Increased Vascular Endothelial Growth Factor-C Expression Is Insufficient to Induce Lymphatic Metastasis in Human Soft-Tissue Sarcomas

Guy Lahat,1 Alexander Lazar,2 Xuemei Wang,4 Wei-Lien Wang,2 Quan-Sheng Zhu,1 Kelly K. Hunt,1 Raphael E. Pollock,1 and Dina Lev3

Abstract Purpose: Unlike carcinomas, soft-tissue sarcoma (STS) rarely exhibit lymphatic spread. Consequently, we examined expression and function of vascular endothelial growth factor (VEGF)-C and STS-associated lymphatic vessel density (LVD) components of this process. Experimental Design: VEGF-C and VEGF-A mRNA and VEGF-C protein expression were evaluated in STS, STS cell lines, and breast cancers (reverse transcription-PCR, quantitative reverse transcription-PCR, and ELISA). STS cell conditioned medium after VEGF-C knockdown was examined for endothelial cell proliferation and migration effects (MTS and migration assays). Paraffin-embedded human lymph node-negative and lymph node-positive STS and lymph node-negative and lymph node-positive breast cancers were examined for VEGF-C, D2-40, and CD31 expression (immunohistochemistry). LVD differences were analyzed by Wilcoxon rank-sum tests. Results: STS and breast cancer VEGF-C expression was comparable and higher than normal tissue levels. STS cells secreted functional VEGF-C: STS conditioned medium induced lymphatic endothelial cell proliferation and migration, which was abrogated by STS cell VEGF-C knockdown. STS and breast cancer intratumoral LVD was similar. STS peritumoral LVD (PT-LVD) was reduced versus breast cancer PT-LVD (P < 0.001). Significantly higher PT-LVD was observed in lymph node-negative versus lymph node-positive STS; lymphatic spreading STS subtypes also had higher LVD. STS VEGF-C expression and PT-LVD lacked correlation, and many lymph node-negative STS had high PT-LVD, suggesting complexity in this metastatic process. Conclusions: Compared with breast cancers, STS exhibited lower PT-LVD independent of VEGF-C expression, which may underlie STS lymph node metastasis rarity. Moreover, lymphatic vessels appear necessary but not sufficient to sustain STS lymphatic spread. Examining STS "nonlymphatic" dissemination may help elucidate mechanisms of lymphatic spread, insights critically important to cancer metastasis control.

Metastatic progression is a hallmark of cancer and the single most important factor affecting patient outcome, hence the remarkable efforts to unravel the molecular mechanisms underlying this complex process (1). Cancer cells metastasize to distant sites via vascular and/or lymphatic systems. Molecular factors underlying hematogenous metastasis are an intensive focus of investigation. Although regional lymphatic metastases are the first step of tumor dissemination in many human cancers, relatively little is known about the mechanisms contributing to this process. Analogous to blood-borne metastasis, lymphatic spread is a multistep process that includes tumor cell detachment, invasion into lymphatic channels, transport to locoregional lymph nodes, extravasation, survival, and growth within the lymph node microenvironment (2). Current thinking is that tumor cells gain access via expansion and invasion of preexisting lymphatics at the tumor periphery (3–5). The formation and invasion of new lymphatics within the tumor (lymphangiogenesis) may also be relevant, although this is not definitively resolved (6–9). Pertinently, recent preclinical studies suggest that lymphatic spread is an active process promoted by tumor-stroma crosstalk rather than one occurring passively when infiltrating tumor cells happen to encounter lymphatic vessels (10). Of many potential prolymphangiogenic factors, vascular endothelial growth factor (VEGF)-C is identified as a major effector (10). VEGF-C is a ligand of VEGF receptor-3 known to promote proliferation, migration, and survival of cultured human lymphatic endothelial cells (HLEC; refs. 11, 12). Genetically engineered mice conditionally overexpressing VEGF-C show

Authors' Affiliations: Departments of 1Surgical Oncology, 2Pathology, and 3Cancer Biology and 4Division of Quantitative Sciences, The University of Texas M. D. Anderson Cancer Center, Houston, Texas

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Requests for reprints: Dina Lev, Department of Cancer Biology, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Unit 1104, Houston, TX 77030. Phone: 713-792-1637; Fax: 713-563-1185; E-mail: dlev@mdanderson.org.

