Vascular Endothelial Growth Factor Overexpression by Soft Tissue Sarcoma Cells: Implications for Tumor Growth, Metastasis, and Chemoresistance

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Abstract
To better elucidate the role of vascular endothelial growth factor (VEGF) in soft tissue sarcoma (STS) growth, metastasis, and chemoresistance, we generated stably transfected human STS cell lines with VEGF to study the effect of VEGF on STS cells in vitro and the effect of culture medium from these cells on human umbilical vascular endothelial cells. Severe combined immunodeficient mice bearing xenografts of transfected cell lines were used to assess the effect of VEGF overexpression and the effect of VEGF receptor (VEGFR) 2 inhibition on STS growth, metastasis, and response to doxorubicin. VEGF-transfected xenografts formed highly vascular tumors with shorter latency, accelerated growth, enhanced chemoresistance, and increased incidence of pulmonary metastases. Blockade of VEGFR2 signaling using DC101 anti-VEGFR2 monoclonal antibody enhanced doxorubicin chemoresponse; this combined biochemotherapy inhibited tumor growth and decreased pulmonary metastases without overt toxicity. Combined therapy reduced microvessel counts while increasing vessel maturation index. VEGF overexpression did not affect the sarcoma cells per se; however, conditioned medium from VEGF transfectants caused increased endothelial cell proliferation, migration, and chemoresistance. Addition of DC101 induced endothelial cell sensitivity to doxorubicin and suppressed the activity of matrix metalloproteinases secreted by endothelial cells. We therefore conclude that VEGF is a critical determinant of STS growth and metastasis and that STS chemoresistance, in our model, is a process induced by the interplay between STS cells and tumor-associated endothelial cells. STS growth and metastasis can be interrupted by combined low-dose doxorubicin and anti-VEGFR2, a strategy that attacks STS-associated endothelial cells. In the future, such therapeutic approaches may be useful in treating STS before the development of clinically apparent metastases.

Introduction
Five-year survival for soft tissue sarcoma (STS) patients is ~50% depending on tumor size, grade, location, the presence of regional or distant disease, and response to therapy (1). Metastases, mainly to the lung, are particularly ominous. The need to successfully control STS dissemination by systemic approaches that can reach total body disease loci remains compelling. However, the use of chemotherapy in STS is problematic due to toxicity, expense, and marked chemoresistance. Even doxorubicin, the single most active agent in STS, has a disappointing overall 30% response rate, with breakthrough tumor progression and frequent recurrence after initial sarcoma chemoresponsiveness (2). Although progression, metastasis, and chemoresistance in STS have been characterized clinically, the molecular mechanisms regulating these tumor processes are less well understood.

Previously, we have examined various molecular mechanisms of chemoresistance in STS (3–5). These efforts have primarily considered processes within the STS cell per se and have not directly addressed the reality that STS are not composed solely of malignant cells but additionally include a diverse ensemble of host cells that are recruited into the tumor. It is likely that therapies targeting both sarcoma cells and otherwise normal tumor-associated cells may lead to improved STS treatment. The likelihood of greater genetic stability in co-opted microenvironment cells than in the STS tumor cells would presumably lead to their being less likely to acquire chemoresistance during the toxic stress selection of chemotherapy (6).

Endothelial cells play a crucial role in tumor development and progression. It is now well established that a tumor must initiate and sustain new blood vessels from preexisting vessels (angiogenesis) and from the recruitment of circulating endothelial progenitor cells (vasculogenesis; refs. 7, 8) if it is to grow beyond a few millimeters (9). This multistep process is regulated by the local balance of endogenous proangiogenic and antiangiogenic factors, where vascular endothelial growth factor (VEGF) plays a critical role. The biological relevance of VEGF in STS has been suggested by clinical studies showing impaired prognosis in patients who have elevated VEGF levels (10). However, the relationship between tumor VEGF levels and either progression of human STS or the therapeutic efficacy of chemotherapy remains elusive.

In light of these considerations, we studied the effect of VEGF expression levels on (a) the progression of human STS and (b) chemotherapeutic efficacy in STS, by generation of VEGF-overexpressing stable transfectants capable of forming metastasizing xenografts in severe combined immunodeficient (SCID) mice. We have been able to show the remarkable effect of VEGF expression levels on both metastatic STS activity and doxorubicin
chemoresistance, the latter process involving VEGF effects on endothelial cell sensitivity to doxorubicin rather than on STS tumor cells per se. An increased understanding of STS tumor cell microenvironment cell interactions may be critical if improved and clinically relevant therapeutics are to be developed.

