A Small Interfering RNA Targeting Vascular Endothelial Growth Factor Inhibits Ewing’s Sarcoma Growth in a Xenograft Mouse Model

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Abstract

Angiogenesis plays an essential role in tumor growth and metastasis and is a promising therapeutic target for cancer. Vascular endothelial growth factor (VEGF) is a key regulator in vasculogenesis as well as in angiogenesis. TC71 human Ewing’s sarcoma cells overexpress VEGF, with a shift in isoform production from membrane-bound VEGF165 to the more soluble VEGF165. Transfection of TC71 cells with a vector-based VEGF targeted small interfering RNA expression system (VEGFsi) inhibited VEGF165 expression by 80% and VEGF165 protein production by 98%, with no alteration in VEGF189 expression. Human microvascular endothelial cell proliferation and migration induced by conditioned medium from VEGFsi-transfected TC71 cells was significantly less than that induced by conditioned medium from TC71 cells and control vector-transfected TC71 cells. Furthermore, after s.c. injection into athymic nu/nu mice, the tumor growth of VEGFsi-expressing TC71 cells was significantly less than that of parental or control vector-transfected cells. Vessel density as assessed by CD31 immunohistochemical analysis and VEGF165 expression as assessed by Northern blotting were also decreased. Intratumor gene therapy with polyethylenimine/VEGFsi also resulted in tumor growth suppression. When inoculated into the tibias of nude mice, VEGFsi-expressing TC71 cells induced osteolytic bone lesions that were less severe than those induced by control groups. These data suggest that targeting VEGF165 may provide a therapeutic option for Ewing’s sarcoma.

Ewing’s sarcoma is a primitive neuroectodermal tumor that most often affects children and young adults in the first two decades of life. It is the second most common malignant bone tumor and accounts for 10% to 15% of all primary bone tumors (1). Patients presenting with localized disease have a significantly better chance of survival than patients who present with metastases (2). Despite the use of multimodal therapy (chemotherapy, radiation therapy, and surgery), the long-term disease-free survival rate of Ewing’s sarcoma patients is still disappointingly low, particularly in the high-risk groups (3, 4). The identification of new therapeutic targets is therefore needed.

Angiogenesis has been specifically linked to increased growth and metastatic potential in human tumors (5). Although numerous growth factors are involved, vascular endothelial growth factor (VEGF), particularly VEGF-A, has been shown to play a pivotal role in tumor angiogenesis (6). Binding of VEGF-A to its receptors induces mitogenesis and chemotaxis of normal endothelial cells and increases vascular permeability, all of which contribute to new vessel formation and tumor growth (7). VEGF also contributes to neovascularization by mobilizing bone marrow–derived endothelial progenitor cells (8). To date, five isoforms of human VEGF have been identified (VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206; ref. 9). Increased levels of VEGF expression have been found in most human tumors, including those of the lung, gastrointestinal tract, kidney, thyroid, bladder, ovary, and cervix (10). Pediatric tumors have also been shown to be very vascular, with a high proliferation rate (11).

Several lines of evidence point to a role for VEGF in the pathogenesis of Ewing’s sarcoma. We recently reported elevated VEGF expression in three of four Ewing’s sarcoma cell lines and in human primary tumor specimens (12). Serum VEGF levels were found to be significantly higher in children with Ewing’s sarcoma than in healthy controls (11, 13). These elevated VEGF levels declined after tumor regression (11, 13). High levels of VEGF165 and VEGF121 expression were detected by immunohistochemical analysis in 17 of 31 Ewing’s tumor samples taken at initial biopsy (14). Positive staining for VEGF at the time of diagnosis correlated with a poor prognosis (14, 15). Finally, EWS-ETS, the specific oncoprotein in Ewing’s sarcoma, has been shown recently to be a transcription factor for the VEGF promoter, stimulating the expression of VEGF (14). Taken together, these data indicate that inhibiting the expression or function of VEGF may lead to improvements in disease outcome.

