Vascular Endothelial Growth Factor: A Therapeutic Target for Tumors of the Ewing’s Sarcoma Family

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ABSTRACT

Purpose: We have reported previously that intratumoral microvessel density (MVD) is a significant prognostic indicator of event-free survival in the Ewing’s sarcoma family of tumors (ESFT). Here, the angiogenic growth factor expression profile and its relationship with MVD has been investigated in ESFT.

Experimental Design and Results: Using ESFT model systems, the potential of these factors as therapeutic targets has been evaluated. A significant correlation ($P = 0.02$) was observed between vascular endothelial growth factor (VEGF) expression and MVD, consistent with the hypothesis that VEGF regulates the development of microvessels in ESFT. There was no correlation between MVD and any of the other growth factors studied. All six ESFT cell lines studied produced and secreted VEGF; five of six cell lines also secreted placental growth factor, one cell line (A673) at high levels. Tumor conditioned medium induced proliferation of human umbilical vein endothelial cells. Expression of VEGF receptors Flt-1 and Flk-1/KDR was heterogeneous across the cell lines. Both receptor tyrosine kinase inhibitors SU6668 (targets Flk-1/KDR, platelet-derived growth factor receptor-β, and fibroblast growth factor receptor-1) and SU5416 (targets Flk-1/KDR) as well as anti-VEGF agents rhuMAb-VEGF (bevacizumab) and VEGF Trap delayed s.c. growth of ESFT in mice compared with untreated groups: SU6668 (100 mg/kg/d), SU5416 (25 mg/kg/d), rhuMAb-VEGF (10 mg/kg twice weekly), and VEGF Trap (2.5 or 25 mg/kg twice weekly).

Conclusions: These data suggest that VEGF is the single most important regulator of angiogenesis in ESFT and may be exploited for therapeutic advantage.

INTRODUCTION

The Ewing’s sarcoma family of tumors (ESFT) includes classic Ewing’s sarcoma, extraskeletal Ewing’s sarcoma, Askin tumor, and peripheral primitive neuroectodermal tumors. ESFTs are small round cell tumors predominantly affecting young people between ages 10 and 20 years. This family of tumors has been defined following the identification of nonrandom chromosome translocations involving rearrangement of the EWS gene on chromosome 22q12 with a member of the ETS gene family of transcription factors. These rearrangements provide a powerful diagnostic tool (1). The site of the primary tumor, presence or absence of metastatic disease at diagnosis, and age at diagnosis are important prognostic indicators (2, 3). At diagnosis, ~25% of ESFT patients present with metastatic disease (4). Although aggressive treatment regimens of multimodal therapy have improved relapse-free survival (4–6), the outcome for these patients remains poor; the 5-year disease-free survival rates are only 10% to 20% in patients with metastatic disease. Therefore, there is an urgent need to identify new targets for the development of novel therapeutic strategies.

Angiogenesis, the neovascularization or formation of new capillaries from preexisting vessels, is a rate-limiting factor for the growth and expansion of tumors (7). Tumors of 1 to 2 mm3 obtain oxygen and nutrients by passive diffusion from neighboring blood vessels. However, to grow beyond this size, tumors must recruit blood vessels to nourish and oxygenate the tumor cells (i.e., angiogenesis must occur). Several features of the microenvironment regulate the formation of the vasculature, including angiogenic factors such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), platelet-derived growth factors (PDGF), and nonangiogenic influences such as hypoxia, necrosis, and metabolic rate of tumor (8, 9). The vasculature of normal tissues is reported to closely reflect such as hypoxia, necrosis, and metabolic rate of tumor. Consequently, some proangiogenic factors may be constitutively expressed in tumors at high levels. The angiogenic nature of a tumor is the sum of positive and negative regulators of angiogenesis, which may arise from both tumor and normal cells of the cellular environment. The key regulator and hence most frequently studied proangiogenic growth factor is VEGF; however, angiogenesis may also be regulated by other growth factors, such as placental growth factor (PIGF; ref. 12), bFGF (13), and PDGF (14), depending on the tumor type.

Assessing microvessel density (MVD) is an established method for measuring the degree of neovascularization within a tumor (15). MVD within isolated regions, or so-called
hotspots, was initially described as a clinically significant prognostic factor in breast (16) and prostate (17) cancer. In these tumors, it can aid in the assessment of disease stage, prediction of metastasis, recurrence, or survival (18, 19). Since then, the prognostic value of MVD has been evaluated in many different cancers (10). In ESFT, we have shown previously that MVD is prognostically significant in these tumors (20).

Inhibition of angiogenesis, by targeting the comparatively homogenous and genetically stable endothelium, represents a promising approach to cancer therapy (21). There are currently >75 agents that target tumor vasculature either directly or indirectly in clinical trials, including 12 that have entered or completed phase III trials (22). Unlike the more conventional chemotherapeutic approaches, little or no acquired drug resistance has been reported following treatment with inhibitors of angiogenesis (23). With the recent success of the first antiangiogenic agent (bevacizumab, Genentech, Inc., San Francisco, CA) in a phase III randomized trial in metastatic colorectal cancer, such inhibitors represent promising adjunct therapy with chemotherapeutic agents or radiotherapy (24).

The primary aim of this study was to examine the expression profile of the angiogenic growth factors VEGF, PlGF, bFGF, PDGFA, and PDGFB and the relationship between their expression and MVD in ESFT. Having identified VEGF as the single angiogenic growth factor most frequently associated with expression of MVD, we sought to investigate the effect of antiangiogenic agents targeting the VEGF pathway in an ESFT s.c. growth mouse model. Two different classes of antiangiogenic agents were investigated: anti–tyrosine kinase receptor agents and inhibitors of VEGF. The receptor tyrosine kinase inhibitors examined (SU6668 and SU5416) are ATP site-directed agents and inhibitors of VEGF. The receptor tyrosine kinase inhibitors examined (SU6668 and SU5416) are ATP site-directed compounds that inhibit growth factor–stimulated receptor tyrosine phosphorylation of Flk-1/KDR alone (SU5416) or FGF receptor 1, PDGF receptor (PDGFR)-β, and Flk-1/KDR (SU6668; refs. 25–27). Two anti-VEGF agents were studied: rhMuAB-Vegf2 (bevacizumab), a humanized VEGF neutralizing antibody, and VEGF Trap, a composite receptor consisting of portions of the human Flt-1 and Flk-1/KDR extracellular domains fused to the Fc portion of human IgG1 (28–30).