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Translational Relevance

Metastatic progression is the hallmark of cancer and the single most important factor affecting outcome. Although lymph node metastasis is a common first step of tumor dissemination in many human cancers, relatively little is known about the mechanisms contributing to this process. It is intriguing that, in contrast to the more common epithelial tumors, soft-tissue sarcomas (STS) only rarely exhibit lymphatic spread. We assessed STS lymphatic vessel density and vascular endothelial growth factor-C expression in a relatively large cohort of human samples and cell lines to evaluate their potential contribution to lymph node metastasis paucity. Our findings suggest that STS exhibit reduced lymphatic vessel density compared with breast carcinoma, a finding not attributable to decreased vascular endothelial growth factor-C expression and/or function. Further examination of STS could prove useful in elucidating the molecular basis of lymphatic metastasis, a process whose understanding will be critical to advancing cancer patients’ clinical care.

hyperplasia of lymphatic vessels, whereas VEGF-C-null mouse embryos completely lack lymphatic vasculature (13). Recent preclinical studies provide direct evidence that increased levels of VEGF-C promote active intratumoral and peritumoral lymphangiogenesis and lymphatic tumor spread to regional nodal basins (14, 15). Correlation between tumor-associated lymphatic vessel number and lymph node metastases has been postulated in several human malignancies including breast cancer, colon cancer, pancreatic cancer, prostate cancer, and melanoma (4, 16–18).

Soft-tissue sarcoma (STS) is a cohort of mesenchymal-origin malignancies that have unfavorable prognosis chiefly due to marked (especially pulmonary) metastatic propensity (19). It is biologically intriguing that, in contrast to the far more common epithelial malignancies, STS rarely exhibit lymphatic spread, a process observed in <5% of STS (20, 21). Although STS are a highly heterogeneous group of malignancies in which the more common subtypes, liposarcoma, leiomyosarcoma, and pleomorphic high-grade sarcoma, only rarely metastasize to lymph nodes, several subtypes exhibiting histologic patterns resembling epithelial (e.g., epithelioid sarcoma) or endothelial cancers (e.g., angiosarcoma) have a higher propensity for lymphatic spread with reported nodal metastatic rates ~40% (21). Manifestly, an enhanced molecular understanding of this well-established STS clinical observation could shed important light on the forces driving/blocking lymph node metastasis development.

One possible explanation for the lack of lymphatic metastasis is that STS exhibit insufficient VEGF-C expression and/or reduced lymphatic conduits. To critically examine this hypothesis, VEGF-C expression and function, lymphatic vessel density (LVD), and possible correlations with lymph node metastasis were evaluated in a cohort of human STS samples.

Materials and Methods

Cell lines and reagents. SKLMS1 (leiomyosarcoma), HT1080 and SW684 (fibrosarcoma), RD and RH30 (rhabdomyosarcoma), SW872 (liposarcoma), MES-SA (uterine sarcoma), A673 (Ewing sarcoma), and A204 (unclassified sarcoma) human STS cell lines as well as human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection. Primary cultures of HLEC and normal human fibroblasts were obtained from Promocell. Cells were cultured based on the providers’ recommendations. Commercially available monoclonal antibodies were used for immunohistochemical studies, including D2-40 (DAKO) to detect lymphatic endothelial vessels, CD31 (Santa Cruz Biotechnology) for vascular endothelial cell detection, anti-VEGF-C antibody (Santa Cruz Biotechnology), and appropriate secondary horseradish peroxidase-conjugated antibodies (Universal kit HRP; Biocare Medical).

Human tissue specimens. Under an institutional review board-approved protocol, formalin-fixed, paraffin-embedded STS and breast carcinoma specimens were retrieved from The University of Texas M. D. Anderson Cancer Center pathology archives, including 45 primary STS from patients with no evidence of lymphatic spread, 10 synovial sarcomas, 12 undifferentiated pleomorphic sarcomas, 11 clear cell sarcomas, 6 leiomyosarcomas, 2 rhabdomyosarcomas, and 4 liposarcomas. In addition, 11 primary STS specimens from patients with pathologically proven lymphatic metastasis were retrieved, including 7 undifferentiated pleomorphic sarcomas, 1 clear cell sarcoma, 1 synovial sarcoma, 1 liposarcoma, and 1 leiomyosarcoma. Ten lymph node-negative and 11 lymph node-positive primary breast cancer specimens were also stained and analyzed. Frozen specimens of human STS and breast cancer were also identified for further evaluation, including matching normal tissue when available. H&E-stained slides of all selected tumors were re-reviewed by an experienced STS pathologist (A.J.L.) to confirm presence of tumor and histologic subtype.

Reverse transcription-PCR. Quantitative and semiquantitative reverse transcription-PCR (RT-PCR) was done as described previously using relevant primers (22).

ELISA. VEGF-C levels were measured in tumor cell collected conditioned medium using ELISA done as per manufacturer’s instructions (R&D Systems).