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RNA-mediated interference is a conserved gene silencing mechanism that recognizes dsRNA as a signal to trigger the sequence-specific degradation of homologous mRNA (16). It was first studied in Caenorhabditis elegans and plants in 1995. RNA-mediated interference was adapted for work with mammalian cells in 2001, with the discovery that the introduction of small interfering RNA (siRNA) <30 nucleotides long in mammalian cells avoids the induction of an IFN response that activates protein kinase R (17). The high efficiency and specificity of RNA-mediated interference has made it a powerful and widely used tool for the analysis of gene function. In this report, we used a vector-based siRNA expression system, which overcomes the limitations of transience and high cost in synthetic siRNAs, to specifically inhibit VEGF<sub>165</sub> expression in Ewing’s sarcoma cells. The siRNA that we synthesized inhibited Ewing’s sarcoma tumor growth in a nude mouse model.

Materials and Methods

Expression plasmids. siRNA expression vector pSilencer2.1-U6 hygro was purchased from Ambion (Austin, TX). siRNA-expressing plasmids targeting human VEGF (VEGFsi) were constructed according to the manufacturer’s instructions. Briefly, four pairs of CDNA oligonucleotides targeting human VEGF mRNA at different locations were synthesized by Integrated DNA Technologies (Coralville, IA). Each pair of oligonucleotides was annealed at 90°C for 3 minutes, cooled to 32°C, and incubated for 1 hour. The annealed dsDNA oligonucleotides were ligated between the BamHI and HindIII sites on the pSilencer2.1-U6 hygro vector. The control vector (si) was constructed by inserting a sequence that expresses a siRNA with limited homology to sequences in the human and mouse genomes. The targeted VEGF sequences were VEGFsi-2: TCATCAC-GAAGTGGTGAAG; VEGFsi-4: GTGGTGAAGTTCATGGATG; VEGFsi-6: GTTACATGGATGTCTATCAG; VEGFsi-7: GATAAGCAGAGACAAGAA; and si control: CACGCTGTCTTATAGGTGCTTGAACACTATCAAACGGTATG. All inserted sequences were verified by DNA sequencing.

Cell culture and transfection. TC71 human Ewing’s sarcoma cancer cells were cultured as described previously (12). MDA-MB-231 human breast cancer cells were obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM with 10% fetal bovine serum. Human microvascular endothelial cells (HMVECs) were purchased from Cambrex (East Rutherford, NJ) and grown in microvascular endothelial cell medium (5% fetal bovine serum in endothelial basal medium with 12 μg/mL bovine brain extract, 10 μg/mL human epidermal growth factor, 1 μg/mL hydrocortisone, and 1 μg/mL GA-1000). HMVECs (passage 3 or 4) were −80% confluent were used for most experiments. All cells were free of Mycoplasma, as screened by Mycoplasma Plus PCR Primer Set (Stratagene, Inc., La Jolla, CA), and were tested for VEGF expression by Northern blotting or ELISA. VEGFsi-7-transfected TC71 cell clone 7-1 (TC/VEGFsi) and control vector-transfected TC71 cell clone (TC/si) were used for the in vivo experiments.

Northern blot analysis. Cultured cells or tumor tissue was lysed in Trizol reagent (Life Technologies, Inc., Grand Island, NY). Total RNA was purified according to the manufacturer’s instructions. VEGF and topoisomerase IIα (topo IIα) gene expression was determined as described previously (18). Densitometric analysis was done using Personal Densitometer SI (Molecular Dynamics, Sunnyvale, CA) and adjusted by glyceraldehyde-3-phosphate dehydrogenase internal control.

Vascular endothelial growth factor protein quantitation. TC71, TC/si, and TC/VEGFsi cells (2.5 × 10<sup>6</sup>) were seeded into 24-well plates. Fresh medium was added after overnight culture. The cultured supernatants were collected 24 hours later and centrifuged at 13,000 rpm for 10 minutes to eliminate cellular fragments. Phenylmethylsulfonyl fluoride was then added to the cultured supernatants at 2 mmol/L and stored at −20°C. The cells were incubated with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide at 1 hour and lysed in equal amounts of DMSO (Sigma Chemical Co., St. Louis, MO). The relative cell density was assessed by A<sub>450</sub> nm. VEGF protein concentration was quantified using an anti-human VEGF<sub>165</sub> ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s protocol.

Migration assay. Cultured supernatants from TC71, TC/si, and TC/VEGFsi cells were collected. Transwells (Costar, Cambridge, MA) were assayed for VEGF by ELISA.

