Inhibition of Angiogenesis by Blocking Activation of the Vascular Endothelial Growth Factor Receptor 2 Leads to Decreased Growth of Neurogenic Sarcomas

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ABSTRACT

Neurogenic sarcomas are incurable, common malignant human peripheral nerve tumors subject to local recurrence and systemic metastasis. In this study, the vascularity, vascular endothelial growth factor (VEGF) expression, and effects of inhibiting VEGF receptor on growth of neurogenic sarcomas were examined. Vascularization and VEGF expression were 6.4- and 15-fold higher in tumors than in normal nerves. The small molecule inhibitor (SU5416) of VEGF receptor 2 had no effect on neurogenic sarcoma cell lines in vitro, but the growth of a human tumor explant xenograft model was reduced by 54.8% compared to vehicle. Reduction in tumor growth was due to decreased tumor angiogenesis, leading to reduction of tumor cell proliferation and increased apoptosis. Inhibiting VEGF function may therefore be a useful adjuvant therapy for neurogenic sarcomas.

INTRODUCTION

NF-1 is the most common human cancer-predisposing familial syndrome, with an incidence of 1 in 3500 live births (1). NF-1 is characterized by a plethora of clinical manifestations including dermatological, vascular, musculoskeletal, and neurological disorders. The most common malignant tumors are neurogenic sarcomas, which occur in 3–5% of NF-1 patients and arise from plexiform neurofibromas of the large peripheral nerves. The overall prognosis for patients with neurogenic sarcomas is poor, with only 30% of the patients remaining disease free 5 years after radical local surgery. Systemic metastasis, especially to the lungs, is often the ultimate cause of death in these patients (2).

Angiogenesis is a crucial process in solid tumor growth and metastasis (3–8). The entire process of neangiogenesis can be induced by VEGF acting as an endothelial cell mitogen and a survival factor by activating its receptors, which are specifically expressed by the endothelial cells (5, 9, 10). VEGF also contributes to metastasis and peritumoral edema by inducing endothelial cell fenestrations in blood vessels (10–13). VEGF is regulated by several tumor mitogens including Ras (14–21); cytokines such as interleukin 1 and interleukin 6 (22, 23); growth factors such as fibroblast growth factor 4, platelet-derived growth factor, tumor necrosis factor α, transforming growth factor β, insulin-like growth factor I, and KGF (24–29); and the inactivation of the p53 tumor suppressor gene (30). In addition, VEGF is stimulated by several physiological factors, of which hypoxia and hypoglycemia are the most important and pertinent in the growth of solid tumors (31–35).

There are several VEGFRs, including VEGFR-1 and VEGFR-2, that are expressed mainly by endothelial cells (36–40). Among the VEGFRs, activation of VEGFR-2 is the most biologically relevant, leading to changes in endothelial cell morphology, actin reorganization, membrane ruffling, chemotaxis, and proliferation (41, 42). Inhibition of VEGFR-2 activation by several different strategies including using the VEGFR-2 dominant negative mutant (6, 43), neutralizing antibodies directed at VEGF-2 or VEGF (5, 44), ribozymes against VEGFR-2 or antisense strategies against VEGF (45, 46) all lead to decreased tumor angiogenesis and overall tumor growth. Pharmacological strategies, such as the use of SU5416 (SUGEN Inc.), a small molecule that specifically inhibits VEGFR-2, are currently the most feasible clinical strategies. In this study, we demonstrate that SU5416 inhibits tumor angiogenesis and the overall growth of NF-1 neurogenic sarcomas. This mode of antiangiogenic therapy may be useful as an adjunct to surgery in controlling the local recurrence and the terminal metastasis of disease in patients with NF-1 neurogenic sarcomas and other soft tissue sarcomas.

MATERIALS AND METHODS

Evaluation of Angiogenesis and VEGF Expression in NF-1 Neurogenic Sarcomas. Five-μm paraffin sections of four normal nerves and six NF-1 neurogenic sarcomas were evaluated. The sections were stained with an anti-factor VIII antibody (DAKO; 1:2000) and a polyclonal anti-VEGF antibody that recognizes all VEGF isoforms (Santa Cruz Biotechnology, Inc.; 1:50), followed by detection with an avidin-biotin complex method (3,3′-diaminobenzidine (VectorStain Elite; Vector Laboratories, Burlingame, CA) system. Microvessel density counts were derived by averaging the number of factor VIII-positive vessels per four to six high-powered fields. VEGF expression was determined on adjacent serial sections and scored semiquantitatively with the MicroComputer Image Device image analysis system (Imaging Research, Inc., Ontario, Canada). Microscope fields (using a ×400 magnification) were acquired and digitized, and positive staining was scored in four to six fields/tissue section, and the results were expressed as the mean ± SE of the means.

