Analysis of Hypoxia-Related Gene Expression in Sarcomas and Effect of Hypoxia on RNA Interference of Vascular Endothelial Cell Growth Factor A

Kara Y. Detwiller, Namali T. Fernando, Neil H. Segal, Sandra W. Ryeom, Patricia A. D’Amore, and Sam S. Yoon

Division of Surgical Oncology, Department of Surgery, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114. Phone: 617-726-4241; Fax: 617-724-3905; E-mail: syoon@partners.org.

Note: K.Y. Detwiller and N.T. Fernando contributed equally to this work.

Requests for reprints: Sam S. Yoon, Division of Surgical Oncology, Department of Surgery, Massachusetts General Hospital, 55 Fruit Street, Boston, MA 02114. Phone: 617-726-4241; Fax: 617-724-3905; E-mail: syoon@partners.org.

© 2005 American Association for Cancer Research.

Abstract
Vascular endothelial cell growth factor A (VEGF-A) and hypoxia play important roles in tumor angiogenesis. VEGF-A gene expression is up-regulated in tumors under hypoxic conditions, yet it is unclear how such up-regulation will affect the efficacy of RNA interference strategies targeting VEGF-A. Four potential short interfering RNA (siRNA) sequences for the VEGF-A gene were cloned into expression plasmids and transfected into HT1080 human fibrosarcoma cells. Stable transfection of these plasmids decreased VEGF-A mRNA levels and protein secretion by up to 99%. Our analysis of >100 hypoxia-related genes using oligonucleotide microarrays of 38 human sarcoma samples and 14 normal tissues identified distinctly different patterns of expression between sarcomas and normal tissues as assessed by hierarchical clustering analysis. Numerous hypoxia-related genes were significantly up-regulated in sarcomas including hypoxia-inducible factor 1α (HIF-1α). Exposure of wild-type HT1080 cells to 1% oxygen resulted in HIF-1α up-regulation and a 74% increase in VEGF-A secretion as compared with secretion under normoxic conditions. Surprisingly, stable cell lines expressing VEGF-A siRNAs silenced VEGF-A expression equally well in hypoxia and normoxia. S.c. injection of cells with VEGF-A siRNAs into athymic nude mice led to slower-growing tumors, decreased blood vessel density, and greater apoptosis when compared with controls. Immunofluorescence analysis of tumor sections revealed areas of HIF-1α nuclear expression, suggesting areas of hypoxia, in both control tumors and VEGF-A-suppressed tumors. We conclude that hypoxia plays an important role in human sarcomas but has no effect on up-regulation of VEGF-A expression in normoxic tumors. (Cancer Res 2005; 65(13): 5881-9)

Introduction
RNA interference (RNAi) is an ancient antiviral mechanism that has been rapidly emerging in both the research and therapeutic applications (1–5). This process was first described by Fire et al. in nematodes (5), and has subsequently been elucidated as a multistep mechanism (2, 6). Double-stranded RNAs are processed by the enzyme Dicer into short interfering RNAs (siRNA), 21 to 25 nucleotides in length (7). These siRNAs are bound to RNA-induced silencing complexes and mediate the degradation of their complementary RNA (7, 8).

Vascular endothelial growth factor A (VEGF-A) is critical for angiogenesis (9–12), and few studies have investigated the use of RNAi in silencing VEGF-A expression (13–16). Two studies have shown inhibition of tumor growth by VEGF-A siRNAs in mouse models, with one study using transient transfection of VEGF-A siRNAs prior to tumor inoculation (13), and the other study using direct intratumoral injection (15).

It remains unknown whether factors that increase mRNA expression or stability will affect RNAi-mediated mRNA degradation. In the case of VEGF, gene expression is potently augmented under hypoxic conditions by both increasing mRNA transcription and stabilization (9, 17). Transcriptional regulation of VEGF-A under hypoxic conditions is primarily mediated by hypoxia-inducible factor 1 (HIF-1), a heterodimeric protein transcription factor, composed of α and β subunits (18). HIF-1α is rapidly degraded under normoxic conditions by the ubiquitin pathway (19). During hypoxia, HIF-1α is stabilized and in concert with HIF-1β, binds to the VEGF-A promoter and transactivates gene expression (20). Hypoxia also increases the stability of VEGF-A mRNA via the binding of RNA-binding proteins to specific 5’ sequences (21).