Fig. 1. Effect of VEGFsi on VEGF expression in TC71 Ewing’s sarcoma or MDA-MB-231 breast cancer cells. A, TC71 or MDA-MB-231 cells were stably transfected with different VEGFsi constructs. VEGF mRNA was quantified by Northern blot analysis. Densitometric analysis was done and the relative expression for each band was normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). B, different TC71 cell clones stably transfected with VEGFsi-7 or the control vector were assayed for VEGF<sub>165</sub> and VEGF<sub>200</sub> expression by Northern blotting. C, cultured supernatants from different TC71 clones stably transfected with VEGFsi-7 or the control vector were collected and assayed for VEGF by ELISA.

www.aacrjournals.org Clin Cancer Res 2005;11(7) April 1, 2005 2663
pretreated with serum-free medium at 37°C for 1 hour before seeding with HMVECs at \(1 \times 10^5\) per well in 100 μL endothelial basal medium with 0.1% fetal bovine serum. The transwells were then inserted into 24-well plates containing 600 μL conditioned medium and incubated at 37°C for 6 hours to allow HMVEC cells to migrate. Cells on the upper side of the filter were removed with cotton swabs. Migrated cells on the lower side of the filter were fixed and stained with H&E. The number of migrated cells was counted under a binocular microscope.

**Proliferation and cytostasis assay.** HMVEC cells (3 \(\times 10^5\)) were seeded into 96-well cell culture plates and allowed to adhere for 5 hours before the addition of conditioned medium from either TC71, TC/si, or TC/VEGFsi cells. The proliferative activity was determined 48 hours later by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. Cytostasis was also quantified 48 hours after adding different concentrations of trastuzumab.

**Western blotting.** Cells were pipetted into 100 mm dishes. When cells reached 80% confluence, cell lysate was collected and protein concentrations were determined using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). The protein (50 μg) was boiled for 5 minutes before being loaded onto a 7.5% SDS-polyacrylamide gel and then transferred to a nitrocellulose membrane (Amersham, Piscataway, NJ). The specific protein bands were detected with monoclonal anti-human HER-2/neu antibody (Ab-3; Oncogene, San Diego, CA) and β-actin antibody (Sigma Chemical) using the enhanced chemiluminescence Western blotting analysis system (Amersham) according to the manufacturer's instructions. Densitometric analysis was done, and values were normalized with β-actin loading control.

**Plasmid/polyethyleneimine formulations.** Polyethylenimine (25 kDa, branched form, Aldrich Chemical, Milwaukee, WI) was prepared at a concentration of 0.1 mol/L in water. A polyethylenimine/plasmid mixture (1.29:1 polyethylenimine/DNA weight ratio) was prepared as described previously (20) by slowly adding the plasmid to the polyethylenimine solution while vortexing vigorously. The solution was then allowed to incubate at room temperature for 15 to 20 minutes before use.

**In vivo studies.** Four- to 5-week-old specific pathogen-free athymic (T-cell deficient) nude mice were purchased from Charles River Breeding Laboratories (Kingston, MA). TC71, TC/si, and TC/VEGFsi Ewing's sarcoma cells in mid-log-growth phase were harvested by trypsinization. Single-cell suspensions (2 \(\times 10^6\) cells in 0.1 mL HBSS) were injected s.c. into the nude mice. The tumors were measured every 4 days with a caliper, and the diameters were recorded. Tumor volume was calculated by the formula: \(V = \frac{a \times b^2}{2}\), where \(a\) and \(b\) are the two maximum diameters. When tumors reached 2 \(\times 2\) cm, the duration of survival was recorded, the mouse was euthanized, and the tumor tissue was collected for analysis of CD31, apoptosis, basic fibroblast growth factor (bFGF), platelet-derived growth factor-β (PDGF-β), transforming growth factor-β (TGF-β), and interleukin (IL)-8 using immunohistochemical analysis and terminal deoxynucleotidyl transferase-mediated diUTP nick end labeling.

For the VEGFsi gene therapy experiments, 2 \(\times 10^6\) TC71 cells were injected s.c. into nude mice. Three days later, when the tumors were palpable, the mice were divided into three groups. Group 1 mice were used as untreated controls. Group 2 received intratumor injections (20 μg/mouse) of polyethylenimine/control vector twice weekly for various times. Group 3 received intratumor injections with 20 μg polyethyleneimine/VEGFsi twice weekly as described for group 2. Tumor size was measured and tumor tissue was examined as described above.