Small interfering RNA knockdown of VEGF-C. SKLMS1 cells (2 × 10⁵ per well) were plated in 6-well plates and incubated overnight at 37°C. The following morning, SmartPool VEGF-C small interfering RNA (siRNA; Dharmacon and Santa Cruz Biotechnology) or non-targeting siRNA constructs was transfected using Lipofectamine 2000 (Invitrogen) reagents per manufacturer’s instructions. Mock-transfected cells were treated with Lipofectamine 2000 only. Incubation time for transfection reagents was 24 h, at which time medium was replaced with fresh regular medium. The next day, cells were harvested for RT-PCR, cell growth, and migration assays; conditioned medium was collected for ELISA.

Measurement of cell proliferation. Cell growth assays were done using CellTiter96 Aqueous Nonradioactive Cell Proliferation Assay kits (Promega) per manufacturer’s instructions. STS, HLEC, and HUVEC cell lines after VEGF-C knockdown with siRNA were plated at concentrations of 1.5 × 10³ to 5 × 10³ per well in 96-well plates. The growth rate was analyzed after 24 h. Absorbance was measured at a wavelength of 490 nm; absorbance values of treated cells are presented as a percentage of the absorbance of untreated cells.

Migration assay. The migration of SKLMS1, HLEC, and HUVEC cells through polycarbonate membranes (8 mm diameter pores) in 24-well BD Biocoeel cell culture chambers (BD Biosciences) was quantified as described elsewhere (23) with minor modifications.

Immunohistochemistry. Immunohistochemistry was done as described previously (22). Briefly, 4 μm thick sections were prepared from the formalin-fixed, paraffin-embedded tumor tissue block and were dried in a 60°C oven for at least 60 min. D2-40 and CD31 staining were conducted at The University of Texas M. D. Anderson Cancer Center clinical core laboratory using incubation in citrate buffer for 60 min at 100°C as antigen retrieval. The sections were placed in the automated Bond Max stainer, which pretreated the slides with additional enzyme-induced epitope retrieval for 2 min followed by

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incubation with relevant antibodies. Appropriate secondary antibodies and the Refine Polymer Detection kit (Leica) were used for immunostaining, with 3,3′-diaminobenzidine serving as chromagen. Appropriate positive and negative controls were run in parallel. For VEGF-C, staining sections were incubated with pepsin for 10 min in room temperature and overnight in 4°C with the primary antibody. Slides were incubated with a goat anti-rabbit horseradish peroxidase-conjugated (Universal kit HRP; Biocare Medical) secondary antibody for 1 h in room temperature. A positive reaction was detected by exposure to stable 3,3′-diaminobenzidine for 5 to 10 min.

Fig. 1. Human STS express VEGF-C to varying levels. A, quantitative RT-PCR measured relative VEGF-C expression levels in human breast cancer ([lymph node-negative (LN−) and lymph node-positive (LN+) and STS (lymph node-negative) specimens [values were normalized as fold change compared with the BRC5 (lymph node-negative) breast cancer sample]. B, VEGF-C mRNA levels in human STS specimens as above examined via RT-PCR (two leiomyosarcoma samples are missing for lack of sufficient RNA). VEGF-A expression is also shown (three VEGF-A isoforms: bottom band, VEGF121; middle band, VEGF165, and top band, VEGF189). C, VEGF-C mRNA expression is higher in human STS specimens than in adjacent normal tissue (RT-PCR). D, lower VEGF-C expression (assessed via immunohistochemistry) was shown in lymph node-negative breast cancer specimens compared with lymph node-positive. STS specimens exhibited similar VEGF-C expression as breast cancer samples. No difference in VEGF-C expression was observed between lymph node-negative and lymph node-positive STS. All original images were captured at ×200 magnification. BRC, breast cancer; FS, fibrosarcoma; MFH, malignant fibrous histiocytoma; LPS, liposarcoma; LMS, leiomyosarcoma; SS, synovial sarcoma; RMS, rhabdomyosarcoma; unclassified, unclassified sarcoma.
Microvessel density assessment. Immunohistochemical reactions for CD31 and D2-40 antigen were interpreted independently by an expert STS pathologist (A.J.L.). Up to five of the most vascularized areas within a tumor (“hot spots”) were chosen at low magnification (×20–×40) and vessels were counted in a representative high-magnification (×200) field in each of these areas. Blood vessel density for CD31 and LVD for D2-40 were calculated as the summation of all counts by two of the authors using a multiheaded microscope.