PATIENTS AND METHODS

Clinical Samples. Tumor material taken at the time of diagnostic surgery from 34 patients with ESFT was analyzed. Patients were from St. James’s University Hospital (Leeds, United Kingdom; $n = 14$), Stanmore Orthopaedic Hospital (Stanmore, United Kingdom; $n = 4$), Royal Marsden Hospital (Sutton, United Kingdom; $n = 3$), Addenbrookes Hospital (Cambridge, United Kingdom; $n = 2$), Royal Victoria Infirmary (Newcastle upon Tyne, United Kingdom; $n = 1$), and Royal Orthopaedic Hospital (Birmingham, United Kingdom; $n = 10$). Median age of patients at diagnosis was 14 years (range, 2–49 years). Diagnosis was made by conventional pathology, including examination of morphology and immunohistochemistry; in some cases, this was supported by G banding for rearrangements of chromosome 22q12 (characteristic of this tumor group). Tissue was confirmed as diagnostic tumor by reverse transcription-PCR (RT-PCR) for the EWS-ETS gene rearrangements (26 of 34) or immunohistochemistry for MIC-2 (CD99; 8 of 34). Primary tumor volume and presence of metastases were detected by conventional imaging and examination of bone marrow by light microscopy. Informed consent was obtained for the use of tumor material for research at each center. Ethical approval was obtained from the Leeds Teaching Hospital Trust Ethics Committee and Trent Multi-Research Ethics Committee.

Human umbilical vein endothelial cells (HUVEC) were isolated from human umbilical cords obtained following cesarean sections conducted at the delivery ward at St. James’s University Hospital. Ethical approval was obtained from the Leeds Teaching Hospital Trust Ethics Committee and informed consent was obtained from each donor. Table 1 contains further patient information.

Immunohistochemistry. Immunohistochemistry and immunofluorescent staining was carried out on serial cryostat sections (5 μm) of primary tumor taken at diagnosis. Histology of each tumor was visualized by light microscopy after staining with H&E. For each method, a serial section was processed without the addition of primary antibody to test for nonspecific binding (negative control). Table 2 contains information on the antibodies, dilutions, and controls used.

For CD31 immunohistochemistry, a three-stage peroxidase method was used. All incubations were carried out at room temperature. Sections were fixed in methanol/acetone (50:50) for 2 × 2 minutes, allowed to air dry, and endogenous peroxidase activity blocked using 0.6% hydrogen peroxide (Sigma, Dorset, United Kingdom) in methanol for 10 minutes. Sections were then washed for 10 minutes in running water. Endogenous biotin or biotin-binding proteins were blocked using the avidin-biotin blocking kit (Vector Laboratories, Peterborough, United Kingdom) according to manufacturer’s instructions. Nonspecific antibody binding sites were blocked by incubation with normal rabbit serum (DAKO Ltd., Cambridgeshire, United Kingdom) in methanol/acetone (50:50) for 1:10 in TBS for 5 minutes. The sections were then incubated with primary antibody for 1 hour (Table 2). After rinsing twice in PBS, the sections were incubated with secondary antibody for 30 minutes. After two further washes in PBS, sections were incubated with avidin-biotin-peroxidase complex (DAKO). Sections were rinsed twice with PBS. The staining pattern was developed by incubating sections with 3,3’-diaminobenzidine substrate for 15 minutes; staining was visual as brown precipitate. Sections were rinsed for 1 minute in running water and counterstained using hematoxylin.

The saponin method (31) was used for immunohistochemistry detection of VEGF, PlGF, PDGFA, and PDGFB. Sections were permeabilized, fixed, and endogenous peroxidase blocked in 1% hydrogen peroxide in balanced salt solution containing Mg2+ and Ca2+ (Life Technologies, Paisley, United Kingdom) supplemented with 0.1% saponin (Sigma) and 0.02% sodium azide for 30 minutes. After rinsing twice with balanced salt solution containing 0.01 mol/L HEPES (Life Technologies) buffer, endogenous biotin or biotin-binding proteins were blocked using the avidin-biotin blocking kit according to manufacturer’s instructions. The sections were then washed thrice in balanced salt solution containing 0.1% saponin and incubated with primary antibody overnight at 4°C (Table 2). Following three washes in balanced salt solution containing 0.1% saponin, the sections were incubated with 1% normal serum (same species as
secondary) for 15 minutes and then with secondary antibody for 30 minutes at room temperature. After rinsing twice with balanced salt solution containing 0.1% saponin, sections were incubated with avidin-biotin-peroxidase complex for 30 minutes at room temperature. Sections were rinsed twice with PBS, and the brown precipitate was developed by incubating sections with 3,3'-diaminobenzidine substrate for 15 minutes. Sections were rinsed for 1 minute in running water and counterstained using hematoxylin.

bFGF was detected by immunofluorescence. Sections were fixed in methanol/acetone (50:50) for 2/2 minutes and left to air dry. The sections were then incubated with primary antibody for 1 hour (Table 2). After rinsing twice in TBS, the sections were refixed in methanol/acetone (50:50) for 2/2 minutes and

<table>
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<th>Patient</th>
<th>Age at diagnosis</th>
<th>Primary tumor site</th>
<th>Metastases at diagnosis</th>
<th>Time to first event (mo)</th>
<th>Follow-up or time to death from diagnosis (mo)</th>
<th>Status</th>
<th>Fusion type</th>
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<td>Sacroiliac joint</td>
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<td>Right fibula</td>
<td>Lung</td>
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<td>60</td>
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</table>
again left to air dry. The sections were then incubated with secondary antibody for 30 minutes. Sections were rinsed twice with 0.1% Tween 20 (Sigma) in TBS followed by rinsing in running water for 1 minute. Sections were mounted in faramount (DAKO) and viewed using a Zeiss Axioplan microscope with fluorescent filter.