As tumors increase in size, hypoxic areas often develop and VEGF-A expression is up-regulated (22, 23). Human sarcomas in particular often show areas of central necrosis and hypoxia (24, 25). Thus, the efficacy of VEGF-A RNAi under hypoxic conditions has important ramifications for the use of this technology as an antiangiogenic strategy against tumors. The aim of this study was (a) to identify effective siRNAs for VEGF-A, (b) to analyze hypoxia-related gene expression including expression of HIF-1α in sarcomas compared with normal tissues, and (c) to determine the efficacy of VEGF-A siRNAs under hypoxic conditions in vitro and in vivo.

Materials and Methods
Cell culture. HT1080, 10T1/2, SVR, and 293HEK cells were obtained from American Type Culture Collection (Manassas, VA). T241 cells were kindly provided by Dr. Richard Mulligan (Boston Children’s Hospital, Boston, MA). ISO-1 cells were kindly provided by Dr. Mikio Masuzawa (Kitasato University School of Medicine, Sagamihara, Japan). All cell lines were maintained in DMEM supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin in a humidified 5% CO2 incubator at 37°C. Hypoxic conditions were achieved by culturing cells in a sealed hypoxia chamber (Billups-Rothenberg, Del Mar, CA) after flushing with 1% O2, 5% CO2, and 94% N2.
Tumor specimens, RNA isolation, and gene expression analysis. Tissue specimens were obtained from patients with soft tissue sarcoma undergoing surgery under an Institutional Review Board–approved protocol. Representative tumor tissue was embedded in ornithine carbamyl transferase compound and frozen as a tissue block using liquid nitrogen. Tumor specimens were selected for analysis after validation of histologic diagnosis. Cryopreserved tumor sections were homogenized under liquid nitrogen by mortar and pestle. Total RNA was extracted from Trizol reagent (Invitrogen, Carlsbad, CA) and purified using the Qiagen RNeasy kit (Valencia, CA). RNA quality was assessed on ethidium bromide agarose gel electrophoresis. cdNA was synthesized from 2 to 5 μg of RNA in the presence of oligo(dT)24-17 (Genset Corp., La Jolla, CA). cRNA was prepared using biotinylated UTP and CTP and hybridized to HG-U133A oligonucleotide arrays (Affymetrix, Santa Clara, CA). Fluorescence was measured by laser confocal scanner (Agilent, Palo Alto, CA) and converted to signal intensity by means of Affymetrix Microarray Suite v4.0 software. For complete expression data, go to GEO (www.ncbi.nlm.gov/geo/+).

Hierarchical clustering analysis based on hypoxia-related genes. A list of >130 hypoxia-related genes was comprised by reviewing the literature (26, 27). One-hundred and seven genes of these genes were represented on the HG-U133A GeneChip array by 147 probe sets. Hierarchical clustering analysis was done using Cluster 2.11 software (http://rana.lbl.gov/ EisenSoftware.htm). Hierarchical clustering was done using an uncentered Pearson correlation coefficient distance metric and average linkage to measure cluster distance during partitioning (28). The mean expression of hypoxia-responsive genes was compared for sarcomas and normal tissues using InStat 3.05 software (GraphPad, San Diego, CA) by unpaired t test.