Statistical analysis. Cell culture-based assays were repeated at least three times and mean ± SD was calculated. Cell lines were examined separately. Microvessel densities were compared between different STS histologic subtypes and breast cancer using Wilcoxon rank-sum tests. Using median as a cutoff value, the association between the dichotomized peritumoral LVD (PT-LVD; above versus below median) and tumorsubtypewasassessedusingFisher’sexacttest. P

Results

STS express VEGF-C. STS are unique in that they rarely metastasize to lymph nodes. One potential molecular explanation for this intriguing observation is that these human tumors lack sufficient VEGF-C expression to sustain lymphangiogenesis. To consider this hypothesis, VEGF-C mRNA expression was examined in a panel of human STS. Frozen lymph node-positive and lymph node-negative breast cancer samples were used as controls. As expected, lymph node-positive breast cancers expressed higher VEGF-C mRNA levels than lymph node-negative samples. VEGF-C mRNA expression was detected at varying levels in a panel of 20 human STS tumors, none of which were derived from patients harboring lymph node metastasis (Fig. 1A and B). For several samples, normal adjacent tissue was also available; significantly higher VEGF-C mRNA expression was evident in the tumor compared with its normal tissue counterpart (Fig. 1C). As shown in Fig. 1, STS expressed an average normalized VEGF-C level similar to that of breast cancer (3.3 ± 4.1 and 3.4 ± 2.2 normalized expression levels in STS and breast cancer, respectively); several of the STS lymph node-negative exhibited higher VEGF-C levels than lymph node-positive breast cancers. Five of the samples examined were derived from patients who developed lung metastases; no correlation was observable between VEGF-C expression levels and hematogenous metastasis activity. Interestingly, the highest level of VEGF-C expression was observed in leiomyosarcoma, a STS subtype that has one of the lowest rates of lymphatic spread.

Next, the expression of VEGF-C protein was evaluated in human STS specimens. A large panel of clinically annotated primary human STS (n = 56) was subjected to immunohistochemical analysis; 11 of the samples were STS subtypes that exhibited lymphatic spread (Fig. 1D). Twenty-one primary breast carcinoma specimens were also used for comparison; of them, 11 were acquired from patients with pathologically proven lymph node metastases and 10 were from lymph node-negative patients. VEGF-C was scored as high in 57% of breast cancer samples (n = 12) and low in 43% of breast cancer samples (n = 9). A trend toward increased VEGF-C expression in lymph node-positive breast cancer samples was identified: 81.1% (9 of 11) of lymph node-positive samples expressed high VEGF-C, whereas only 33% (3 of 10) of lymph node-negative specimens exhibited high VEGF-C (P = 0.065). As a group, VEGF-C expression levels in STS specimens were comparable with those of breast cancer samples; high expression was shown in 55.3% (n = 31), whereas low expression was seen in 44.7% (n = 25). Most importantly, no correlation in STS was seen between VEGF-C expression and lymph node metastasis: 47.7% (21 of 44) of lymph node-negative samples expressed high VEGF-C levels, whereas 54.5% (6 of 11) of lymph node-positive highly expressed VEGF-C. These results refute the hypothesis that STS do not express VEGF-C at levels sufficient to sustain lymphangiogenesis; VEGF-C is highly expressed in many human STS, yet expression per se does not correlate with STS lymphatic spread.

Recently, several publications suggested that VEGF-A can also potentially promote lymphangiogenesis and lymphatic metastasis (24, 25). STS are highly angiogenic and a variety of studies have shown that these tumors exhibit increased VEGF-A expression (26, 27). To examine whether decreased VEGF-A expression accounted for lack of lymphatic metastasis in STS samples studied above, expression of VEGF-A mRNA was also considered. As shown in Fig. 1B, STS samples exhibited significant VEGF-A expression (VEGF-A 121, 165, and 189 isoforms). As depicted in this figure, the expression of VEGF-A isoforms varied between different specimens as well as within each sample; no correlation was found between VEGF-A (any and all isoforms) expression and outcome.

Human STS-secreted VEGF-C is functional and induces lymphatic endothelial cell proliferation and migration. Human VEGF-C cDNA encodes for the proVEGF-C that undergoes