**Determination of Microvessel Density.** In this study, CD31 antibodies were used for immunohistochemical staining of endothelial cells in a tumor section. This was found to be a more reliable method of detecting small immature vessels than CD34 or von Willebrand factor (data not shown), consistent with previous studies (32). Following staining with CD31, each section was scanned on a Zeiss Axioplan microscope at ×160 magnification to identify three areas with the greatest MVD. The vessel count was done at ×250 magnification for an overall area of 0.79 mm² for each of the three “hotspots” identified. The mean microvessel count was calculated for each section. MVD was expressed as the number of microvessels per millimeter square. Tumor MVD separated into two clear groups: negative/low MVD (MVD < 100 per mm²) and high MVD (MVD > 100 per mm²).

**Cell Culture.** HUVEC cells were isolated from human umbilical cords obtained from the delivery ward at St. James’s University Hospital (see Clinical Samples). The cords were collected in cord buffer [137 mmol/L NaCl, 4 mmol/L KCl, 10 mmol/L HEPES-HCl (pH 7.4), 11 mmol/L glucose] and isolated by an enzymatic technique (33). Once isolated, cells were grown in M199 medium (Sigma) containing 20% FCS to be used for all experiments.

The characterized ESFT cell lines TC-32, RD-ES, and TTC-466 were grown in RPMI 1640 (Sigma) containing 10% FCS; medium for TTC-466 was supplemented with 10% conditioned medium. The ESFT cell line A673, breast cancer cell line MCF-7, and human foreskin fibroblasts were grown in DMEM (Sigma) and 10% FCS. ESFT cell lines SK-ES1 and SK-N-MC were grown in McCoy’s medium (Sigma) plus 15% FCS and DMEM/F-12 medium (Sigma) plus 10% FCS, respectively. Cell lines were purchased from the American Type Culture Collection (Rockville, MD), except for the TC-32 and RD-ES cells (kind gifts from Dr. J. Toretsky, Division of Pediatrics, University of Maryland, Baltimore, MD) and TTC-466 cells (kind gift from Dr. P. Sorenson, British Columbia Children’s Hospital, Vancouver, British Columbia, Canada). All cells were maintained in a humidified atmosphere of 5% CO₂/95% air at 37°C (Sanyo Gallenkamp, Loughborough, United Kingdom).

**Reverse Transcription-PCR.** Total RNA was isolated from ESFT cell lines using Ultraspec RNA (AMS Biotechnology, Oxon, United Kingdom). The quality of isolated RNA was confirmed by separation of RNA (1 µg) in 1% agarose gel containing ethidium bromide (0.5 µg/mL) in 1× Tris-borate EDTA and visualized under UV light using a transilluminator. First-strand cDNA was synthesized from total RNA (1 µg) using 5 units of murine leukemia virus reverse transcriptase (Pharmacia Biotech, St. Albans, United Kingdom) in 1× PCR buffer [10 mmol/L Tris-HCl (pH 8.3), 50 mmol/L KCl, Perkin-Elmer Applied Biosystems, Warrington, United Kingdom], 1 mmol/L deoxyribonucleotide triphosphates (Pharmacia Biotech), 8 mmol/L MgCl₂ (Perkin-Elmer Applied Biosystems), 0.3 µg random hexamer primers (Life Technologies), and 8 units of RNA guard (Pharmacia Biotech). cDNA (10 µL) was then amplified for the angiogenic growth factors and receptors VEGF, Flt-1, Flk-1/Flt-2, bFGF (BD PharMingen, Santa Cruz, CA, SC-152), VEG (BD PharMingen, Oxford, United Kingdom, 55036), PDGFR-α, and PDGFR-β (Table 3). The efficiency of amplification was controlled by amplification for the housekeeping gene β2-microglobulin (Table 3). Amplification was carried out using 1.25 units of Amplitaq gold (Perkin-Elmer Applied Biosystems) and primer pairs (40 pmol) for the above growth factors and receptors in

<table>
<thead>
<tr>
<th>Target</th>
<th>Primary antibody type and source</th>
<th>Dilution</th>
<th>Secondary antibody type and source</th>
<th>Dilution</th>
<th>Staining method used</th>
<th>No. tumors analyzed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD31</td>
<td>Mouse anti-human endothelial cell, CD31 (DAKO, M0823)</td>
<td>1:10</td>
<td>Biotinylated rabbit anti-mouse IgG (DAKO, E0413)</td>
<td>1:100</td>
<td>Three stage peroxidase</td>
<td>34</td>
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<tr>
<td>bFGF</td>
<td>Rabbit anti-human bFGF (Santa Cruz Biotechnology, Santa Cruz, CA, SC-152)</td>
<td>1:300</td>
<td>Goat anti-rabbit Alexa Fluor 594 nm (Molecular Probes, Leiden, the Netherlands, A11012)</td>
<td>1:10,000</td>
<td>Immunofluorescence</td>
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<tr>
<td>VEGF</td>
<td>Mouse anti-human VEGF (BD PharMingen, Oxford, United Kingdom, 55036)</td>
<td>1:50</td>
<td>Biotinylated rabbit anti-mouse IgG (DAKO, E0413)</td>
<td>1:100</td>
<td>Saponin method</td>
<td>30</td>
</tr>
<tr>
<td>PDGFA</td>
<td>Rabbit anti-human PDGFA (Santa Cruz Biotechnology, SC-128)</td>
<td>1:800</td>
<td>Biotinylated goat anti-rabbit IgG (DAKO, E0432)</td>
<td>1:200</td>
<td>Saponin method</td>
<td>28</td>
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<tr>
<td>PDGFB</td>
<td>Rabbit anti-human PDGFB (Santa Cruz Biotechnology, SC-7878)</td>
<td>1:400</td>
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<td>1:200</td>
<td>Saponin method</td>
<td>28</td>
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<tr>
<td>PIGF</td>
<td>Goat anti-human PIGF (Santa Cruz Biotechnology, SC-1880)</td>
<td>1:80</td>
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<td>Saponin method</td>
<td>28</td>
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</tbody>
</table>

NOTE. Sufficient material was not available to analyze each tumor for all markers. Conditions for use of antibodies were determined empirically.
1 × PCR buffer (as above), 0.2 mmol/L deoxynucleotide triphosphates, and 1.6 mmol/L MgCl2. AmpliTaq gold was activated by heating at 94 ℃ for 10 minutes and amplification of the target cDNA was done by PCR (Table 3). Positive controls for each target RNA were included (Table 3). Negative controls included reverse transcriptase–negative controls in which reverse transcriptase enzyme was replaced with water as well as water-negative controls containing all components for the RT-PCR reaction but no target RNA.