Evaluation of SU5416 on NF-1 Neurogenic Sarcoma Cell Lines in Vitro. The human NF-1 neurogenic sarcoma cell line ST88-14 (47, 48) was plated in 96-well plates at a density of 1 × 10⁴ cells/well in RPMI 1640-based media supplemented with 15% FCS. The cells were grown overnight and then treated with SU5416 (12.2–200 nM) or vehicle for 48 h, and cell viability was assessed with the Cell Titer 96 Aqueous One Solution kit (Promega, Madison, WI). Each experiment was repeated three times, with each dosage being tested in quadruplicate in each experiment. Plates were read on a MR600 Dynatech microplate reader at a wavelength of 490 nm, subtracting reference readings at a wavelength of 630 nm. Results were compared to those from cells treated with media alone and expressed as a percentage relative to untreated cells to generate a dose-response curve. BedUrd incorporation was used to measure the effects of SU5416 on
ST88-14 cell proliferation. ST88-14 cells were plated at a density of $2.4 \times 10^4$ cells, grown on 6-well Lab-Tek Chamber Slides (Nunc, Inc., Naperville, IL), treated with SU5416 (100 μM to 10 nM) for 48 h, and then fixed. A mouse monoclonal anti-BrDUrd antibody (Boehringer Mannheim, Indianapolis, IN) was used as per the manufacturer’s protocol, and the proliferative index was determined by scoring four high-powered fields ($\times 400$) and determining the percentage of BrDUrd-positive cells with the aid of a computer image analysis system. The experiment was repeated twice with two sets per experiment for each SU5416 dose. The means and SE of the means were calculated for each treatment group. The TUNEL assay was used to determine the effect of SU5416 (100 μM to 10 nM) on the ST88-14 apoptotic index as per the manufacturer’s protocol (Kit POD; Boehringer Mannheim). The apoptotic index was determined by light microscopy as described above.

Evaluation of the Effects of SU5416 on Growth and Tumor Vascularity of NF-1 Neurogenic Sarcoma Xenografts. An operative specimen from a neurogenic sarcoma originating from the musculocutaneous nerve of a NF-1 patient was passaged s.c. in male NOD/SCID mice for 18 months. Throughout the multiple passages, the xenografted tumor maintained an immunohistochemical profile similar to the original tumor, with sarcomatous differentiation, a high mitotic index, vimentin positivity, and neurofibromin negativity. Tumor volume ($V$) was calculated as $V = (\text{length} \times \text{width} \times \text{height})/6$. Animals bearing tumors 200–700 mm$^3$ (average volume, 365 mm$^3$) at 7 days after implantation were randomized into three experimental groups: (a) SU5416 in DMSO at 25 mg/kg/day i.p.; (b) an equivalent volume of PBS i.p. daily; and (c) PBS i.p. daily. The mice were treated daily, and the tumor volumes and weights were recorded for 8 days, followed by the i.p. injection of 100 mg/kg BrdUrd (Sigma-Aldrich Canada Ltd., Oakville, Canada) before sacrifice by cervical dislocation. The harvested tumors and visceral organs were fixed in 10% buffered formalin, and tumor samples were also flash-frozen in liquid nitrogen for later analysis.

The tumors were analyzed for neovascularization, proliferation, and apoptosis. Neovascularization was evaluated in 5-μm cryosections as described previously (50), with the following modifications. The primary antibody used was rat antimonoclonal CD31 (platelet/endothelial cell adhesion molecule 1) monoclonal antibody at a 1:10 dilution (PharMingen International, San Diego, CA).

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**Fig. 1.** Quantitative image analysis demonstrating that NF-1 neurogenic sarcomas have increased vascularity and VEGF expression compared to normal human nerves. A, the six NF-1 neurogenic sarcomas showed much higher levels of factor VIII-positive vessels than the four normal human peripheral nerves. B, VEGF expression paralleled the vascularity with much higher levels in the NF-1 neurogenic sarcomas. SE (bars) are derived from quantifying four to six high-power fields for each sample and then taking the average for each group.