Materials and Methods

Cell lines and stable transfectant generation. Human SKLMS-1 leiomyosarcoma, RD rhabdomyosarcoma, and the human umbilical vascular endothelial cell (HUVEC) lines were purchased from the American Type Culture Collection (ATCC; Rockville, MD) and cultured according to ATCC recommendations. The full-length human VEGF165 cDNA was cloned into pcDNA3 expression vector with transcription driven by a cytomegalovirus enhancer-promoter (kindly provided by Dr. Lee M. Ellis, M.D. Anderson Cancer Center, Houston, TX) and also contained the neomycin resistance gene. Subconfluent SKLMS-1 or RD cells were transfected; individual G418-resistant colonies were cloned and expanded as described previously (11). Sarcoma cell conditioned medium was prepared and stored as described previously (12).

Northern blot and Western blot analyses. Northern and Western blot analyses were done as described previously (13). Western blot primary antibodies included monoclonal mouse anti-human-VEGF (R&D Systems, Minneapolis, MN) and goat polyclonal anti-human angiopoietin-2 (Ang-2; Santa Cruz Biotechnology, Santa Cruz, CA). To detect Ang-2 expression in HUVECs, cells were treated with VEGF (10 ng/ml) and CoCl₂ (50 μmol/L) for 24 hours.

Proliferation, chemosensitivity, migration, and chemoinvasion assays. Endothelial cell growth assays were done as described previously (5). For in vitro treatment experiments, HUVECs were treated with control rat IgG (10 μg/ml), doxorubicin (0.0625 μmol/l), DC101 (10 μg/ml), or DC101 (10 μg/ml) plus doxorubicin (0.0625 μmol/l) for 1 hour. Conditioned media from VEGF165 transfectants and controls were added to the HUVECs. Migration and proliferative abilities were determined. Tumor cell growth assays were done using 96-well plates (2,000 cells per well). Cell growth was quantitated using crystal violet staining (5). Growth rate was analyzed 24, 48, and 72 hours after treatment with doxorubicin (doses range, 0-500 μmol/l), DC101 (10 μg/ml), or DC101 (10 μg/ml) plus doxorubicin (0.1 μmol/l). Tumor cell invasion was assessed as described previously (14).

cDNA microarray. Total RNA was extracted from SKLMS-1, SKLneo, and SKLSE-10 cells using Trizol reagent (Life Technologies, Gaithersburg, MD). RNA quality was checked and then used to synthesize cDNA as a template for generating biotin-16-UTP (nonradioactive)–labeled cRNA target using TrueLabelingAMP linear RNA amplification kit (SuperArray Bioscience Corp., Frederick, MD). Oligo GEArray Human Toxicology and Drug Resistance Microarray (SuperArray Bioscience) was used to characterize expression of 263 genes. Prehybridization (2 hours) and hybridization (overnight) was done at 60°C in a hybridization oven using 6-μg labeled cRNA target. SDS membranes (0.5%) underwent chemiluminescent detection. Array data were analyzed using the GEArray Expression Analysis Suite.

Zymography. HUVECs were incubated with no therapy, IgG, DC101, and/or doxorubicin in VEGF-enriched serum-free medium for 48 hours. Culture supernatants were collected, and equimolar amounts of concentrated samples were subjected to 10% SDS-PAGE gel. After electrophoresis and 16 to 24 hours of incubation, the gels were stained with Coomassie brilliant blue and destained. Equimolar loading was verified by comparisons of staining patterns on the gel.

Animal studies. Female SCID mice were obtained from Taconic Farms (Germantown, NY). Animals received humane care as per the Animal Welfare Act and the NIH "Guide for the Care and Use of Laboratory Animals." Experiments were carried out under a protocol approved by the Institutional Animal Care and Use Committee at The University of Texas M.D. Anderson Cancer Center (Houston, TX). Trypan blue staining–confirmed viable sarcoma cells, 1 x 10⁶ in 0.1 ml HBS, were injected s.c. into the flank of 5- to 6-week-old SCID mice. Tumors were measured twice weekly and resected on reaching 1.5 cm in diameter, weighed and frozen at −140°C, or fixed in a 10% formalin solution and paraffin embedded. Following tumor resection, mice were kept alive for up to 13 weeks to allow lung metastasis development. Metastases were enumerated after India ink instillation (15); only nodules that exceeded 0.5 mm in diameter were counted. Animals