RT-PCR products (30 μL) were size separated on a 2% agarose gel in 1 × Tris-borate EDTA. A 50-bp molecular weight ladder (Life Technologies) was used to estimate product size. Amplified bands were identified following staining with ethidium bromide (0.5 μg/mL) and visualization under UV light using a transilluminator. The identity of amplified product was confirmed by direct sequence analysis; excised bands were collected after 24 hours; medium was centrifuged at 3,000 g for 5 minutes to remove any cellular fragments. Importantly, for these experiments, the growth of the ESFT cell line A673 was unaffected by culture for up to 72 hours into HUVEC medium (M199 plus 10% or 20% FCS; data not shown).

HUVEC cells (2 × 10^5 per well) were seeded in gelatin-coated (0.2%, Sigma) 96-well plates and left to adhere overnight. On the following day, cells were rinsed with serum-free medium and then stimulated with M199 medium containing 10% or 20% FCS (control) or A673 TCM for 72 hours. All conditions were carried out in triplicate. During the last 18 hours of the 72-hour incubation, bromodeoxyuridine (1:750, Biotrack Cell Proliferation ELISA System, Amersham Pharmacia Biotech, Little Chalfont, Bedfordshire, United Kingdom) was added to cells and proliferation was assayed according to manufacturer’s instructions.

**Receptor Tyrosine Kinase Inhibitors.** For *in vitro* studies looking at viable cell counts, ESFT cells (TC-32, RD-ES, or TTC-466) were seeded in Primaria six-well plates (2 × 10^3 per well) and left to adhere overnight and medium was replaced. SU6668 (0.01-10 μmol/L), SU5416 (0.01-10 μmol/L), SUGEN (now Pfizer), San Francisco, CA], or DMSO (10 μL; vehicle for SU6668 or SU5416) was added to cells. After 24 and 48 hours, cells were harvested and viable cell number was counted using the trypan blue exclusion assay. For cell proliferation assays, ESFT cells TC-32, RD-ES, or TTC-466 were seeded (1 × 10^5 per well) in Primaria 96-well plates and treated as above. After 24 and 48 hours, cell proliferation was assayed using the Biotrack Cell Proliferation ELISA as above. In addition to the vehicle negative control (DMSO), viable cell number and proliferation were measured in cells under normal growth conditions.

The effect of SU6668 (100 mg/kg/d) and SU5416 (25 mg/kg/d) on RD-ES growth was examined in *nu/nu* mice. Mice (*n* = 22 and 20, respectively) were injected s.c. in one flank with RD-ES cells (2.5 × 10^6 in 0.2 mL medium). On day 8, following the development of a palpable tumor, mice were injected daily with either vehicle alone (DMSO), SU6668 (100 mg/kg), or SU5416 (25 mg/kg). The effect of SU6668 (100 mg/kg/d) on A673 growth was also examined in *nu/nu* mice. As described above, mice (*n* = 4) were injected with A673 cells. On day 15, following the development of a palpable tumor, mice were injected daily with either vehicle alone (DMSO) or SU6668 (100 mg/kg). Tumors were measured twice weekly by caliper measurements in two dimensions, the largest diameter (*a*) and its perpendicular (*b*): tumor size = *a* × *b*. Mice were sacrificed when s.c. tumors reached a size of 1.4 cm^2, at the end of the experiment, or if the mouse showed signs of distress.

**Table 3**  
Primer sequences, PCR cycle characteristics, and positive controls used for amplification of angiogenic growth factors and their receptors and the housekeeping gene δ2-microglobulin

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF</td>
<td>TCGGGGCTTCGAAAACATG</td>
<td>CTCGTTGAGAGTCTGTTTC</td>
</tr>
<tr>
<td>Fli-1</td>
<td>GTCAACGAGAGTGAAGTTGCTTA</td>
<td>CAGTGTCAGAAGTGGTTATT</td>
</tr>
<tr>
<td>Flk-1/KDR</td>
<td>ACCGCGCTGAAGTTGGGACCC</td>
<td>TGGTTGGTCACCTGATTGGAGG</td>
</tr>
<tr>
<td>PDGFA</td>
<td>TCACCCGGTTCCACCCAACTA</td>
<td>TGGCGCTCATCCTCACCCTAC</td>
</tr>
<tr>
<td>PDGFB</td>
<td>CTCTGTTGCTTCTGCTGCTG</td>
<td>GCGGACCTTCAAAACAAATG</td>
</tr>
<tr>
<td>PDGFR-α</td>
<td>AACTGATCCCCGAGACTCTCTG</td>
<td>CAGGTGATGGTCCCCAGATCT</td>
</tr>
<tr>
<td>PDGFR-β</td>
<td>ACCATCTCATGCGAGTAAACA</td>
<td>TGGCGAGATGGAAACCCAG</td>
</tr>
<tr>
<td>δ2-Microglobulin</td>
<td>CTCGGGCTACTCTCTCTTTCT</td>
<td>TGGCGAGATGGAAACCCAG</td>
</tr>
</tbody>
</table>
Table 3  Primer sequences, PCR cycle characteristics, and positive controls used for amplification of angiogenic growth factors and their receptors and the housekeeping gene β2-microglobulin (Cont’d)