**Fig. 2.** Experiments demonstrating that SU5416 does not alter the in vitro proliferation of NF-1 neurogenic sarcoma ST88-14 cells. A, cell viability MTS (Owens reagent) assay after 48 h with serial dilutions of SU5416 in DMSO done in quadruplicate for three different experiments. No significant effect on cell viability was observed at doses up to 3.7 log-fold higher than those reported to inhibit human endothelial cell proliferation. B, percentage of BrdUrd-positive ST88-14 cells was also not altered over a similar large dose range of SU5416. C, the apoptotic index, as measured by the TUNEL assay, was also not altered by SU5416. SE (bars) is derived from duplicate measurements from two different experiments.
This was followed with a tetramethyl rhodamine isothiocyanate-labeled secondary antibody (donkey antirat antibody; The Jackson Laboratory; dilution, 1:50). Microvessel density was determined by fluorescence microscopy and image analysis. Cell proliferation was determined on the paraffin-embedded sections using anti-BrdUrd (Boehringer Mannheim) antibody. The apoptotic index was determined using the TUNEL assay on the paraffin-embedded tissue samples according to the manufacturer’s protocol (Boehringer Mannheim). Both cell proliferation and the apoptotic index were scored by determining the percentage of positive cells in four high-powered fields (×400) per section using the computer-aided image analysis system, with the means and SE calculated for each treatment group.

RESULTS

Angiogenesis and VEGF Expression in NF-1 Neurogenic Sarcomas. Histological sections were analyzed to determine angiogenesis and whether it correlated to VEGF expression in a spectrum of peripheral nerve lesions, a feature not previously studied in this tissue. The normal nerve had an average of 14 vessels/4 high-power fields, which was much lower than NF-1 neurogenic sarcomas with 90 vessels/4 high-power fields (Fig. 1A). Similarly, VEGF expression was significantly elevated at 45% field positivity in neurogenic sarcomas, in contrast to the 3% field positivity observed in normal nerves (Fig. 1B). These data suggest that tumor angiogenesis is related to the malignant potential of peripheral nerve tumors, which correlates to VEGF expression. Whereas this correlation has been demonstrated in several other solid tumors (51–54), this is the first report in human peripheral nerve tumors.

Effect of SU5416 on NF-1 Neurogenic Sarcoma Cell Lines in Vitro. Cell viability, cell proliferation, and apoptosis were determined for the ST88-14 cell line, a well-characterized NF-1 human neurogenic sarcoma cell line, after stimulation for 48 h with SU5416. The MTS (Owens reagent) colorimetric assay (49) demonstrated that at concentrations between 12.2 nM and 200 μM of SU5416, cell viability was unchanged from the vehicle or PBS controls (Fig. 2A). This dose range is well above the IC_{50} of 40 nM (3.7 log higher) observed in human endothelial cells treated with SU5416 for 48 h. Furthermore, SU5416 did not alter the proliferation of ST88-14 cells as evaluated by BrdUrd incorporation (Fig. 2B). SU5416 also did not alter the apoptotic index measured by the TUNEL assay of ST88-14 cells in vitro (Fig. 2C). Therefore, proliferation indices of NF-1 neurogenic sarcoma cells treated with SU5416 were not affected in vitro, and this reflects the fact that SU5146 acts by inhibiting VEGFR-2 expressed by endothelial cells (36–38, 40).

Effect of SU5416 on NF-1 Neurogenic Sarcoma Xenograft Tumor Growth and Angiogenesis. NOD/SCID mice were implanted s.c. with tumor obtained from a serially passaged NF-1 neurogenic sarcoma xenograft 7 days before drug treatment. After ensuring that the tumors were growing, the mice were randomized into the three experimental arms. The 22 mice treated with SU5416 (25 mg/kg/day) showed a rapid and significant regression in tumor volume (Fig. 3A). By day 8 of treatment, mice treated with SU5416 had a tumor volume of 200 mm^3, while those treated with PBS or DMSO showed a much higher tumor volume of 450 mm^3. This result suggests that SU5416 acts by inhibiting VEGFR-2 expressed by endothelial cells, thereby inhibiting tumor growth.
burden that was 54.8% \( (P < 0.02) \) lower than either DMSO (21 mice)- or PBS (11 mice)-treated animals, which did not differ from each other \( (P < 0.76; \text{Fig. 3B}) \). The animals tolerated all treatments equally well, with only an occasional but nonsignificant weight drop noted in some animals treated with SU5416 (data not shown). Furthermore, examination of the liver, heart, lungs, and brain did not reveal any pathological abnormalities or significant differences between SU5416-treated group and control groups. One of the kidneys examined from the SU5416-treated animals exhibited a small area of old hemorrhage and granular casts within the renal medulla, and the significance of this finding remains uncertain.