Fig. 2. Human STS cell lines express VEGF-C to varying levels. A, RT-PCR showing VEGF-C mRNA expression in a panel of STS cell lines. VEGF-A expression is also shown. B, VEGF-C protein expression in STS cells as measured by ELISA. A correlation between mRNA and protein expression is seen. PC3 cells (prostate cancer) were used as control as they have been shown previously to express high VEGF-C levels. Several STS cell lines were shown to exhibit VEGF-C similar to those expressed by PC3 cells. Minimal expression was observed in normal human fibroblasts (NHF). H711089, fibrosarcoma; RD, rhabdomyosarcoma; SKLM1, leiomyosarcoma; SW584, fibrosarcoma; SW872, liposarcoma; RH30, rhabdomyosarcoma; A673, Ewing’s sarcoma; A204, unclassified sarcoma; MESSA, uterine sarcoma.
additional processing steps to become the mature, functional cytokine. Data above show the VEGF-C mRNA is highly expressed in a variety of STS; however, it is possible that inefficient processing results in a nonfunctional VEGF-C protein. To examine the activity of STS-expressed VEGF-C, we first evaluated VEGF-C expression in vitro in a large panel of human STS. VEGF-C mRNA expression was detected at varying levels in several STS cell lines tested; STS also exhibited high levels of VEGF-A mRNA (Fig. 2A). Next, we evaluated VEGF-C protein expression and secretion using STS conditioned medium examined by ELISA; PC3, a prostate cancer cell line known to express high levels of VEGF-C, served as control (Fig. 2B). Concordance between VEGF-C mRNA and protein levels in STS cell conditioned medium was observable. Only minimal expression was seen in normal human fibroblast conditioned medium.

A knockdown approach was used to evaluate the functional effects of STS-secreted VEGF. SKLMS1 (human leiomyosarcoma) cells were transiently mock-transfected (Lipofectamine 2000 only) or transfected with nontargeting siRNA or with an anti-VEGF-C siRNA. VEGF-C knockdown was confirmed via RT-PCR and ELISA (Fig. 3A); no effect on VEGF-A expression was shown after anti-VEGF-C siRNA transfection. To confirm the activity of STS-secreted VEGF-C, conditioned medium was collected from the SKLMS1 cells transfected as above and evaluated for effects on lymphatic endothelial cell proliferation and migration. A significant decrease in HLEC growth was shown when incubated with conditioned medium from VEGF-C knocked down SKLMS1 cells compared with mock-transfected and nontargeting siRNA SKLMS1 conditioned medium controls (P < 0.05; Fig. 3B), potentially reflecting the previously described effect of VEGF-C on endothelial cell proliferation as well as survival. Similarly, whereas HLEC cells were migratory when grown in SKLMS1 mock and nontargeting siRNA conditioned medium, a significant decrease in migration was shown when conditioned medium from SKLMS1 cells after VEGF-C silencing was used for HLEC incubation (P < 0.05; Fig. 4). In addition to its effects on lymphatic endothelial cells, VEGF-C has been shown previously to affect the growth and migration of vascular endothelial cells. To further confirm that STS-secreted VEGF-C is functional, the effect of SKLMS1 conditioned medium on the growth and migration of HUVEC cells was evaluated in a set of experiments as done above. Incubation of HUVEC in conditioned medium from SKLMS1 VEGF-C knockdown cells resulted in significant inhibition of cell growth and migration (P < 0.05; Figs. 3B and 4). To rule out the possibility that the results of the migration assays reflect the effect of decreased VEGF-C on endothelial cell proliferation/survival rather than direct effect on motility, we determined the number of viable endothelial cells after exposure to the different conditioned medium for 8 h. Equal numbers of endothelial cells were grown in conditioned medium from mock-transfected SKLMS1 cells compared with mock-transfected and nontargeting siRNA SKLMS1 conditioned medium controls (P > 0.05; Fig. 4).
medium corresponding to those used for the migration assays in a 24-well plate, and after 8 h, the number of viable cells was counted. Up to 20% reduction in number of cells was observed in wells containing anti-VEGF-C SKLMS1 conditioned medium. Overall, the decrease in number of viable cells at this period (<20%) was much less pronounced than the decrease in number of migrating cells (>50%). These results thus confirmed that reduced STS-secreted VEGF-C directly inhibited the migration of endothelial cells. This entire set of experiments was further repeated using a second unrelated set of siRNAs for VEGF-C knockdown in SKLMS1 and results were reproducible (data not shown), thus significantly reducing the possibility that the findings are secondary to siRNA off-target effects.

Recently, it has been shown that VEGF-C can potentially exert biological effects on cancer cells per se, resulting in tumor progression by regulating migration and invasion, proliferation, and resistance to chemotherapy (15, 28). To examine whether VEGF-C exhibits similar effects in STS, the effect of VEGF-C silencing on SKLMS1 growth (Fig. 3B), migration, and chemosensitivity was evaluated (data not shown). Although SKLMS1 cells express high level of VEGF-C, these autocrine functions were not observed. Taken together, these data suggest that VEGF-C secreted by STS cells growing in culture is functional and has paracrine effects on both lymphatic and vascular endothelial cells yet is not sufficient to induce lymphatic spread of STS.