**Fig. 2.** Effect of VEGF siRNA on HMVEC cell chemotaxis and proliferation. Cultured supernatants from TC71, TC/si, and TC/VEGFsi were collected and assayed for their ability to induce HMVEC cell migration and proliferation. A, HMVEC cell migration was quantified after 6 hours as described in Materials and Methods. *, \(P < 0.01\), compared with TC/si supernatants. B, HMVEC cells were incubated with the indicated supernatant for 48 hours. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. **, \(P < 0.01\), compared with TC/si supernatants.

**Fig. 3.** Effect of VEGFsi on HER-2/neu and topo IIα expression. Total protein or RNA was extracted from TC71, TC/si, or TC/VEGFsi cells. A, HER-2/neu protein was quantified by Western blotting. B, topo IIα expression was quantified by Northern blotting.
To assess bone tumor formation, $2 \times 10^5$ TC71, TC/si, or TC/VEGFsi clone 7-1 cells were injected into the right tibia under anesthesia. Three weeks later, digitized radiographic images were taken using a MX-20 Specimen Radiograph System (Faxitron X-ray Co., Wheeling, IL). A grading system for bone lysis with numerical values ranging from 0 to 4 was used to determine the extent of bone destruction (21). A grade of 0 represented no bone lysis; grade 1 was minimal but visible bone lysis within the medullary canal; grade 2 was moderate bone lysis in the medullary canal with preservation of the cortex; grade 3 was severe bone lysis with cortical disruption; and grade 4 was massive destruction.

**Immunohistochemical analysis.** Tumor sections were stained with H&E. Frozen sections were fixed with acetone, incubated in 3% H$_2$O$_2$ in methanol for 10 minutes to block endogenous peroxidase, and then incubated in 5% normal horse serum plus 1% normal goat serum in PBS for 20 minutes to block nonspecific protein. Expression of CD31 was detected using a rat anti-mouse CD31 as the primary antibody (PharMingen, San Diego, CA); a goat anti-rat horseradish peroxidase was the second antibody followed by incubation with chromogen diaminobenzidine. The expression of bFGF, PDGF-$\beta$, TGF-$\beta$, and IL-8 protein was detected by incubating tissue sections with rabbit anti-human bFGF antibody (Sigma Chemical), rabbit anti-human PDGF-$\beta$ antibody, rabbit anti-human TGF-$\beta$ antibody, or rabbit anti-human IL-8 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) as the primary antibody and horseradish peroxidase–labeled goat antibody against rabbit IgG as the second antibody (Jackson Immunoresearch Laboratory, Inc., West Grove, PA). Gill’s hematoxylin was used as a counterstain.

Apoptotic and necrotic cells were quantified using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining. Formalin-fixed paraffin-embedded sections were dewaxed before being permeabilized with proteinase K for 15 minutes at room temperature. After blocking endogenous peroxidase in 3% H$_2$O$_2$, the fragmented DNA was labeled with biotin-16-dUTP with terminal transferase at 4°C overnight.

**Statistical analysis.** Two-tailed Student’s $t$ test was used to statistically evaluate the tumor volumes, migration, and proliferation of HMVECs. $P \leq 0.05$ was considered statistically significant.

**Results**

**Effect of vascular endothelial growth factor small interfering RNA on vascular endothelial growth factor expression.** Four VEGF siRNA-expressing plasmids (plasmid 2, 4, 6, and 7) were constructed using the pSilencer2.1-U6 hygro vector to target different regions of human VEGF mRNA. TC71 Ewing’s sarcoma and MDA-MB-231 breast cancer cells were stably transfected with these plasmids, and the VEGF mRNA levels were measured using Northern blotting. As shown in Fig. 1A, VEGF expression was significantly inhibited by VEGFsi-6 and VEGFsi-7. In TC71 cells, VEGF levels were reduced by 60% and 40%, respectively, compared with nontransfected and control vector-transfected TC71 cells. In MDA-MB-231 cells, transfection of VEGFsi-7 resulted in a 75% reduction in VEGF RNA compared with both nontransfected and control vector-transfected cells. Transfection...
of plasmids 2 and 4 resulted in no significant alteration of VEGF RNA expression in either cell line. Single-cell clones of TC71 cells transfected with plasmid 7 were then isolated and VEGF mRNA levels were examined by Northern blotting (Fig. 1B). VEGF<sub>165</sub> expression in clones 7-1 and 7-17 was inhibited by 75% and 80%, respectively, compared with wild-type and control vector-transfected cells. By contrast, expression of VEGF<sub>189</sub> was not altered. VEGF protein production was >98% lower in clones 7-1 and 7-17 than in untransfected and control vector-transfected cells (Fig. 1C).