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**Figure 1.** Establishment and characterization of VEGF165-overexpressing sarcoma cell lines. A, Western blot screening of a panel of STS cells for the expression of VEGF. B, Northern blot analysis of VEGF165 mRNA expression. Total RNA (20 μg) was analyzed for VEGF165-transfected clones, vector control clones, and parental cell lines. An ~1 kb exogenous VEGF transcript was detected in the VEGF165-transfected leiomyosarcoma cell lines SKLSE1O, SKLSE13, and SKLSE 23 and the rhabdomyosarcoma cell lines RDSE14, RDSE17, and RDSE26. The indicated glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was probed as a loading control. C, Western blot analysis of VEGF protein expression in the conditioned medium of sarcoma cells. The concentrated conditioned medium from VEGF165-transfected cell lines and control cell lines was analyzed under nonreduced condition. D, Biological activity of overexpressed VEGF165 protein as evaluated by a proliferation assay. Conditioned medium of the VEGF165-overexpressing cells and control cells was added to HUVEC, and assays were done as described in Materials and Methods. Assays were done in triplicate. Bars, SD. **, *P < 0.001 versus control.**
bearing sarcoma xenografts (eight mice per group) began receiving treatment when the tumor diameters reached 5 mm. DC101 (ImClone Systems, Inc., New York, NY), an anti-VEGF receptor (VEGFR) 2 rat IgG1 monoclonal antibody (16), was given i.p. at a dose of 400 \( \mu \)g every 3 days for a total of seven times. Doxorubicin (6 mg/kg; American Pharmaceutical Partners, Los Angeles, CA; conventional schedule) was given twice every 2 weeks, and doxorubicin (1.2 mg/kg; continuous low-dose schedule) was given every 3 days for a total of eight times. Combined treatment (400 \( \mu \)g DC101 plus 1.2 mg/kg doxorubicin) was given once every 3 days for a total of eight times. Control animals bearing s.c. flank tumors were treated with normal rat IgG (400 \( \mu \)g) or saline following the same schedules.

**Histologic and immunohistochemical analysis.** Conventional H&E staining was done and examined by light microscopy. Immunohistochemistry was done using anti-mouse CD31 (1:50; PharMingen, San Diego, CA), anti-mouse \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA; 1:80, Neomarkers, Fremont, CA), anti-mouse Ang-2 (1:50), and anti-Ki67 (1:500; DAKO Carpinteria, CA). For microvessel counts, frozen tissues were sectioned and stained with anti-CD31 antibody and then counted at \( \times 200 \) magnification. For pericyte analysis, frozen sections were stained for \( \alpha \)-SMA. Blood vessels associated with \( \alpha \)-SMA-positive cells were considered mature (17). Sections were stained with \( \alpha \)-SMA and with anti-CD31, which stains both mature and immature vessels. The CD31+ and \( \alpha \)-SMA-positive versus CD31+ vessels were scored as percent SMA-positive vessels (vessel maturation index).

**Results**

SKLMS-1 and RD cell lines expressing low and medium levels of endogenous VEGF, respectively (Fig. 1A), were stably transfected with VEGF\(_{165}\) and resultant cell lines were designated SKLSE and RDSE. Transfection with the pcDNA3 plasmid containing a neomycin-resistant selection marker gene alone resulted in SKLneo and RDneo used as controls.
Northern blots of VEGF\textsubscript{165} mRNA expression (Fig. 1B) and Western blots for VEGF\textsubscript{165} protein expression (Fig. 1C) showed increased VEGF\textsubscript{165} mRNA and protein levels in the selected transfectants. Conditioned medium from the parental cells, vector control cells, and the VEGF\textsubscript{165}-overexpressing transfectants was compared for ability to stimulate HUVEC proliferation (Fig. 1D). HUVEC proliferation stimulated by the conditioned medium of VEGF\textsubscript{165}-overexpressing cells was significantly higher, showing that the overexpressed VEGF\textsubscript{165} from the transfectants was biologically active.