**STS exhibit decreased PT-LVD compared with breast cancer.** Although STS express highly active VEGF-C, the possibility still remains that this cytokine is not sufficient to induce enough tumor-associated lymphatics needed to sustain metastasis. To evaluate STS LVD, the panel of primary human STS and breast cancer specimens used above was subjected to further immunohistochemical analysis. LVD and blood vessel density were quantified after immunostaining with D2-40 (which recognizes human podoplanin, a mucin transmembrane glycoprotein expressed by lymphatic endothelial cells but not by vascular endothelial cells) and CD31, respectively (Fig. 5). STS and breast cancer samples exhibited high vascularity (Supplementary Table S1; Fig. 5); interestingly, CD31 counts were significantly higher in lymph node-positive samples compared with lymph node-negative specimens. No significant difference in intratumoral LVD (IT-LVD) was shown comparing STS and breast cancer samples (median, 12 versus 18, respectively; Supplementary Table 1). IT-LVD did not differ significantly between lymph node-negative and lymph node-positive breast cancer; however, a trend toward significantly higher IT-LVD was observed in samples derived from lymph node-positive STS ($P = 0.09$). Median PT-LVD calculated for all STS examined was 23 (range, 0-86) compared with 43 (range, 5-65) for breast cancer specimens, suggesting that as a group STS harbor a statistically significant ($P < 0.001$) lower number of lymphatic vessels at the tumor margin, despite similar amounts of VEGF-C expression, than do breast cancers (Supplementary Table S1; Fig. 6A). Interestingly, tumor cell emboli within tumor-associated lymphatic vessels were commonly observed in breast cancer specimens, especially in the lymph node-positive tumors (Fig. 5); in contrast, lymphovascular invasion was rarely seen in STS samples. Finally, PT-LVD was higher than IT-LVD in both STS and breast cancer samples ($P = 0.01$).

Previously published data suggest that PT-LVD levels correlate with breast cancer lymphatic spread (4, 5). Comparable results were observed in our cohort of breast cancer samples: median PT-LVD of lymph node-negative samples was 30 compared with 52 in lymph node-positive samples ($P = 0.002$; Fig. 6B). Although the median PT-LVD level in STS was significantly lower than that of breast cancer, PT-LVD counts in lymph node-positive STS were higher than in lymph node-negative specimens (median, 31 versus 19, respectively; $P = 0.02$; Supplementary Table S1). However, in contrast to breast cancer, a large proportion of STS lymph node-negative samples exhibited PT-LVD rates above the median (Fig. 6B): 18
of STS versus 1 (10%) of breast cancer specimens with high LVD counts (LVD count > median) were found in lymph node-negative specimens ($P = 0.04$).

STS are a heterogeneous group of tumors; although lymphatic spread is unusual for most histologies, several uncommon STS subtypes (e.g., clear cell sarcoma) exhibit a higher propensity for lymph node metastasis. To evaluate whether such rarer STS exhibit a higher rate of tumor-associated lymphatics, we compared the PT-LVD of the 11 primary lymph node-negative clear cell sarcoma specimens included in our cohort with the other 34 lymph node-negative STS specimens. The median PT-LVD of clear cell sarcoma specimens was significantly higher compared with that of other nonmetastatic STS subtypes [29 (range, 5-51) versus 17 (range, 0-45), respectively; $P = 0.05$]. Lastly, we examined if IT-LVD and PT-LVD of all STS specimens correlated with VEGF-C expression levels using the same collection of samples as described above; no statistically significant correlation between VEGF-C expression level and LVD could be identified in these human STS specimens. Median PT-LVD within the group of specimens expressing low levels of VEGF-C was 28 (range, 0-86) compared with 29 (range, 0-51) within the group of specimens expressing high levels of VEGF-C ($P = 0.09$).

Taken together, our data suggest that although STS express VEGF-C at levels comparable to breast cancer, they exhibit significantly lower levels of tumor-associated lymphatic vessels, potentially contributing to their reduced propensity for lymphatic spread. This hypothesis is strengthened by the observation that lymph node-positive STS as well as STS histologic subtypes with a higher lymph node metastasis rate do exhibit higher tumor-associated LVD. However, the complexity of the metastatic process is further illustrated by our studies, which show that peritumoral lymphatics might be necessary but not sufficient for lymph node metastasis development, with many lymph node-negative STS exhibiting relatively high PT-LVD rates. Using STS as an investigative model to elucidate additional contributing factors and mechanisms may enhance the understanding of solid tumor lymphatic spread.

**Discussion**

The lymphatic system is a main route of tumor metastasis, especially for tumors of epithelial origin such as breast, lung, and the gastrointestinal tract as well as melanoma, where tumor cells frequently colonize draining regional lymph nodes. Although the physiologic importance of the lymphatic system has been recognized for centuries, its involvement in the metastatic cascade has been less extensively evaluated than the intensive investigations of hematogenous tumor spread.
Lymphatic metastasis is a complex process controlled by multiple factors produced by a variety of cell types. The functional consequences of this complex regulation no doubt depend on the interplay between these factors. A better understanding of the molecular events underlying interactions between tumor cells and host factors resulting in lymphatic dissemination will hopefully lead to effective therapies capable of preventing such adverse occurrences.