<table>
<thead>
<tr>
<th>PCR cycle characteristics</th>
<th>Positive control</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>40 cycles of 30 s at 94°C, 30 s at 60°C, 1 min at 72°C; final cycle of 7 min at 72°C</td>
<td>MCF-7 (34)</td>
<td></td>
</tr>
<tr>
<td>35 cycles of 1 min at 94°C, 1 min at 62°C, 1 min at 72°C; final cycle of 10 min at 72°C</td>
<td>HUVEC (35)</td>
<td></td>
</tr>
<tr>
<td>35 cycles of 1 min at 94°C, 1 min at 60°C, 1 min at 72°C; final cycle of 10 min at 72°C</td>
<td>HUVEC (36)</td>
<td></td>
</tr>
<tr>
<td>35 cycles of 1 min at 95°C, 30 s at 52°C, 45 s at 72°C; final cycle of 5 min at 72°C</td>
<td>HUVEC (37)</td>
<td></td>
</tr>
<tr>
<td>35 cycles 1 min at 94°C, 1 min at 62°C, 1 min at 72°C; final cycle of 10 min at 72°C</td>
<td>HUVEC (38)</td>
<td></td>
</tr>
<tr>
<td>35 cycles of 30 s at 94°C, 30 s at 55°C, 1 min at 72°C; final cycle of 7 min at 72°C</td>
<td>Human foreskin fibroblasts (40)</td>
<td></td>
</tr>
<tr>
<td>33 cycles of 30 s at 94°C, 1 min at 60°C, 1 min at 74°C; final cycle of 7 min at 74°C</td>
<td>Human foreskin fibroblasts (40)</td>
<td></td>
</tr>
</tbody>
</table>

**Anti–Vascular Endothelial Growth Factor Agents.** The effect of rhuMAb-VEGF (bevacizumab; 10 mg/kg twice weekly) and VEGF Trap (2.5 or 25 mg/kg twice weekly, Regeneron Pharmaceuticals, NY) on RD-ES or A673 growth was examined in 
uu/nu mice. Mice [rhuMAb-VEGF n = 5-8 (RD-ES) and n = 5 (A673) per group; VEGF Trap n = 5 (RD-ES) and n = 3 (A673) per group] were injected s.c. in one flank with RD-ES or A673 cells (5 × 10⁶ in 0.2 mL medium); cell numbers injected s.c. were doubled compared with that in previous experiments to increase the frequency of tumor take. On day 15, following the development of a palpable tumor, mice were injected twice weekly with either rhuMAb-VEGF (10 mg/kg)/control vehicle (0.9% NaCl) or VEGF Trap (2.5 or 25 mg/kg)/control vehicle (Fc control protein). Both rhuMAb-VEGF and VEGF Trap were given for up to 4 weeks. Tumor size was measured twice weekly as above and mice were sacrificed when tumors reached a size of 1.4 cm², at the end of the experiment, or if the mouse showed signs of distress. All procedures with mice were done as per United Kingdom guidelines and carried out under a project license issued by the Home Office (London, United Kingdom).

**Histologic Analysis.** Tumors removed from mice were divided into two, half of which was fixed in zinc fixative (BD Biosciences, Oxford, United Kingdom) and embedded in paraffin and the other half was embedded in OCT compound and frozen. Histology of tumors was examined by light microscopy following staining of tumor sections (4 µm) with H&E.

**Statistical Analyses.** Associations between MVD and expression of angiogenic factors were evaluated using Fisher’s exact test. A two-stage regression approach was used for analyzing data from the in vivo model system with receptor tyrosine kinase inhibitors and anti-VEGF agents. Here, data consisted of repeated tumor growth measurements on each mouse. A linear regression was fitted to each mouse-specific growth curve. The slope coefficients estimate the average growth rate of tumor in each mouse and these were then used as the dependent variable in an analysis of covariance comparing treatments with and without the starting tumor size as a covariate. \( P < 0.05 \) was considered significant for all studies.

**RESULTS**

**Microvessel Density and Its Relationship with Angiogenic Growth Factors in Ewing’s Sarcoma Family of Tumors.** Microvessels were readily detected in ESFT following immunohistochemistry for the endothelial cell marker CD31 and examination by light microscopy (Fig. 1). The frequency of MVD identified two distinct tumor groups, those with a MVD > 100 per mm² (high MVD) and those with a MVD < 100 per mm² (low MVD). Of the 34 samples analyzed, 8 (24%) had high MVD.

VEGF was expressed at high levels in 18 of 30 (60%); Fig. 24) of the tumors examined; the remaining 12 tumors were negative (40%; Fig. 2B). There was no gradation of VEGF expression across the tumor group. In contrast, PDGFA or PDGFβ was heterogeneous with no negative tumors. Expression of PDGFA and PDGFβ was low (Fig. 2C and F), intermediate (Fig. 2D and G), or high (Fig. 2E and H). For statistical analyses, expression was classified as either strongly positive (homogeneous staining across all tumor; 18 of 28 (64%) PDGFA and 13 of 28 (46%) PDGFβ) or focally positive (10 of 28 (36%) PDGFA and 15 of 28 (54%) PDGFβ). PDGFA and bFGF expression was also heterogeneous; PDGFA expression was either absent [11 of 28 (39%); data not shown], focally positive [9 of 28 (32%); Fig. 2F], or strongly positive across the whole tumor [8 of 28 (29%); Fig. 2J]. Expression of bFGF was either negative [6 of 33 (18%); data not shown], low positive [10 of 33 (30%); data not shown], intermediate positive [3 of 33 (9%); Fig. 2M], or strongly positive [14 of 33 (43%); Fig. 2N]. For statistical analyses, staining with PIg and bFGF was classed as negative or positive. For all growth factors investigated, levels of expression were not dependent on site of primary tumor (data not shown).

Expression of VEGF correlated with MVD (\( P = 0.02; \) 7 of 7 tumors with high MVD and 11 of 23 tumors with low MVD were positive for VEGF). This suggests that VEGF may be an important regulator of MVD in ESFT. There was no correlation between MVD and expression of bFGF (\( P = 0.14; \) 5 of 8 tumors with high MVD and 22 of 25 tumors with low MVD were positive for bFGF), PIg (\( P = 0.42; \) 6 of 8 tumors with high MVD and 11 of 20 tumors with low MVD were positive for PIg), PDGFA (\( P = 0.67; \) 4 of 7 tumors with high MVD and 14 of 21 tumors with low MVD were positive for PDGFA), or PDGFβ (\( P = 0.40; \) 2 of 7 tumors with high MVD and 11 of 21 tumors with low MVD were positive for PDGFβ).