Xenografts from VEGF\textsubscript{165}-transfected cell lines SKLSE\textsubscript{10}, SKLSE\textsubscript{13}, SKLSE\textsubscript{23}, and RDSE\textsubscript{14}, RDSE\textsubscript{17}, and RDSE\textsubscript{26} had average latency periods of 14, 16, 17, days, and 17, 15, and 18 days, respectively. In contrast, xenografts of control and parental cell lines SKLneo, SKLMS-1, RDneo, and RD had much longer average latency periods of 26, 27, 29, and 31 days. Tumors from VEGF\textsubscript{165}-overexpressing SKLSE and RDSE cells grew faster than those from vector control or parental cells ($P < 0.001$; Fig. 2A) as can also be seen visually (Fig. 2B). During tumor removal, we noted that VEGF\textsubscript{165}-overexpressing xenografts adhered more tightly to adjacent normal tissues. H&E staining of primary tumor margins

Table 1. Incidence of pulmonary metastases in SCID mice harboring a human STS

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Pulmonary metastases</th>
<th>Frequency*</th>
<th>No. nodules mean (range)</th>
<th>Size (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKLMS-1</td>
<td>0/8</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKneo</td>
<td>0/8</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SKLSE\textsubscript{10}</td>
<td>5/8 ($^b$ &lt; 0.02)</td>
<td>9 (0-16)</td>
<td>0.6-3.2</td>
<td></td>
</tr>
<tr>
<td>SKLSE\textsubscript{13}</td>
<td>3/8 $^b$</td>
<td>4 (0-12)</td>
<td>0.8-2.4</td>
<td></td>
</tr>
<tr>
<td>SKLSE\textsubscript{23}</td>
<td>4/8 $^b$</td>
<td>5 (0-12)</td>
<td>0.5-2.2</td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>0/8</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDneo</td>
<td>0/8</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>RDSE\textsubscript{14}</td>
<td>4/8 ($^c$ &lt; 0.01)</td>
<td>6 (0-12)</td>
<td>0.5-2.1</td>
<td></td>
</tr>
<tr>
<td>RDSE\textsubscript{17}</td>
<td>5/8 $^b$</td>
<td>8 (0-17)</td>
<td>0.6-2.6</td>
<td></td>
</tr>
<tr>
<td>RDSE\textsubscript{26}</td>
<td>4/8 $^b$</td>
<td>8 (0-13)</td>
<td>0.5-1.8</td>
<td></td>
</tr>
</tbody>
</table>

NOTE: The overexpression of VEGF\textsubscript{165} in cell lines was significantly associated with pulmonary metastasis compared with control lines.

* Mice with metastasis/mice with xenograft.

$^b$ Lung nodules $>0.5$ mm in diameter were counted.

$^c$ SKLSE group: $P < 0.02$.

$^b$ RDSE group: $P < 0.01$, Fisher’s exact test.

Figure 3. Overexpression of VEGF\textsubscript{165}-enhanced tumor angiogenesis. Histologic sections of xenografts of VEGF\textsubscript{165}-transfected, vector control, and parental cell lines were immunostained for endothelial cells with antibody against CD31 (A and B) and Ang-2 (D). A, microvessel counts in sections of xenografts from the indicated STS lines show a significant ($^*$, $P < 0.001$) increase in microvessels relative to the control cells. B, examples of microvessel staining with anti-CD31 in sections of SKLSE\textsubscript{10} cells and SKLneo. Ang-2 expression is increased in endothelial cells in vitro (C) and in vivo (D) following exposure to VEGF\textsubscript{165}. C, Western blot of conditioned medium from HUVECs showed greatly increased Ang-2 secretion in vitro following treatment with VEGF\textsubscript{165} and cobalt chloride. D, immunohistochemical staining for VEGF and Ang-2 in xenograft tumor sections showed high Ang-2 expression in tumor vessel endothelial cells of tumors overexpressing VEGF\textsubscript{165}.

Figure 3.
revealed that parental and control cell line xenografts abutted normal tissues, whereas VEGF165-overexpressing tumors directly invaded adjacent fat and muscle tissue (Fig. 2C).

Because high levels of VEGF are prognostic for chemoresistance in patients bearing certain malignancies (18), we asked if high VEGF165 expression levels could negatively affect doxorubicin efficacy in the human STS xenograft model. Doxorubicin inhibited the xenograft growth of controls much more than the growth of SKLSE10 (Fig. 2D, left) and RDSE17 (Fig. 2D, right); response rates for SKLSE10 and RDSE17 tumors were 58.4% and 66.3% of those for SKLneo and RDneo, respectively.

Animal body weights were measured at regular intervals to evaluate chemotoxicity (19). Significant weight loss was observed in all STS xenograft–bearing mice treated with conventional schedule doxorubicin (data not shown).

As shown in Table 1, pulmonary metastases were not detected in any of the mice bearing parental or control cell xenografts. In contrast, significant metastatic activity was detected in mice bearing VEGF165-overexpressing xenografts, suggesting that increased VEGF165 expression in SKLSE and RDSE cells enhanced their metastatic potential in vivo.