**Effect of vascular endothelial growth factor small interfering RNA on vascular endothelial growth factor–induced endothelial cell proliferation and migration.** TC71 cells secrete significant amounts of VEGF (ref. 12; Fig. 1C). Because VEGF has been shown to induce the migration and proliferation of endothelial cells, we hypothesized that VEGF siRNA would reduce the chemotactic and mitogenic effect of TC71 cultured supernatants on endothelial cells. We collected cultured supernatants from TC71, TC/si, and TC/VEGFsi clones 7-1 and 7-17. As shown in Fig. 2A, the culture supernatants from untransfected and control vector-transfected cells induced robust HMVEC cell migration. By contrast, few HMVEC cells migrated when conditioned medium from TC/VEGFsi clone 7-1 or 7-17 was used as the chemotactic stimulus. HMVEC cell proliferation was also significantly lower when cells were cultured for 48 hours in conditioned medium from TC/VEGFsi clone 7-1 and 7-17 cells rather than in conditioned medium from untransfected or control vector-transfected cells (Fig. 2B).

To determine whether VEGF<sub>165</sub> inhibition by siRNA influenced TC71 cell growth in vitro, the doubling time of each cell line was quantified. There was no difference in growth rates before and after VEGFsi transfection. Both Flt-1 and Flk-1 were detected on the TC71 cell membrane by immunohistochemical analysis (data not shown).

**Effect of vascular endothelial growth factor small interfering RNA on HER-2/neu and topoisomerase IIα expression.** We have shown previously that, in addition to VEGF, TC71 cells overexpress HER-2/neu (22). Transfection of E1A resulted in the down-regulation of both HER-2/neu and VEGF and the up-regulation of topo IIα (22). We therefore determined whether VEGF siRNA also affected HER-2/neu or topo IIα expression. As shown in Fig. 3, there was no significant change in either HER-2/neu protein levels or topo IIα expression following transfection with VEGFsi. Furthermore, clone 7-1 and 7-17 cells were as sensitive to herceptin as control vector-transfected cells (data not shown). These data show the specificity of our VEGF siRNA.
Effect of vascular endothelial growth factor small interfering RNA on Ewing’s sarcoma tumor growth in vivo. To determine whether inhibition of VEGF<sub>165</sub> by siRNA had an effect on tumor growth, TC71, TC/si, or TC/VEGFsi clone 7-1 cells were inoculated s.c. into nude mice. TC71 and TC/si cells grew rapidly, resulting in palpable tumors 3 to 4 days following injection (Fig. 4A). By contrast, tumor formation was significantly slower after inoculation of TC/VEGFsi clone 7-1. The TC/VEGFsi clone 7-1 tumors were significantly smaller than those in both control groups. Survival time was also significantly longer for mice inoculated with TC/VEGFsi clone 7-1 cells (Fig. 4B). TC/VEGFsi clone 7-1 tumors were pale, with a massively necrotic center and a thin layer of tumor cells in the periphery. Similar findings were seen with TC/VEGFsi clone 7-17 tumors (data not shown). No significant difference in either tumor growth or macroscopic appearance was detected between the tumors induced by the TC/si control cells and the parental TC71 cells.

To determine the VEGF status in these tumors, RNA was extracted from tumor tissue and Northern blotting for VEGF was done. Both VEGF<sub>165</sub> and VEGF<sub>189</sub> were detected in TC71 and TC/si tumors, but only VEGF<sub>189</sub> was expressed in TC/VEGFsi clone 7-1 tumors (data not shown), indicating that inhibition of VEGF<sub>165</sub> by VEGFsi transfection was stable in vivo. Effect of VEGFsi gene therapy on Ewing’s sarcoma tumor growth. We next investigated whether VEGF siRNA can be used as a gene therapy. We elected to use polyethylenimine as our gene delivery system because of our previous experience with this nonviral vector (20). TC71 cells were injected s.c. into nude mice. Three days later, the palpable tumors were injected with polyethylenimine/VEGFsi-7 or polyethylenimine/si control. As shown in Fig. 4C, polyethylenimine/VEGFsi-7 gene therapy significantly inhibited tumor growth in mice compared with the polyethylenimine/si control. Survival time for the polyethylenimine/VEGFsi-treated mice was also significantly longer (Fig. 4D). Polyethylenimine/VEGFsi-7-treated tumors grew slowly, with ulceration appearing when the tumor reached ~6 mm in diameter.