More than a hundred years ago, Paget first showed that metastases do not occur arbitrarily but instead are highly dependent on interaction between the tumor (seed) and the microenvironment (soil; ref. 29). The well-established and intriguing clinical observation that human STS rarely metastasize to lymph nodes is an example of the seed and soil phenomenon and as such may present a unique model to further elucidate molecular factors regulating lymphatic tumor spread.

Tumor-associated lymphatics are thought to be the major conduit for tumor cell spread into the lymphatic system. A correlation between lymphatic (intratumoral and/or peritumoral) vessel density and the propensity for lymphatic spread has been identified in a variety of human malignancies (4, 16–18). STS are frequently very angiogenic, harboring a large number of tumor-associated blood vessels; an association between STS blood vessel density and systemic spread, especially to the lungs, has been described (30). However, not much is known about STS-associated LVD and whether a paucity or absence of tumor-associated lymphatics could underlie a markedly lower tendency for lymphatic dissemination. In a recently published series, 16 of 32 sarcomas exhibited intratumoral lymphatics and LVD that was comparable or even higher than that of five human carcinoma controls. However, only one of the samples was retrieved from a sarcoma harboring lymph node metastasis (31). Similarly, in the larger panel of sarcomas presented here, no significant difference in IT-LVD was observed comparing STS and breast cancer samples. Interestingly, lymph node-positive STS exhibited a higher IT-LVD compared with lymph node-negative STS, trending toward statistical significance and possibly suggesting a role for lymphangiogenesis in STS lymph node metastasis; no such correlation could be shown for the breast cancer samples evaluated. The importance of intratumoral lymphatics in lymphatic tumor spread is currently an unresolved issue. Immunohistochemical studies have shown previously the existence of proliferating intratumoral lymphatics in several human tumors (6, 7, 9). In addition, several studies have reported IT-LVD as an accurate independent predictor of poor disease-specific survival and increased metastatic propensity in tumors such as melanoma, breast cancer, endometrial cancer, colon, lung, prostate, ovary, pancreas, and head and neck (6, 9, 32–35). Such findings notwithstanding, the functional significance of intratumoral lymphatic vessels is still unresolved. Intratumoral lymphatics may not be able to transport tumor cells because the elevated hydrostatic pressure within the tumor may compress these vessels. Padera et al. showed that the center of tumors do not contain functional lymphatics and that lymphatic vessels at the periphery of tumors are the conduit for metastasis (3). Peritumoral lymphatics are the vessels immediately adjacent to the tumor, and these may represent preexisting vessels compressed into the peritumoral rim by the expanding tumor mass (3). Moreover, peritumoral lymphatic endothelial cells can proliferate, implying that these vessels could possibly arise secondary to lymphangiogenesis (3). Several studies have identified PT-LVD in a variety of malignancies as significantly associated with regional metastasis and poor disease outcome (36–38). To the best of our knowledge, PT-LVD has not been previously evaluated in STS. Our study suggests that, as a group, STS exhibit a significantly lower PT-LVD compared with breast cancer. Moreover, STS with proven lymphatic metastases do express a higher rate of peritumoral lymphatics, as do STS histologic subtypes with a higher propensity for lymphatic spread. These findings highlight the importance of peritumoral lymphatic vessel density in lymphatic progression. The relatively decreased PT-LVD in STS may at least partially explain the rarity of lymphatic spread in STS. However, our data also show that a sizable number of lymph node-negative STS do exhibit peritumoral lymphatics. This observation illuminates the complexity of the metastatic process and
implies that the mere presence of lymphatic vessels in or around STS, although needed for lymphatic spread, are not sufficient per se for this process to occur. Additional factors facilitating the invasion of sarcoma cells into the lymphatic vasculature, survival within the lymphatics, extravasation, and growth in the lymph node microenvironment are also necessary. For example, it is possible that, even when lymphatic vessels are present, STS cells exhibit a reduced capacity for lymphatic invasion. This hypothesis is supported by our finding that intralymphatic tumor cell aggregates are commonly observed in breast cancer samples but are only rarely identified in STS specimens. Several reports have shown that lymphovascular invasion is an independent predictor of lymph node metastasis in various cancers (39, 40). Lymphovascular invasion may reflect the number of tumor cells penetrating into the lymphatic system; theoretically, lack of lymphovascular invasion in STS may reflect a diminished ability of STS cells to invade into the peritumoral lymphatics. This observation should be further evaluated; additional studies examining the capacity of STS tumor cells to adhere and invade into lymphatics are currently ongoing.