Next, we investigated whether the accelerated tumor growth and metastasis could be due to tumor angiogenesis stimulated by VEGF165. Xenografts from VEGF165-overexpressing cell lines had much higher microvessel counts (P < 0.001; Fig. 3A) and microvessel density (data not shown) than did control or parental cell line xenografts. Figure 3B depicts representative examples of xenograft microvessel appearance in tumors from VEGF165-overexpressing SKLSE10 cells and control SKLneo cells, showing that enhanced VEGF165 expression increased intratumoral angiogenesis of human STS.

Ang-2, a potent proangiogenic switch factor, can be induced by tumor-derived VEGF signaling through endothelial cell VEGFR2, resulting in destabilized host vasculature (20, 21). Consistent with these reports, we treated HUVECs with VEGF165, showing induction of Ang-2 expression by these cells (Fig. 3C). Immunohistochemistry studies showed that Ang-2 expression was much greater in the intratumoral endothelium of VEGF165-overexpressing tumors than in the controls (Fig. 3D, bottom), indicating that VEGF165 overexpression in sarcoma xenografts enhanced the proangiogenic state in tumor vasculature.

Assays were done to determine whether the enhanced in vivo growth of SKLSE and RDSE cell xenografts resulted from alterations in their in vitro properties. The growth and invasion rates of VEGF165-transfected cells and control cells were similar (data not shown), indicating that the increased growth and metastasis of SKLSE and RDSE cells was not a result of an alteration in the intrinsic properties of these cells. Finally, we compared the expression of a panel of known chemoresistance genes in the parental and transected SKLMS-1 cells using a cDNA array. No difference in gene expression was detected (data not shown), suggesting that VEGF165 does not act in an autocrine-like manner to affect the tumor cell chemoresistant effectors directly.

Proliferation, migration, and chemosensitivity of HUVECs were assessed after exposure to conditioned medium derived from STS parental cells, vector control cells, and VEGF165-overexpressing transfectants. The proliferation (Fig. 1D) of HUVECs stimulated by VEGF165-overexpressing cell conditioned medium was significantly higher than that stimulated by conditioned medium of controls. Similarly, HUVEC migration was significantly enhanced by VEGF165-overexpressing cell conditioned medium (Fig. 4A). Interestingly, HUVECs exhibited much greater resistance to doxorubicin cytotoxic effects after exposure to VEGF transfectant conditioned medium (Fig. 4B). To the best of our knowledge, the effect of VEGF on endothelial cell chemoresistance has not been described previously; our data show that the increased chemoresistance of VEGF165 STS xenografts is due to the induced chemoresistance of the tumor-associated endothelial cells rather than the tumor cells per se.

Antiangiogenic scheduling of low-dose chemotherapy is an attractive therapeutic strategy to minimize chemotherapy-related toxicity (22). Because signs of conventional-dose doxorubicin toxicity developed in all mice treated, low-dose doxorubicin was combined with DC101 as a chemosensitizer to treat mice bearing VEGF165-overexpressing sarcoma xenografts. Whereas treatment with either continuous low-dose doxorubicin or DC101 alone did not significantly affect growth of SKLSE10 and RDSE17 xenografts (Fig. 5A, left and right), combined continuous low-dose doxorubicin and DC101 was markedly inhibitory. In addition, although animals receiving low-dose doxorubicin experienced some weight loss, the loss was significantly less than that of the conventional-schedule group. Moreover, combined therapy caused no additional host animal toxicity above that seen in the low-dose doxorubicin alone group (data not shown). Taken together, these data suggest that DC101 blockade of VEGFR2 signaling can reverse the endothelial cell chemoresistance caused by human STS xenograft overexpression of VEGF165.

Microvessel counts were significantly lower in tumors treated with DC101 plus doxorubicin than in those treated with either
agent alone (Fig. 5B). The percentage of SMA-positive vessels was significantly greater in VEGF165-overexpressing xenografts from animals treated with the DC101 and low-dose doxorubicin compared with either drug alone or control treatments (Fig. 5C), suggesting that antiangiogenic therapy increased the percentage of mature vessels in VEGF165-overexpressing xenografts.

To determine whether combined therapy more effectively inhibited xenograft proliferation in vivo, xenograft sections were stained with Ki67 (Fig. 5D). Immunohistochemical staining demonstrated less proliferation in tumors overexpressing VEGF when treated with combination therapy.