Immunohistochemical findings. Tumor tissue from mice was excised and subjected to histologic staining. As shown in Fig. 5, CD31-positive vessels were abundant in TC71 and TC/si tumors (A1 and A2). Vessel density was significantly decreased in tumors formed by TC/VEGFsi clone 7-1, although numerous vessels were seen in the normal tissue surrounding the tumor (A3). A similar phenomenon was seen in tumors treated with polyethylenimine/VEGFsi-7 (A5), whereas the vessel density in tumors treated with polyethylenimine/si control was similar to that observed in the untreated TC71 tumors (A4). Terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assay revealed the presence of massive apoptotic and necrosis cells in mice inoculated with TC/VEGFsi clone 7-1 and in wild-type tumors treated with polyethylenimine/VEGFsi-7 (B3 and B5). By contrast, TC71, TC/si, and polyethylenimine/si-treated tumors showed only small areas of necrosis and apoptosis (B1, B2, and B4). Four other important angiogenic factors (bFGF, PDGF-β, TGF-β, and IL-8) remained unchanged in the TC/si, TC/VEGFsi clone 7-1, and polyethylenimine/VEGFsi-7-treated tumors (Fig. 5C and D; data not shown). These data indicated that the antiangiogenic and antitumor effect was secondary to the inhibition of VEGF<sub>165</sub>.

Effect of VEGFsi on bone tumor formation. The data above showed that VEGFsi-7 inhibited s.c. growth of TC71 cells. We next evaluated whether VEGFsi influenced tumor formation in the bone. TC71, TC/si, and TC/VEGFsi clone 7-1 cells were injected into the tibias of nude mice. After 3 weeks, 70% of mice injected with TC71 cells and 80% of mice injected with TC/si cells had developed osteolytic bone tumors, as assessed by radiography, compared with only 29% of mice injected with TC/VEGFsi clone 7-1 cells (Fig. 6; Table 1; <i>P < 0.01</i>). The bone damage was quantified using a numerical grading system defined by Weber et al. (21). The average level of

**Table 1.** Effect of VEGFsi on tumor-induced bone lysis

| Tumor cell injected | Bone tumor incidence* | Average lytic level† |
|---------------------|-----------------------|----------------------|
| TC71                | 7/10 (70%)            | 1.8                  |
| TC/si               | 8/10 (80%)            | 2.5                  |
| TC/VEGFsi clone 7-1 | 2/7 (29%)            | 0.3                  |

*NOTE: TC71, TC/si, or TC/VEGFsi clone 7-1 cells were inoculated into tibias of nude mice. Three weeks later, radiograph images were obtained.

†Mice with bone tumors/total number of mice (% with tumors).

1Osteolytic bone lesions were graded from 0 to 4 (with 4 being the most lytic) as defined in Materials and Methods.

2<i>P < 0.01</i>, compared with either TC71 or TC/si.
bone lysis in TC71 and TC/si injected animals was 1.8 and 2.5, respectively, compared with 0.3 in animals injected with TC/VEGFsi clone 7-1.

Discussion

We showed previously that compared with normal human osteoblasts three of four Ewing's sarcoma cell lines over-express VEGF and that there is a shift in isoform production from the membrane VEGF165 to the more soluble VEGF189 (12, 23). We have also shown that VEGF is abundantly expressed in the primary tumors of Ewing's sarcoma patients (data not shown). In the present study, we show that VEGF165 plays a critical role in the growth of Ewing's sarcoma. This was done by selectively inhibiting VEGF165 expression and protein production using RNA-mediated interference by siRNA. Delivery of siRNA can be achieved through exogenous application of synthetic siRNA or through endogenous expression using plasmid or vector delivery to the target cell. Chemically or enzymatically synthesized siRNA is costly and has been shown to have a relatively short half-life with only transient inhibition of the target gene (24). To overcome these shortcomings, we constructed several vector-based expression systems in which sense and antisense strands of short VEGF sequences were transcribed into hairpin structures under the control of a U6 promoter and then processed into functional siRNAs by double strand–specific RNase called Dicer inside the cells (25). We designed four different VEGFsi plasmids targeting four different VEGF sequences in different regions of the VEGF mRNA. All were located between exons 1 and 5. VEGFsi-7 specifically blocked VEGF165 expression in both Ewing's sarcoma and breast cancer cells. By contrast, VEGF189 expression was unchanged in the transfected cells. VEGF protein secretion was similarly inhibited by VEGFsi-7 as quantitated by ELISA. Reduction in VEGF protein production was also documented by the finding that HMVEC cell proliferation and migration induced by TC71 conditioned medium was almost completely abolished following transfection with VEGFsi-7 but not affected by the VEGFsi control plasmid. The exact mechanism of this selective VEGF165 inhibition is unclear but may involve an effect of the siRNA at the post-transcriptional level (16).