Tumor-associated endothelium labeled with platelet/endothelial cell adhesion molecule 1/CD31 immunofluorescence antibody and quantitatively evaluated with computer-assisted image analysis demonstrated significantly decreased tumor vascularity in the SU5416-treated group. In sixteen \( 5 \times 10^5 \) \( \mu \text{m}^2 \) fields, the average number of vessels in the SU5416 group was 61.5 \( \pm 7.2 \), compared to 119 \( \pm 10.8 \) vessels in the DMSO group and 123.1 \( \pm 11.6 \) vessels in the PBS group (Fig. 4A). Data were analyzed using factorial ANOVA and a Fisher's PLSD post hoc procedure. These differences were significant at \( P < 0.0001 \). Unlike the \textit{in vitro} results in ST88-14 cells, BrDUrd labeling was decreased in the SU5416-treated sarcomas to 1.7 \( \pm 0.2 \) positive cells/\( \times 40 \) in the group, compared to 25.4 \( \pm 2.2 \) and 24.8 \( \pm 1.6 \) positive cells/\( \times 40 \) for the DMSO and PBS control groups, respectively (Fig. 4B). Using Fisher’s PLSD procedure, values were significant to a \( P < 0.0001 \). Furthermore, a TUNEL assay demonstrated a high apoptotic index of 33.9 \( \pm 1.3 \) in the SU5416-treated xenografts, compared to an apoptotic index of 1.8 \( \pm 0.13 \) in the DMSO vehicle control group (Fig. 4C). These data were also shown to be significant using a Fisher’s PLSD test \( (P < 0.0001) \).

**DISCUSSION**

The NF-1 gene is localized to chromosome 17q11.2, with deletions, translocations, insertions, and, most commonly, point mutations leading to nonfunctional truncated proteins (55–57). The NF-1 gene is a typical tumor suppressor gene with germ-line inactivation of one allele and somatic inactivation of the other due to loss of heterozygosity, resulting in benign tumors such as the neurofibromas (58, 59). Malignant conversion of these benign neurofibromas, which occurs with an incidence of 3–5% of NF-1 patients, to neurogenic sarcomas likely requires further genetic alterations, including loss of the \( p53 \) gene (60–62). A common mechanism of malignant conversion of a benign solid tumor is the induction of the angiogenic switch (8, 63–66). This holds true for peripheral nerve tumors as demonstrated by this study, in which a significant increase in vascularity was observed in NF-1 neurogenic sarcomas compared to normal nerves. The tumor angiogenesis in NF-1 neurogenic sarcomas was associated with increased VEGF expression, implicating VEGF as a potential inducer of tumor angiogenesis in peripheral nerve tumors.

We further developed and used an \textit{in vivo} human NF-1 neurogenic sarcoma xenograft explant model in NOD/SCID mice to determine the functional relevance of VEGF-mediated tumor angiogenesis. The xenografts retained the histological appearance of the original sarcoma, including being negative for neurofibromin expression over several passages in mice. The lipid-soluble, small molecule, VEGFR-2 inhibitor SU5416 was used to block VEGF-mediated tumor angiogenesis. Daily injection of SU5416 resulted in an average tumor shrinkage of 54.8% within 8 days of SU5416 treatment, compared to the control animals receiving PBS or the DMSO vehicle only. The inhibition of tumour growth was primarily a result of inhibiting tumor angiogenesis, because there was no direct effect of SU5416 on ST88-14 NF-1 neurogenic sarcoma cell viability, proliferation, or apoptosis \textit{in vitro}. In addition, the much smaller SU5416-treated xenografts were associated with decreased vascular density and accompanying decreased sarcoma cell proliferation and increased apoptosis. These experiments emphasize the crucial role tumor angiogenesis plays in overall tumor growth. Not only is the tumor vasculature vital for the supply of nutrition, oxygen, and metabolite exchange, but also for the endothelial-derived paracrine growth-promoting factors needed to sustain tumor growth (67, 68). Inhibition of angiogenesis, as undertaken with SU5416, would therefore lead to an additional loss of endothelial survival and growth factors, resulting in overall rapid
tumor regression, as demonstrated in this study. We therefore conclude that VEGF-induced tumor angiogenesis is a fundamental requirement for the growth of human neurogenic sarcomas, and that administration of a small molecule VEGFR-2 inhibitor, SU5416, is a novel biological approach toward adjuvant treatment of this highly metastatic sarcoma. A recently published study (69) using this agent also confirms our findings through inhibition of cell line-derived tumor growth and similarly postulates that this is due to the antian- giogenic effect of the drug. If the long-term effects of SU5416 administration on the xenograft models of NF-1 neurogenic sarcomas, the subject of the current study, also demonstrate efficacy and a lack of toxicity, then this agent may be a therapeutic candidate in cases where surgery and local radiation of NF-1 neurogenic sarcomas have failed. Furthermore, SU5416 may be of potential value in a much wider spectrum of solid tumors where VEGF-related angiogenesis has been implicated.

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REFERENCES


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