**Angiogenic Growth Factors and Receptor Expression in Ewing’s Sarcoma Family of Tumor Cell Lines.** The angiogenic profile of ESFT cell lines was examined by RT-PCR for the angiogenic growth factors VEGF and PIg and receptors Flt-1 and Flk-1/KDR, bFGF, PDGFA, PDGFβ, PDGFRα, and PDGFRβ (Fig. 3). The VEGF primers annealed to exons 1 and 8, thus amplifying all five isoforms of VEGF (VEGF121, VEGF145, VEGF165, VEGF189 and VEGF206). However, only...
three bands were identified by RT-PCR in the ESFT cell lines. Two of the three bands were successfully sequenced and identified as VEGF_{121} (516 bp) and VEGF_{165} (648 bp). The size of the third band (588 bp) indicated that it may represent VEGF_{145}; however, this could not be confirmed by sequence analysis and hence may represent a VEGF_{121}/VEGF_{165} heteroduplex (41). Neither VEGF_{189} (720 bp) nor VEGF_{206} (771 bp) were detected in any of the ESFT cell lines. The PlGF primers used amplified two isoforms of PlGF [PlGF-1 (184 bp) and PlGF-2 (248 bp)], both of which were expressed by all six ESFT cell lines. However, the VEGF receptors Flt-1 (417 bp) and Flk-1/KDR (403 bp) were differentially expressed. Expression of the ligands PDGFA (409 bp) and PDGFB (317 bp) was cell line dependent, whereas PDGFR-α (565 bp) and PDGFR-β (437 bp) receptors were detected in all the cell lines studied (Fig. 3). bFGF (92 bp) was detected in all six cell lines; previous studies by our group have shown expression of FGFR receptors 1 to 4 in the same six ESFT cell lines (38).

**Vascular Endothelial Growth Factor and Placental Growth Factor Are Secreted by Ewing's Sarcoma Family of Tumor Cells.** Specific ELISAs for VEGF, PlGF, bFGF, PDGFA, and PDGFB were done to measure the secretion of the above factors in conditioned medium obtained from all six ESFT cell lines. Only VEGF and PlGF were detected in TCM from ESFT cell lines (Fig. 4A and B, respectively). The concentration of VEGF and PlGF in TCM increased with time, consistent with continual secretion by ESFT cells (data not shown). The A673 cell line secreted high levels of both VEGF and PlGF, which were comparable with levels reported in other tumor types (42, 43). Other cell lines also secreted high levels of VEGF but lower levels of PlGF (RD-ES and TTC-466). Because ESFT are thought to arise in several different neural crest-derived cells, consistent with the multiple sites of ESFT, it is not possible to determine whether the levels of VEGF are high in tumor cells compared with those in the normal cell of origin as is the case for some tumors (44, 45). However, these data imply that VEGF and PlGF are the major proangiogenic factors secreted by ESFT cell lines, whereas bFGF and PDGF isoforms may remain cell associated or sequestered in the extracellular matrix.

TCM (24 hours) containing either 10% or 20% FCS enhanced the proliferation of HUVEC compared with control cells (Fig. 4C). The enhanced proliferation of HUVEC is consistent with the hypothesis that VEGF and PlGF secreted by ESFT cells may play a major role in modulating angiogenesis in these tumors. Both receptor tyrosine kinase inhibitors SU6668 and SU5416 had no significant effect on viable cell number or proliferation of TC-32, RD-ES, or TTC-466 cells in vitro after 24 or 48 hours, suggesting that there is no VEGF autocrine or paracrine survival loop in ESFT cells (data not shown).

**Growth Inhibition of Ewing’s Sarcoma Family of Tumors with Receptor Tyrosine Kinase Inhibitors and Anti-Vascular Endothelial Growth Factor Agents In vivo.** S.c. injection of nu/nu mice with the ESFT cells RD-ES and A673 resulted in rapid tumor growth in all injected mice; after ≤40 days, 100% of the mice had tumors of 1.4 cm² and were sacrificed according to experimental protocol. However, the rate of tumor growth was significantly reduced in mice treated with both receptor tyrosine kinase inhibitors SU6668 (100 mg/kg/d) and SU5416 (25 mg/kg/d; RD-ES only) compared with control mice treated with vehicle alone (DMSO; P = 0.001 for all; Fig. 5A and B).

Growth was also significantly inhibited in mice with RD-ES tumors following treatment with the anti-VEGF agent rhuMAb-VEGF (10 mg/kg twice weekly) compared with control mice treated twice weekly with control vehicle alone (0.9% NaCl; P = 0.001; Fig. 6A). In contrast, although some delay in tumor growth was observed following treatment of mice with A673 tumors, this failed to reach significance (P = 0.08; Fig. 6B). This may in part be explained by the delayed A673 tumor growth in one control mouse. As VEGF Trap inhibits VEGF and may also inhibit PlGF (30, 46), we investigated the effect of this agent on the growth of two ESFT tumors (RD-ES and A673) with different VEGF and PlGF profiles (Fig. 4A and B). Both high (25 mg/kg twice weekly) and low (2.5 mg/kg twice weekly) doses of VEGF Trap significantly inhibited growth of RD-ES tumors compared with that of control mice treated with vehicle alone (Fc control protein; P = 0.001 and 0.001, respectively; Fig. 7A), whereas only high-dose VEGF Trap inhibited A673 tumor growth (P = 0.005; Fig. 7B).