One of the drawbacks of siRNA is that other nontargeted genes with as few as 11 continuous nucleotides similar to those of the targeted gene can be affected (26). To confirm the specificity of VEGFsi-7, we examined its effect on the expression of other structurally or functionally related genes, including bFGF, PDGF-β, TGF-β1, IL-8, HER-2/neu, and topo IIα. None were affected following transfection. HER-2/neu is an upstream regulator of VEGF (27), and we have shown previously a link between HER-2/neu and VEGF (12). Down-regulation of HER-2/neu in Ewing's sarcoma cells by herceptin also resulted in decreased VEGF expression and protein production.5 E1A transfection led to a decrease in VEGF and HER-2/neu expression and an increase in topo IIα. By contrast, the inhibition of VEGF165 by VEGFsi-7 had no effect on either HER-2/neu or topo IIα expression, again indicating the specificity of this particular siRNA.

Transfection of VEGFsi-7 into TC71 cells did not alter cell growth in vitro. However, when these cells were injected either s.c. or into the bone of nude mice, tumor growth was slower than in parental and TC/si control-transfected cells. TC/VEGFsi cells produced small tumors that were avascular in appearance with decreased vessel density. Because other proteins have been shown to be involved in tumor angiogenesis, we examined the tumors by immunohistochemical analysis and found no change in bFGF, PDGF-β, TGF-β1, or IL-8. Together, these data confirm once again the specificity of our VEGFsi and indicate that VEGF165 plays a pivotal role in Ewing’s sarcoma angiogenesis and tumor growth.

Our data also indicate that gene therapy targeting VEGF165 may have therapeutic benefit. In this study, we elected to use polyethyleneimine as the vector delivery system because of our prior experience and success with polyethyleneimine/IL-12 gene therapy (20). Polyethyleneimine is a cationic polymer, which is nontoxic when delivered in vivo. This polymer retains its cationic state at physiologic pH levels, prevents endosomal buffering, and does not elicit a significant immune response. The injection of polyethyleneimine/VEGFsi into palpable TC71 murine tumors resulted in the inhibition of tumor growth, increased animal survival, decreased tumor vessel density, decreased tumor VEGF expression, and increased tumor apoptosis and necrosis compared with tumor injected with polyethyleneimine/si control vector. As seen with the TC/VEGFsi-transfected tumors, the levels of bFGF, PDGF-β, TGF-β1, and IL-8 were unchanged and similar in the polyethyleneimine/VEGFsi and polyethyleneimine/si control-treated tumors. Once again, these data indicate that the antiangiogenic and antitumor effect seen was secondary to the inhibition of VEGF165.

In summary, we have shown that siRNA technology can be used to specifically inhibit one VEGF isoform. Both cell transfection and delivery by polyethyleneimine resulted in selective inhibition of VEGF165 expression, leading to decreased tumor vascularity and growth in vivo. These data indicate that VEGF165 plays a central role in Ewing's sarcoma angiogenesis, because PDGF-β, bFGF TGF-β1, and IL-8 were all unchanged. Therefore, targeting VEGF with specific small-molecule inhibitors may have therapeutic benefit. The cure rate for patients with Ewing's sarcoma, particularly those who present with large tumors or metastatic disease, is poor, with a disease-free survival rate of 40% to 50% at 2 years (3, 4). Survival rates have remained stagnant over the past 20 years despite aggressive dose-intensive chemotherapy combined with radiation therapy and surgery. Therefore, it behoves us to consider novel therapeutic approaches in an effort to improve the outcomes of these patients.

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