Table 2. Combined DC101 and doxorubicin therapy effect on the development of pulmonary metastasis in SCID mice harboring a human STS

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>Treatment groups</th>
<th>Rat IgG + saline</th>
<th>DC101</th>
<th>Doxorubicin</th>
<th>Doxorubicin + DC101</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKLMS-1</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>SKLneo</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>SKLSE10</td>
<td>6/8</td>
<td>5/8</td>
<td>4/8</td>
<td>2/8*</td>
<td></td>
</tr>
<tr>
<td>SKLSE13</td>
<td>4/8</td>
<td>4/8</td>
<td>2/8</td>
<td>1/8*</td>
<td></td>
</tr>
<tr>
<td>SKLSE23</td>
<td>3/8</td>
<td>2/8</td>
<td>1/8</td>
<td>0/8*</td>
<td></td>
</tr>
<tr>
<td>RD</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>RDneo</td>
<td>0/8</td>
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<td>0/8</td>
<td>0/8</td>
<td></td>
</tr>
<tr>
<td>RDSE14</td>
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<td></td>
</tr>
</tbody>
</table>

NOTE: Frequency of pulmonary metastasis: mice with metastasis/mice with xenografts. Lung nodules >0.5 mm in diameter were counted. Combination biochemotherapy was more significantly associated with suppression of pulmonary metastasis than was either monotherapy compared with mock therapy.

*SKLSE group: P = 0.005.
† RDSE group: P = 0.002, Fisher's exact test.
conditioned medium from SKLSE10 and RDSE17 cells (Fig. 6A). These activities in HUVECs exposed to high VEGF 165-containing conditioned medium from VEGF165-transfected or control vector-transfected cells was added to treated cells, and assays were done as described in Materials and Methods. Bars, SD. **, P < 0.01 versus doxorubicin alone group.

Figure 6. Antiangiogenic therapy with DC101 and low-dose doxorubicin inhibited the proliferation and migration of HUVECs in vitro. Results of endothelial cell proliferation (A) and migration (B) assays. Cells were treated as indicated. Conditioned medium from VEGF165-overexpressed or control medium of HUVECs cultured with the indicated therapeutic agents as described in Materials and Methods showed decreased activities of secreted MMP-2 and MMP-9 following treatment with combined DC101 and doxorubicin.

stained for Ki67, a nuclear marker of proliferation. As shown in Fig. 5D, combination therapy suppressed proliferation in the VEGF165-overexpressing tumors much more than did either monotherapy or mock therapy, suggesting that the decreased vascularity leads to decreased tumor cell proliferation.

Because inhibition of angiogenesis has been shown before to suppress breast cancer metastasis (23), we were interested to examine whether antiangiogenic treatment with DC101 and low-dose doxorubicin could inhibit lung metastasis of STS. Lungs of mice from the various treatment groups were examined for sarcoma metastases. Metastatic burden was significantly reduced in mice receiving the combined regimen than in those treated by either agent alone (Table 2).

To further elucidate the mechanisms by which the combined therapy was superior in inhibiting tumor growth and metastasis, in vitro endothelial cell migration and proliferation assays were done. Although single-agent doxorubicin or DC101 inhibited the migration and proliferation of HUVECs exposed to control cell conditioned medium, doxorubicin or DC101 alone did not affect these activities in HUVECs exposed to high VEGF165-containing conditioned medium from SKLSE10 and RDSE17 cells (Fig. 6A and B). In addition, combined therapy inhibited endothelial cells more than single agent alone or mock treatments.

Endothelial cell matrix metalloproteinase (MMP) secretion has been associated with tumor metastasis (24). Interestingly, we found that endothelial cell MMP activity was markedly inhibited in the conditioned medium of HUVECs treated with combined DC101 and doxorubicin but not in the conditioned medium treated with either agent alone (Fig. 6C), implying that DC101 and doxorubicin in combination may help decrease STS pulmonary metastasis via the inhibition of MMP secretion by tumor-associated endothelial cells.

Discussion

In this study, we examined the effects of VEGF165 overexpression on human STS progression and chemotherapeutic efficacy. S.C. implantation of VEGF165-overexpressing sarcoma cell lines in SCID mice produced highly vascular tumors that exhibited a much shorter latency, significantly accelerated tumor growth, angiogenesis, and metastasis. However, no effect on sarcoma cell growth and invasion could be seen in vitro. We learned that the inhibitory effects of doxorubicin alone on tumor growth were minimal in VEGF165-overexpressing xenografts, whereas treatment with the anti-VEGFR2 monoclonal antibody combined with doxorubicin markedly improved xenograft responsiveness to doxorubicin.