In comparison with control tumors, large areas of geographic necrosis were observed throughout RD-ES tumors treated with both receptor tyrosine kinase inhibitors and anti-VEGF agents (Fig. 8C-F, G, and J-L). Similar effects were also observed throughout A673 tumors following treatment with...
the receptor tyrosine kinase inhibitor SU6668 and anti-VEGF agent rhuMAb-VEGF (Fig. 8D, E, H, and I). However, in contrast to RD-ES tumors, enhanced necrosis was only observed in mice with A673 tumors treated with the high-dose VEGF Trap (25 mg/kg; Fig. 8O) and not low dose (2.5 mg/kg; Fig. 8N) when compared with corresponding control tumors (Fig. 8M). In all groups that responded to treatment, tumors were generally less vascular and cellular but more vacuolated compared with those in control groups. Toxicity was observed in vivo with both receptor tyrosine kinase inhibitors SU6668 and SU5416, which on postmortem were found deposited in the bowel. No toxicity was observed with VEGF targeting agents.

**DISCUSSION**

The results from the present investigation show a significant positive correlation between VEGF and MVD in ESFT. These results, coupled with in vitro observations of TCM-induced endothelial cell proliferation and in vivo inhibition of ESFT growth following treatment with receptor tyrosine kinase inhibitors and anti-VEGF agents, suggest that VEGF may be a major regulator of angiogenesis in this tumor group.

In primary human ESFT, the expression of the proangiogenic growth factor VEGF positively correlated with MVD, consistent with the hypothesis that VEGF may regulate the formation of microvessels in these tumors. PDGFA, PDGFB, PI GF, and bFGF were also expressed by some ESFT, demonstrating that these tumors can produce several different proangiogenic factors; however, their expression did not correlate with MVD. Consistent with our observations, down-regulation of VEGF expression following treatment of mice with the tumor suppressor adenovirus type 5 E1A gene resulted in a decrease in MVD and s.c. ESFT growth (47). In breast cancer, the number of different proangiogenic factors expressed has been reported to increase as the tumors progress (48). Whether the profile of proangiogenic growth factors in ESFT also correlates with progression remains to be seen. To date, the prognostic

![Fig. 2](clincancerres.aacrjournals.org) Angiogenic growth factor expression in ESFT. VEGF immunohistochemistry: example of a (A) positive tumor and (B) a negative tumor. PDGFA immunohistochemistry: (C) low, (D) intermediate, and (E) high levels. PDGFB immunohistochemistry: (F) low, (G) intermediate, and (H) high levels. PlGF immunohistochemistry: (I) PlGF hotspots within tumor and (J) majority of tumor PlGF positive. Negative controls: (K) negative control primary antibody omitted goat anti-rabbit secondary and (L) negative control primary antibody omitted rabbit anti-goat secondary. All original magnification, ×200. bFGF immunofluorescence: (M) intermediate and (N) high bFGF positivity. Original magnification, ×400.
importance of circulating levels and/or tumor expression of angiogenic factors, such as VEGF and bFGF, in pediatric malignancies, soft tissue sarcomas, and malignant bone tumors have only included analysis on a limited number of peripheral primitive neuroectodermal tumors or Ewing’s sarcoma (49–55). Of these studies, some suggest that serum VEGF may be used to monitor therapeutic response in children with solid malignancies (52). However, in agreement with others (49, 50), we have not found this to be a consistent or reliable marker primarily due to the release of VEGF from activated platelets (data not shown). We are currently conducting a prospective clinical outcome study through the United Kingdom Children’s Cancer Study Group (study no. BS 2002 02) to evaluate the prognostic significance of angiogenic factors in ESFT using a multivariate analysis.

All the ESFT cell lines studied produce and secrete VEGF, providing a useful model to investigate its potential role in this tumor group. These results are consistent with other studies that have also shown high levels of VEGF secretion by ESFT cell lines in vitro (47, 56). Recent reports suggest that the overexpression of VEGF by ESFT cells may in part be regulated by the insulin-like growth factor/insulin-like growth factor receptor-I autocrine loop and/or the synergistic activation of the VEGF promoter following interaction of EWS-ETS fusion proteins with transcription factor Sp1 (55, 56). In addition to VEGF secretion, we have also shown that ESFT cells express VEGF receptors Flt-1 and Flk-1/KDR, suggesting that VEGF may function both as a paracrine and as an autocrine factor in these tumors. Indeed, stimulation of HUVEC proliferation by ESFT TCM was observed, thus supporting the hypothesis that VEGF has a paracrine role in regulating the formation of new vasculature in these tumors. However, we found no evidence to suggest VEGF has an autocrine growth effect (data not shown) as has been described for other tumor types (43, 57, 58). All the ESFT cell lines express predominantly VEGF121 and VEGF165 mRNA. The secretion of these isoforms suggests that ESFT are capable of inducing vascularization by recruiting distal blood vessels as well as expanding the capillary bed within the tumor (59).

In addition to VEGF, PIGF was also produced and secreted by ESFT cell lines. Recent reports indicate that PIGF may play an important role in angiogenesis by increasing endothelial cell survival and enhancing their response to VEGF as well as increasing vessel density, size, and permeability (60–63). Additionally, PIGF may also modulate VEGF activity by forming functional heterodimers with VEGF (63–65). Following activation of Flt-1 by PIGF, reports suggest that
Flt-1 can amplify VEGF signaling by intermolecular transphosphorylation of Flk-1/KDR (63). Together, both VEGF and PlGF may also recruit bone marrow–derived endothelial cells, a process that has been shown to potentiate the neovascularization of tumors including ESFT (12, 66, 67).

ESFT cells also synthesize and express bFGF, PDGF, and PDGFB, although the protein products of these growth factors seem to remain cell-associated or sequestered in the extracellular matrix and hence were not detected by the ELISA assay. These results suggest that they may not have a direct effect on endothelial cell proliferation. However, recently, bFGF and PDGFB have been shown to promote angiogenesis in tumors by enhancing VEGF and/or VEGFR expression (68–70). Thus, we cannot currently exclude a juxtacrine or intracrine role for these factors in enhancing VEGF-dependent angiogenesis. ESFT cell lines also express mRNA for all four FGF receptors (38) and both PDGFR-α and PDGFR-β receptors; whether these growth factors and their receptors have autocrine or paracrine roles in regulating angiogenesis or the survival and proliferation of ESFT is currently being investigated.