Generation of vascular endothelial cell growth factor A short interfering RNA expression plasmids. The following criteria were used to identify targets for siRNAs from the VEGF-A cdNA coding sequence: (a) starts with an AA dinucleotide, (b) 21 nucleotides in length, (c) G/C content of <50%, and (d) no sequence homology to other coding sequences on BLAST search. Using these criteria, four siRNA sequences to human VEGF-A were chosen (Table 1). One negative control siRNA containing a scrambled negative control sequence NC were named pV1, pV2, pV3, pV4, and pNC, respectively. Transient and stable transfection, pV1-pV4 and pNC were transiently transfected into 293HEK cells using 400 ng plasmid and 1.2 μL TransIT-293 Transfection Reagent (Mirus, Madison, WI) following the manufacturer’s instructions. The medium was changed after 24 hours and the supernatant was harvested after 48 hours. For transient transfection of HT1080 cells, cells were transfected using 1 μg plasmid and 1.5 μL Fugene 6 (Roche Diagnostics, Indianapolis, IN) according to the manufacturer’s instructions. The medium was changed after 24 hours, and supernatant was harvested after 48 hours. For transient transfection with hygromycin selection, the day following transfection, new medium with hygromycin 200 μg/mL (293HEK) or 100 μg/mL (HT1080) was added, and supernatant was harvested after 5 days. For stable transfection of HT1080 cells, cells were transfected with pV2, pV3, or pNC and placed under hygromycin selection for 3 weeks. Surviving colonies were isolated and expanded.

ELISA and Western blot analysis. VEGF-A protein levels in the supernatant were measured using the human VEGF-A DuoSet ELISA kit (R&D Systems, Inc., Minneapolis, MN). ELISA plates were read using the Emax Precision Microplate Reader (Molecular Devices, Sunnyvale, CA) and VEGF-A levels quantified against a standard curve from 0 to 4,000 pg/mL plotted with a four-parameter curve. To normalize VEGF-A levels per 10³ cells, cell counts were obtained by counting trypsinized cells using a hemacytometer. To assess the relative degree of VEGF-A protein silencing, VEGF-A levels from stably transfected clones were compared with levels from wild-type HT1080 cells. Data reflects the mean of samples done in triplicate.

For analysis of HIF-1α protein levels, cells were harvested in SDS sample buffer supplemented with Protease Inhibitor Cocktail (Roche Diagnostics). Equal amounts of protein were separated on a 4% to 20% polyacrylamide gel and transferred to a nitrocellulose membrane. After blocking in 5% nonfat milk, the membrane was incubated overnight at room temperature with anti-HIF-1α antibody (BD Biosciences, San Jose, CA) diluted 1:100 and anti-lamin antibody (29) diluted 1:10,000. Horseradish peroxidase–conjugated goat anti-mouse IgG (Zymed Laboratories, San Francisco, CA) diluted 1:2,000 was used as secondary antibody, and proteins were detected using the Enhanced Chemiluminescence kit (Amersham Biosciences, Piscataway, NJ).

Quantitative real-time PCR. Total RNA was isolated in Trizol (Invitrogen), and cdNA was synthesized using the Superscript First-Strand Synthesis System (Invitrogen) with random hexamers. Quantitative real-time PCR analysis was done using the LightCycler Detection System (Roche Diagnostics). cdDNA product (200 ng) was amplified in a 20 μL reaction containing 2 μL SYBR Green PCR Master Mix (Roche Diagnostics) and 5 pmol of each primer. Primers were obtained from the human VEGF-A Relative RT-PCR Kit (Ambion). The PCR program consisted of an initial denaturation at 95°C for 10 minutes followed by amplification for 40 cycles (95°C for 10 seconds, 60°C for 5 seconds, and 72°C for 15 seconds). Data were generated from each reaction, analyzed using LightCycler software (Roche), and normalized to 18S RNA. To assess the relative degree of VEGF mRNA silencing, VEGF mRNA levels were compared with levels from wild-type HT1080 cells.

[H]Thymidine proliferation assay. To assay for proliferation, 10⁵ cells were plated onto 96-well flat-bottomed plates and maintained in medium overnight. Cells were pulsed with 1 μCi of [³H]thymidine for 8 hours, and then harvested for liquid scintillation counting (Wallac, Gaithersburg, MD). Data reflect the mean of triplicates samples.