The significance of VEGF expression in STS patients has been recognized previously in patient-based descriptive studies. In a study of 85 STS patients, serum VEGF levels correlated significantly with grade (25). Others have observed that the magnitude of STS patient circulating or tumor VEGF levels is an important prognostic factor (10, 26). Mutation of p53 is very frequently found in STS and this may also play a role in up-regulating VEGF as we have shown before (12). To the best of our knowledge, the current study is the first to show that overexpression of VEGF165 accelerates STS angiogenesis, growth, metastasis, and chemoresistance to doxorubicin. The effect of VEGF165 overexpression on tumor progression in different tumor types is not entirely consistent. For example, overexpression of VEGF165 enhanced both tumor angiogenesis and growth in a MCF-7 human breast carcinoma cell system (27) but only stimulated tumor angiogenesis without increasing the growth rate of OVCAR-3 human ovarian cancer xenografts (28). Varying degrees of effect were also noticed in preclinical models of β-cell carcinogenesis (29), melanoma (30), and glioma (31). Some of the apparent discrepancies observed in VEGF function in cancer development may be due to different ways VEGF impinges on the tumor-versus-microenvironment interplay and suggest that tumor type and tumor cell variability are important determinants for the role of VEGF.

This report also facilitates an awareness that chemoresistance must be considered in the context of both tumor cells as well as cells recruited from the microenvironment. Examining both of these cellular components in tandem has enabled an initial identification of the critical role of endothelial cell chemoresistance in STS progression and dissemination. We have shown that STS chemoresistance is determined not only by tumor cell response to drugs but also by host cells, although genetically stable, which can also be adapted to become resistant to the toxic effect of chemotherapy. VEGF produced by tumor cells can mediate endothelial cell protection against the effects of chemotherapy by promoting the production and/or activation of cytoprotectors, such as survivin (32). In a similar manner, Kim et al. (33) have
shown that the phosphatidylinositol 3-kinase/Akt pathway is activated in endothelial cells exposed to radiation, which in turn can lead to the activation of other prosurvival pathways. Targeting endothelial cell–based survival pathway components may prevent their production or activation and may be an important strategy to overcome therapy resistance. In the context of tumor angiogenesis, this conceptual framework suggests that abrogating tumor VEGF expression-mediated chemoresistance might be augmented by strategies that attack both the tumor cell as a source of VEGF as well as the microenvironment endothelial cells recruited by the elaboration of VEGF.

Anti-VEGF monotherapy efficacy has been shown in STS animal models (34). We have shown previously that combining anti-VEGF therapy with chemotherapy results in superior STS inhibition (5). The current report extends our previous studies to encompass the more clinically significant issue of controlling metastasis, the most common lethal event in STS. Moreover, our study design uses a preclinical model of supranormal VEGF production that more accurately mirrors the human STS tumor-bearing continuum.

A perceived concern with our model system might be that it lacks true relevance to the clinical situation in patients with STS. Scientific and clinical oncology literature exists, which shows that VEGF expression is significantly elevated in human STS: in tumor specimens at the RNA (35) and protein levels (10, 36, 37) as well as in patient serum samples (25, 26, 35, 38). Elevated serum VEGF levels are reported to correlate with STS tumor grade (25, 36) and size (25, 26), and immunohistochemical assessment of VEGF levels in STS tumors correlates high-intensity VEGF staining with advanced tumor grade (36, 37). These reports indicate clearly that VEGF is overexpressed in STS. In several series studying circulating VEGF levels in patients with STS, VEGF levels were found to be elevated in the vast majority of patients compared with healthy controls (25, 26, 35, 38). For example, Graeven et al. (25) reported mean serum VEGF levels to be 137 pg/mL (22-404 pg/mL; n = 18) for healthy controls compared with 672 pg/mL (44-2,500 pg/mL; n = 62) in STS patients with macroscopic tumor lesions. In generating the model system used in our article, parental STS cell line VEGF secretion levels were compared with those of an immortalized normal human fibroblast cell line. Two cell lines, with low to moderate expression levels (by Western blot comparison among the cell lines; Fig. 1A), were stably transfected with VEGF to assess the effect of VEGF on STS biology with the minimum of genetic disruption. We reported previously quantitated VEGF levels secreted by at least one of these STS cell lines into tissue culture medium (12) using the same ELISA methodology used for patient sample analysis in the previously referred published study. The tissue culture VEGF levels indicated that parental cell line VEGF secretion approximated to moderate serum levels in patient samples. Tissue culture VEGF levels are, admittedly, a debatable surrogate for equivalency to patient serum levels; however, the shortcoming in the comparison is that the measured levels in the medium would be an overestimate compared with in vivo-secreted VEGF levels. Taking this into consideration, combined with our ELISA measurements falling into the same range as moderate patient serum levels and Western blot results showing secreted VEGF levels to be the same or less than that released by immortalized fibroblast cell lines, we generated VEGF-overexpressing lines to more closely mimic the clinical STS scenario because elsewhere serum concentrations of VEGF have been reported as being 10 (35) and 12 (26) times higher than normal in patients with STS. The need for a model that expresses abundant VEGF to mimic the clinical situation additionally stems from the reality that the high expression of VEGF observed in clinical STS is most likely secondary to the hypoxia generated in these tumors, which frequently grow to very large dimensions before detection. A recently published report supports the need for such a model in STS by showing that STS cells in culture, when exposed to hypoxic conditions, significantly increase VEGF expression compared with normoxic controls (39). These issues, taken together with the previously mentioned reports, indicate that STS overexpress VEGF and that our laboratory-generated model represents an acceptable investigative model of the actual clinical situation.