Recent clinical studies in metastatic colorectal cancer have shown that bevacizumab in combination with cytotoxic therapy has positive effects on patient survival (24). This proof of principal study has restimulated interest in the exploitation of VEGF as a target for antitumor growth strategies. Because VEGF expression correlates with MVD in ESFT and high levels

![Graph](image-url)
of this factor were detected in ESFT cell conditioned medium, we have investigated the effects of two different antiangiogenic strategies that disrupt the VEGF pathway on s.c. growth of ESFT in mice. These included the receptor tyrosine kinase inhibitors SU6668 (inhibits FGF receptor 1, PDGFR-h, and Flk-1/KDR) and SU5416 (inhibits Flk-1/KDR; refs. 25–27) and the VEGF targeting agents rhuMAb-VEGF (bevacizumab; refs. 28, 29) and VEGF Trap (30). In contrast to the VEGF targeting agents that mainly inhibit secreted VEGF [rhuMAb-VEGF inhibiting human (tumor-derived) VEGF; VEGF Trap inhibiting human (tumor-derived) and host (murine-derived) VEGF (also PlGF)], the receptor tyrosine kinase inhibitors are also capable of targeting angiogenic growth factor receptors expressed by host cells (25–27). Compounds, such as SU6668, may also play a role in inhibiting the FGF-induced and/or PDGF-induced recruitment and proliferation of angiogenesis, promoting host-derived stromal cells, such as fibroblasts and pericytes, which are known to express VEGF (70–73).

Consistent with previous reports on other tumor types, both class of agents reduced the vascularity and growth of ESFT in nude mice (25–28, 30, 74, 75). Interestingly, as observed in mice with RD-ES tumors, the degree of inhibition induced by SU5416 (Flk-1/KDR inhibitor) was similar to that observed for SU6668 (inhibitor of Flk-1/KDR, FGF receptor 1, and PDGFR-h), implying that the VEGF signaling pathway is the key regulator of angiogenesis in ESFT, consistent with the profile of angiogenic factors we observed in the primary ESFT. Results also suggest that in ESFT the influence of FGF and PDGF signaling pathways on stromal cell recruitment, as discussed above, are minimal. These receptor tyrosine kinase inhibitors have, however, been removed recently from clinical trials following unacceptable toxicity when given in combination with chemotherapeutic agents (76).

Delay in tumor growth was observed in mice with RD-ES and A673 tumors after treatment with rhuMAb-VEGF and VEGF Trap. However, in rhuMAb-VEGF-treated mice, this delay was only significant in mice with RD-ES but not A673 tumors. The lack of a significant effect in A673 tumors may reflect the delayed growth in one of the control mice. Alternatively, as rhuMAb-VEGF only inhibits human VEGF, significant inhibition of A673 tumor growth may require the neutralization of both human and murine VEGF (77). Unlike rhuMAb-VEGF, VEGF Trap is capable of inhibiting both
Fig. 8  Comparative histology by H&E of tumors treated with or without receptor tyrosine kinase inhibitors and anti-VEGF agents. Representative examples of RD-ES tumors treated with (A) SU6668 and SU5416 control, (B) SU6668 (100 mg/kg/d), and (C) SU5416 (25 mg/kg/d); A673 tumors treated with (D) SU6668 control and (E) SU6668 (100 mg/kg/d); RD-ES tumors treated with (F) rhuMAb-VEGF control and (G) rhuMAb-VEGF (10 mg/kg twice weekly); A673 tumors treated with (H) rhuMAb-VEGF control and (I) rhuMAb-VEGF (10 mg/kg twice weekly); RD-ES tumors treated with (J) VEGF Trap control, (K) VEGF Trap (2.5 mg/kg twice weekly), and (L) VEGF Trap (25 mg/kg twice weekly), and (O) VEGF Trap (25 mg/kg twice weekly). Large areas of geographic necrosis (arrows) were observed in RD-ES tumors treated with SU6668, SU5416, rhuMAb-VEGF, and VEGF Trap when compared with histology of corresponding control tumors. Similarly, large areas of geographic necrosis were also observed in A673 tumors treated with SU6668 and rhuMAb-VEGF. However, in contrast to RD-ES tumors, enhanced necrosis was only observed following treatment of A673 tumors with high-dose VEGF Trap (25 mg/kg twice weekly; O) when compared with histology of control tumors (M). Original magnification, ×100 (A–C) and ×40 (D–O).
human-derived and mouse-derived VEGF, thus making it a more effective agent in experimental mouse models (30, 46, 77). Additionally, as VEGF Trap is a composite decoy receptor consisting of both Flt-1 and Flk-1/KDR extracellular domains, this agent may also bind PI GF (46, 72). To evaluate the potential importance of PI GF in ESFT, we investigated the efficacy of this agent using cell lines with different VEGF and PI GF profiles (RD-ES, VEGF\textsuperscript{high}PI GF\textsuperscript{low}; A673, VEGF\textsuperscript{high}PI GF\textsuperscript{high}). Compared with RD-ES tumors, A673 tumors were rapidly growing with large areas of necrosis and high vascularity and did not respond to low doses of VEGF Trap. As PI GF has a higher affinity for Flt-1 than VEGF, this may reflect the reduced availability of VEGF Trap for sequestering VEGF (60). Alternatively, consistent with the hypothesis that PI GF enhances VEGF-induced neovascularization in comparison with VEGF alone, higher concentrations of VEGF Trap may be required to inhibit the synergistic effects of these growth factors when both are highly expressed within tumors. Thus, inhibition of both growth factors may be critical for delaying growth of tumors in which these factors are both overexpressed. These hypotheses may also in part explain the lack of significant growth delay observed following treatment of A673 tumors with rhuMAb-VEGF. We are currently investigating these hypotheses.

In summary, results from our studies show that expression of VEGF alone correlates with MVD, suggesting that it may be the single most important regulator of neovascularization in ESFT. Moreover, the significant inhibition of ESFT growth in the s.c. mouse model following treatment with Flk-1/KDR receptor tyrosine kinase inhibitors and anti-VEGF agents strongly supports the further evaluation of antiangiogenic agents for the development of new therapeutic strategies in ESFT.

ACKNOWLEDGMENTS

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Vascular Endothelial Growth Factor: A Therapeutic Target for Tumors of the Ewing's Sarcoma Family

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