Table 1. Selected sequences of human VEGF siRNAs

<table>
<thead>
<tr>
<th>Name</th>
<th>Targeted sequence</th>
<th>G/C content</th>
<th>Location*</th>
<th>Exon†</th>
<th>VEGF isoform*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human VEGF siRNA 1 (V1)</td>
<td>AATCATCACGAAAGTGGTGAAG</td>
<td>42.9</td>
<td>106</td>
<td>2, 3</td>
<td>121, 165, 189</td>
</tr>
<tr>
<td>Human VEGF siRNA 2 (V2)</td>
<td>AATGGTGATAAGTCTGTGAGT</td>
<td>42.9</td>
<td>116</td>
<td>2, 3</td>
<td>121, 165, 189</td>
</tr>
<tr>
<td>Human VEGF siRNA 3 (V3)</td>
<td>AACATCACCAGCAGATTATAG</td>
<td>38.1</td>
<td>301</td>
<td>3, 4</td>
<td>121, 165, 189</td>
</tr>
<tr>
<td>Human VEGF siRNA 4 (V4)</td>
<td>AAATCCCGGTTAATGCTGCTGG</td>
<td>47.6</td>
<td>469</td>
<td>6</td>
<td>189</td>
</tr>
</tbody>
</table>

*Location in relation to start codon.
†Location in relation to exons.
*VEGF isoforms targeted by siRNA.
Animal studies. All mouse protocols were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care. After isoflurane anesthesia, 5 × 10^6 cells were resuspended in 100 μL of HBSS and injected s.c. into the right flank of athymic nude mice. Six mice were used for each group. Tumors were measured thrice per week for 3 weeks, and tumor volume (TV) was calculated by using the following formula: TV = length \times width^2 \times 0.52. Mice were sacrificed after 18 days, and tumors were excised.

Immunohistochemistry and immunofluorescence. Tumors were bivalved after collection. One-half of each tumor was fixed in 10% buffered formalin for 24 hours, embedded in paraffin, and processed into 5 μm sections. The other half of each tumor was snap-frozen in liquid nitrogen and processed into 5 or 10 μm sections. CD31 staining was done on 5 and 10 μm frozen sections. Sections were fixed in cold acetone for 5 minutes, 1:1 chloroform and acetone for 5 minutes and then cold acetone again for 5 minutes. Sections were then blocked with diluted rabbit serum, incubated overnight at 4°C with rat anti-CD31 (PharMingen, San Jose, CA) primary antibody diluted 1:100 followed by rabbit anti-rat biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) diluted 1:500. The remaining steps were done using the Vectastain Elite ABC kit for rat IgG (Vector Laboratories) according to manufacturer's instructions. At low magnification (50×), regions of highest vessel density were identified. Then under high power (200×), blood vessels were counted in at least four fields and a masked observer.

For HIF-1α and VEGF immunofluorescence, paraffin sections were rehydrated in xylene followed by decreasing concentrations of ethanol. Slides were then placed in 10 mmol/L citrate buffer (pH 6.0) and heated for 30 minutes at 95°C. Sections were then blocked with 1% bovine serum albumin in PBS. Goat anti-VEGF-A antibody (R&D Systems) diluted 1:100 or mouse anti-HIF-1α antibody (BD Biosciences) diluted 1:100 was then applied followed by AlexaFluor 594 rat anti-goat or AlexaFluor 564 goat anti-mouse (Molecular Probes, Eugene, OR) secondary antibody, respectively. Hoechst nuclear dye (2 μg/mL) was then applied, VEGF fluorescence intensity was determined by identifying four areas of highest fluorescence per tumor sample, obtaining a photograph with 100 ms exposure time, and quantifying red fluorescence.

For proliferating cell nuclear antigen (PCNA) immunohistochemistry, paraffin sections were deparaffinized and rehydrated in xylene followed by decreasing concentrations of ethanol. Slides were then blocked with 2% bovine serum albumin in PBS. Goat anti-VEGF-A antibody in 10 mmol/L citrate buffer (pH 6.0) and heated for 30 minutes at 95°C. Sections were then blocked with 1% bovine serum albumin in PBS. Goat anti-VEGF-A antibody (R&D Systems) diluted 1:100 or mouse anti-HIF-1α antibody (BD Biosciences) diluted 1:100 was then applied followed by AlexaFluor 594 rat anti-goat or AlexaFluor 564 goat anti-mouse (Molecular Probes, Eugene, OR) secondary antibody, respectively. Hoechst nuclear dye (2 μg/mL) was then applied, VEGF fluorescence intensity was determined by identifying four areas of highest fluorescence per tumor sample, obtaining a photograph with 100 ms exposure time, and quantifying red fluorescence.

