroxidase (Vector, Burlingame, CA); and (f) with 3,3′-diaminobenzidine and H$_2$O$_2$ (Vector). The tissues were then stained with Gill’s hematoxylin and NH$_4$OH, dehydrated, and mounted. The rabbit antihuman vWF antibody was purchased from Dako (Carpinteria, CA) and used at 1 μg/ml. Negative controls included staining with mouse IgG1 (Becton Dickinson, San Jose, CA). Secondary antibodies were used at 1:200 dilution and included biotinylated antirabbit IgG made in horse and biotinylated antirabbit IgG made in goat (Vector).

Positively stained vessels were counted in five separate fields for each specimen using an Olympus model BH2 microscope. In keeping with the protocol developed by Weidner et al. (5), fields containing the highest density of vWF-positive vessels, i.e., “bursts,” were identified at scanning power and then counted at ×400. The numbers for the five fields were averaged. However, the vessel counts used to calculate the SD values depicted in Fig. 3 were obtained from 10 representative fields of the tumor specimen, which would include both “bursts” as well as areas with sparse vessels. Fig. 3 represents the SD in individual cases averaged together.

Statistical Analysis. Descriptive statistics, such as mean and SD, were used to summarize the results. The χ$^2$ and Wilcoxon tests were used for univariate analysis. Statistical significance was defined by $P < 0.05$.

Western Analysis of Tumor Matrix. Five sarcoma and five breast carcinoma specimens were used for matrix extraction according to a protocol described previously (10). These specimens were obtained either fresh or fresh frozen. Briefly, the tissue fragments were pelleted and homogenized in −3 volumes of high salt extraction buffer (3.5 M NaCl and 50 mM Tris-HCl, pH 7.4) containing protease inhibitors. The homogenate was centrifuged at 12,000 × g for 15 min, and the high salt extraction was repeated. The insoluble material was again pelleted and extracted overnight in an equivalent volume of 2 M urea, 50 mM Tris-HCl buffer (pH 7.4) with constant stirring. The urea extract was centrifuged at 12,000 × g for 15 min, and the supernatant was dialyzed against several changes of Tris-buffered saline (TBS; 150 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing protease inhibitors. High salt extracts were also dialyzed against TBS. Proteins in the media were precipitated overnight at 4°C with ammonium sulfate at 50% saturation. The precipitate was collected by centrifugation at 10,000 × g for 1 h and dissolved in 1 ml of TBS with protease inhibitors. The high salt and urea extracts and the media fractions were stored at −80°C.

Subsequently, the protein contents were measured and normalized for Western analysis. Tissue extracts were resolved on nonreducing SDS-PAGE mini-gels and transferred to nitrocellulose membranes by electrophoretic blotting. Membranes were blocked for 2 h at room temperature in PBS-Tween (PBS and 0.1% Tween 20) containing 1% BSA and 1% gelatin and then incubated for 1 h with primary antibody in PBS-Tween. The primary antibodies used were against VEGF, bFGF, aFGF, TGF-β, TGF-α, PD-ECGF, angiogenin, and IFN-α obtained from R&D (Minneapolis, MN). Other sources were used for the antibodies to TIMP-1 (Chemicon, Temecula, CA), thrombospondin (Dr. Luisa Iruela Arispe, UCLA), and plasminogen fragment (American Diagnostica, Inc., Greenwich, CT). The concentration of these primary antibodies was 1 μg/ml. Membranes were then washed three times over a 30-min period in PBS-Tween, incubated for 1 h with the appropriate horseradish

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4 The abbreviations used are: vWF, von Willebrand factor; VEGF, vascular endothelial growth factor; bFGF, basic fibroblast growth factor; aFGF, acidic FGF; TGF, transforming growth factor; PD-ECGF, platelet-derived endothelial cell growth factor; TIMP, tissue inhibitor of metalloproteinase.
peroxidase-conjugated secondary antibody (mouse monoclonal antirabbit IgG or sheep antimouse IgG; Amersham, Piscataway, NJ) diluted 1:5000 in PBS-Tween, and washed in PBS-Tween as before. The membranes were then developed using the ECL Western blotting detection system according to the manufacturer’s instructions (Amersham).

**In Situ Hybridization.** In situ hybridization studies were conducted with riboprobes made from the full-length TIMP-1 cDNA (provided by Dr. Judith Gasson, UCLA). The pBluescript SK plasmid (Stratagene, La Jolla, CA) containing a 0.5-kb EcoRI fragment of the human TIMP-1 gene was linearized with XbaI for antisense strand preparation from the T7 promoter and with HindIII for sense strand preparation from the T3 promoter (11). [35S]UTP-labeled RNA transcripts were synthesized at concentrations of 4 to 6 × 10⁵ cpm/μl. Paraffin-embedded sections were deparaffinized, fixed in 4% paraformaldehyde in PBS, rehydrated, washed in 0.5× SSC, and digested with proteinase K at room temperature for 10 min. The sections were acetylated using 0.25% acetic anhydride and 0.1M triethanolamine HCl rinsed with 0.5× SSC and dehydrated with ethanol and finally dried. A TIMP-1 riboprobe was applied in a hybridization mixture containing deionized formamide (50%), dextran sulfate (10%), tRNA (0.5 mg/ml), salmon sperm DNA (1 mg/ml), 10 mM DTT, 5 mM EDTA, 20 mM Tris-HCl, and 10 mM NaPO₄ (pH 6.8). The mixture was heated at 65°C for 15 min and chilled on ice. Fresh DTT was added to achieve a concentration of 20 mM. Then 120 μl of the mixture were applied to each section, and Parafilm coverslips were applied. Hybridizations were carried out in humidified chambers overnight at 55°C. Coverslips were removed in 5× SSC, 10 mM DTT at 55°C. Sections were washed with 2× SSC containing 2-mercaptoethanol and EDTA, treated with RNase A and washed in 0.1× SSC at 62°C for 3 h (high stringency). Slides were then washed for 5 min at room temperature in 0.5× SSC without 2-mercaptoethanol or EDTA. The sections were dehydrated with ethanol and air dried. The slides were exposed to Ilford K5D emulsion and stored in the dark at 4°C until developed, which took at least 10–14 days. Digital image analysis was then used to compare the signal intensities with the antisense probe with the signal intensities of the sense probe (background) over the corresponding areas of tumor cells and stroma in the carcinomas. Digital image analysis was composed of a Leitz Dialux microscope linked to a Vidicon camera, an IBM PC with PCVision digitizer, and Microscience software.