Vulnerability of established tumor vasculature to therapeutic manipulation of VEGF/VEGFR2 seems to occur selectively only in those vessels that are immature and lack the contact or presence of pericytes (40). Conversely, the presence of pericytes as an integral component of mature tumor neovasculature may provide a basis for surviving antiangiogenic treatments. These considerations may help explain the results of our experiment where the percentage of cells that stained positive for SMA, a marker of pericytes, was highest in VEGF transfectants treated with the combined regimen. This treatment, compared with single-agent or no treatment controls, was much more effective in eliminating SMA-negative immature cells, leaving a much higher percentage of cells that were SMA positive. A similar finding has been reported in orthotopic models of human gastric (41) and pancreatic cancers (42, 43) treated with VEGFR tyrosine kinase inhibitors. Although immature blood vessels rely on VEGF-induced survival signals (44), inhibition of both VEGF and platelet-derived growth factor signaling was found to be superior for the elimination of pericytes (40). It has been suggested previously that eliminating immature and “leaky” neovasculature while leaving intact the more mature vasculature can provide a more effective conduit for chemotherapy to reach the tumor cells (45). However, apparently, combinations of treatments that will also target endothelial support cells, such as pericytes, might result in even better tumor control and thus merit further investigation.

Two clinical trials have been conducted in STS using antiangiogenic systemic treatments (46, 47). A phase II study examined the effect of SU5416, a tyrosine kinase VEGFR2 inhibitor, on 13 patients with locally advanced or metastatic STS (46). Although no objective tumor responses were observed, the drug was generally well tolerated. Pretreatment and post-treatment tumor biopsies showed no significant alteration in VEGFR2 phosphorylation, suggesting that therapeutic levels of the drug were not achieved. Furthermore, the drug was used as monotherapy, and per our observations above, VEGF inhibition seems to work better as a chemosensitizing agent than as stand-alone therapy. An additional trial combined doxorubicin with bevacizumab, a human monoclonal antibody that binds VEGF (47). A 12% response rate was observed, which was not greater than the single-agent doxorubicin response rate. Although 65% of the study patients enjoyed disease stabilization, this came at the expense of increased cardiomyopathy. Apparently, the discrepancy between this clinical study and our own preclinical results has to do with the extent of disease at time of therapy initiation. The patients in the clinical trial already had extensive metastatic disease when treated. Established metastases contain mature blood vessels against which the described antiangiogenic therapy may be ineffective as we have shown above. In contrast, the animals in our study were treated with a preventive intent (i.e., before the development of
metastases). A second difference is that the clinical trial used conventional dose of chemotherapy, resulting in major toxicity. In our studies, low-dose chemotherapy was effective as conventional doses but with much less toxicity. Taken together, these results suggest the possibility that combining VEGF-therapy with low-dose doxorubicin might be most useful in stage III high VEGF-secreting STS patients in whom there is a high risk of eventual metastatic spread. Clinical trials pertaining to this question may be a relevant next step.

In conclusion, combination chemotherapy/angiogenesis treatment algorithms targeting both endothelial and tumor cells should be considered for stage III STS patients so that both primary tumor and subclinical metastases can be addressed. Such combinations, if successful, may offer new vistas of tumor control for STS patients burdened by this devastating malignancy.

Acknowledgments


Cancer Research 2006; 66: (17). September 1, 2006 8778 www.aacrjournals.org

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Vascular Endothelial Growth Factor Overexpression by Soft Tissue Sarcoma Cells: Implications for Tumor Growth, Metastasis, and Chemoresistance

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