Recent investigations suggest that tumor cells as well as tumor-associated stromal cells produce a variety of cytokines that potentially affect lymphatic spread of malignancy (10). Of these, VEGF-C and its signaling through VEGF receptor-3 expressed on lymphatic endothelium may play a seminal role in this process. VEGF-C demonstrably promotes lymphangiogenesis and lymphatic endothelial hyperplasia in preexisting peritumoral vessels, leading to an increased contact area between neoplastic cells and lymphatic conduits (41). Moreover, VEGF-C enhances lymphatic vessel permeability, modulates the adhesion properties of lymphatic endothelium, regulates the expression of surface chemotactic factors on these cells, and promotes tumor cell entry into the lymphatic system (41, 42). Multiple studies have shown that VEGF-C expression level correlates with lymphatic spread in a variety of cancers (3, 43–46). Due to its fundamental role in lymph node metastasis induction, lack of or reduced VEGF-C expression and/or function could be a candidate explanation for the lower LVD identified in our study. In support of this hypothesis, several previously published studies using the T241 murine fibrosarcoma cell line have shown that, by forcefully overexpressing VEGF-C, this fibrosarcoma regained the ability to metastasize to lymph nodes without a change in the proliferation, migration, or tumor growth characteristics of these cells (3). Increased intratumoral and peritumoral lymphatics were noted, although intratumoral vessels were deemed nonfunctional (3). However, data presented in this current study tend to argue against a model that high levels of VEGF-C are sufficient to cause PT-LVD and lymph node metastasis. We find high VEGF-C expression levels in human STS specimens relative to normal tissues, levels that are comparable with those of breast cancer. In addition, human STS cell lines were shown to highly express functional VEGF-C in vitro.

To the best of our knowledge, only limited information concerning VEGF-C expression in human STS has been previously published. An initial assessment published after the characterization of VEGF-C included a small number of human STS specimens; VEGF-C was shown to be overexpressed (11). In a recently published correspondence, immunohistochemistry identified increased VEGF-C in a panel of human STS specimens (31). A large-scale gene expression analysis using a 12,601-feature cDNA microarray platform was used to examine 181 tumors representing 16 classes of human bone and STS; VEGF-C was overexpressed in several STS histologic subtypes, especially in high-grade pleomorphic sarcoma (malignant fibrous histiocytoma) and liposarcoma (47). Taken together, results of these studies show that VEGF-C is highly expressed in several STS histologic subtypes that lack the propensity to metastasize to lymph nodes. Therefore, it can be concluded that, unlike a variety of other cancers, VEGF-C expression in STS does not correlate with the development of lymph node metastasis, cannot be used as a prognostic factor for lymphatic spread, and that the paucity of STS lymph node metastasis cannot be attributed to a lack of this important cytokine.

Recently, it has been shown that tumor-derived VEGF-A promotes expansion of lymphatic networks within the tumor and within draining lymph nodes even before actual tumor metastasis (48). VEGF-A has been shown to induce a lymphangiogenic response in tumors expressing this factor at high levels, and these tumors show increased rates of lymph node metastases (25, 48). Our results, as well as several previously published reports, rule out the lack of VEGF-A as a factor contributing to the rarity of STS lymphatic spread in that these tumors “do” express high levels VEGF-A and are highly vascular (27).

Taking into account that VEGF-C and VEGF-A are not sufficient to induce lymphatic metastases in STS, it is possible that a deficiency in other essential factors may be the underlying paucity of lymphatic metastasis in this tumor system. Among such potential factors are those inducing tumor cell homing to lymphatics/lymph nodes. For example, the chemokine CCL21 is secreted by lymphatic endothelium but not by blood vascular endothelium; it interacts with the chemokine receptor 7 (49, 50). CCL21 mediates homing of lymphocytes and migration of antigen-stimulated dendritic cells from tissues into lymphatic vessels and secondary lymphatic organs, thereby playing an important role in immune-related and inflammatory processes (49). Recently, CCL21 has been shown to enhance lymph node metastasis of CCR-7-expressing malignant melanoma xenografts (50). Using lymph node-negative and lymph node-positive STS to further evaluate the expression of CCR-7 as well as the identification of other such factors could further our understanding of the metastatic process.

In summary, presented here are initial molecular insights into the intriguing clinical observation that STS rarely metastasize to lymph nodes. Further use of STS as a model for the study of lymphatic dissemination could help elucidate the molecular forces driving the development of lymphatic metastases. Understanding mechanisms essential for this process may be highly relevant to achieving advances in the management of cancer patients.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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