**RESULTS**

**Clinical and Statistical Analysis.** The sarcomas were assigned histological grades including low, moderate, and high. We found that the histological grade of the tumor correlated with survival (P = 0.03) but not with metastases (Fig. 1). The
vessel counts ranged from 10 to 110 per high power field. For purposes of analysis, the cases were divided into three groups: 1–29, 30–59, and ≥60 vessel count/field. There was no correlation found between metastatic disease (present and future) or survival and the number of “burst” vessels stained by vWF (Fig. 2). When all representative microscopic fields were taken into account, we found that there was a larger SD among the vessel counts of 10 breast carcinoma cases than in those of the present 25 cases (Fig. 3).

Vessel Patterns. At scanning power, we observed that the vessels in sarcomas were in general more diffusely distributed, whereas those in breast carcinomas would often cluster in “bursts” (Fig. 4). In carcinomas, malignant cells were juxtaposed to stromal cells including fibroblasts and myofibroblasts

![Fig. 4](image-url) Immunohistochemical staining of leiomyosarcoma (A) and breast carcinoma (B) with vWF, ×400.

![Fig. 5](image-url) H&E staining of sarcoma (A) and breast carcinoma (B).

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<th>Table 1</th>
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<td>Sarcoma</td>
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<td>Angiogenic stimulators</td>
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<td>VEGF</td>
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(Fig. 5). In contrast, the sarcoma specimens contained mainly malignant cells with a relatively acellular matrix.

**Tumor Matrix Analysis.** We used Western blotting to analyze the extracted matrix from five representative leiomyosarcomas and five breast carcinoma cases (Table 1). The sarcoma matrix contained VEGF, bFGF, aFGF, TGF-β, PD-ECGF, angiogenin, and TIMP-1. The carcinoma matrix contained VEGF, TGF-α, PD-ECGF, angiogenin, and TIMP-1. Overall, there were no significant differences in the angiogenic profile of the soluble matrix components of sarcomas and carcinomas.

In *in situ* hybridization studies with the antisense TIMP-1 probe, strong signals were observed corresponding to TIMP-1 expression within the stromal cells of the carcinomas (Fig. 6). The TIMP-1 signal was observed only within the stromal cells and not the carcinoma cells. Furthermore, there was a gradient of TIMP-1 expression being more greatly expressed within the stromal cells near the invading carcinoma cells. Signal intensities with the antisense TIMP-1 probe were 5–10-fold greater than those with the sense probe (background; Fig. 7).

**DISCUSSION**

In the present study, we have statistically analyzed a group of our sarcoma patient population for any correlation between vessel count and clinical outcome. This small group of patients was carefully chosen, because the tissues were obtained prior to any treatments such as surgery, radiation, and chemotherapy. The data here did confirm the importance of grade as a prognostic indicator in sarcomas. However, neovascularity, as determined by counting bursts of vessels stained with vWF antibody, had no prognostic significance. This conclusion is in agreement with two studies published previously of sarcoma patients. Ohsawa *et al.* (7) saw no correlation in 42 cases of malignant fibrous histiocytoma in Japan, and Saenz *et al.* (8) discovered the same result in 119 cases of soft tissue sarcomas at the Memorial Sloan-Kettering Cancer Center. We are confident that we adhered to the techniques described previously by Weidner *et al.* (5). We had used this technique successfully in the past to study a group of breast carcinoma cases and had at that time found a correlation between vessel count and known prognostic factors.
Angiogenesis is thought to be regulated by the balance of positive and negative factors in a particular microenvironment. The Western analysis performed showed that the stroma of the two tumor types contained both angiogenic stimulators and inhibitors. However, in situ hybridization with one angiogenic inhibitor, TIMP-1 (23), showed that it localized predominantly to the stromal fibroblasts and myofibroblasts and not within the carcinoma cells. In carcinomas, two compartments contribute to this balance. Angiogenesis may occur when the influence of one compartment exceeds the other. In areas near the malignant epithelial cells, angiogenic stimulation predominates and results in "bursts" of vessels. On the other hand, the region near the stromal fibroblasts and myofibroblasts would contain more angiogenic inhibitors and would be less vascular. Angiogenesis thus becomes an all-or-none phenomenon, dependent upon the contributions of both compartments. Classic clustered bursts of angiogenesis in carcinoma are thus the example of this phenomenon. Sarcomas, on the other hand, are governed predominantly by the contributions of a single compartment, the sarcomal cells themselves. Hence, their contribution would be more constitutive, stable, and predictable and not confused by the presence of another compartment. The diffuse vessel density of sarcomas that we have observed in the present study would be the consequence of the influence of a single compartment.

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**REFERENCES**


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