For proliferating cell nuclear antigen (PCNA) immunohistochemistry, paraffin sections were deparaffinized and rehydrated in xylene followed by decreasing concentrations of ethanol. Slides were then placed in 10 mmol/L citrate buffer (pH 6.0) and heated for 30 minutes at 95°C. Sections were then blocked with 1% bovine serum albumin in PBS. Goat anti-VEGF-A antibody (R&D Systems) diluted 1:100 or mouse anti-HIF-1α antibody (BD Biosciences) diluted 1:100 was then applied followed by AlexaFluor 594 rat anti-goat or AlexaFluor 564 goat anti-mouse (Molecular Probes, Eugene, OR) secondary antibody, respectively. Hoechst nuclear dye (2 μg/mL) was then applied, VEGF fluorescence intensity was determined by identifying four areas of highest fluorescence per tumor sample, obtaining a photograph with 100 ms exposure time, and quantifying red fluorescence.

Results

Inhibition of vascular endothelial cell growth factor A secretion by RNA interference in 293HEK and HT1080 cells. We first examined VEGF-A production in a panel of five different sarcoma cell lines and the human embryonic kidney cell lines 293HEK (Fig. 1A). The fibrosarcoma cell line HT1080 secreted 3,150 pg/mL of VEGF-A after a 48-hour incubation period. ISO-1 and T241 cells secreted intermediate levels of VEGF-A (797-910 pg/mL) following the manufacturer’s instructions. Nuclei were stained with Hoechst nuclear dye (2 μg/mL) and processed into 5 or 10 μm sections. CD31 staining was done on 5 and 10 μm sections. TMR Red (Roche Applied Science, Indianapolis, IN) following the manufacturer’s instructions. Nuclei were stained with Hoechst nuclear dye (2 μg/mL). Images were obtained on a Zeiss microscope and analyzed using AxioVision 4.0 software (Carl Zeiss Vision).

Sequences were ligated into an expression plasmid to create VEGF-A siRNA plasmids, pV1-pV4, and a negative control plasmid, pNC. To examine the efficacy of these plasmids in silencing VEGF-A expression, plasmids were transiently transfected into 293HEK cells, and the supernatant was tested for VEGF-A levels after 48 hours. VEGF-A siRNAs showed variable efficacy in decreasing VEGF-A protein secretion, with the most effective siRNA, V2, decreasing VEGF-A secretion by 74% (Fig. 1B). V3, and V4 siRNAs decreased VEGF-A by 32%, 59%, and 24%, respectively. Because these were transient transfections, a significant proportion of VEGF-A protein secretion may have been from untransfected cells. To reduce VEGF-A secretion by these untransfected cells, transiently transfected 293HEK cells were exposed for 5 days to selection with hygromycin, and VEGF-A levels in the supernatant were then measured. Again, the V2 siRNA was the most effective siRNA, decreasing VEGF-A levels by 87% (Fig. 1C). The other siRNAs decreased VEGF-A levels by 33% to 80%.

VEGF-A siRNAs were then transiently transfected into HT1080 cells. HT1080 cells were significantly more difficult to transfect compared with 293HEK cells based on transfection efficiencies calculated with the enhanced green fluorescent protein reporter gene (data not shown). After transient transfection and selection with hygromycin for 5 days, levels of VEGF-A were decreased by up to 65% (Fig. 1D). Similar to 293HEK cells, the V2 siRNA was most effective at reducing VEGF-A expression in HT1080 cells.

Stable RNA interference of vascular endothelial cell growth factor A in HT1080 cells. To generate HT1080 clones with stable knockdown of VEGF, VEGF siRNA plasmids pV2 and pV3 were stably transfected into HT1080 cells along with the negative control plasmid pNC. Stable cell lines were tested for VEGF-A secretion after a 48-hour incubation (Fig. 2A). Three clones, V2-3, V2-4, and V3-4, showed decreased secretion of VEGF-A by 97%, 95%, and 72%, respectively, and these clones were chosen for further study along with one negative control clone, NC-2. To confirm inhibition of VEGF-A at the mRNA level, VEGF-A mRNA was measured in the selected stably transfected lines using quantitative real-time PCR. The range of VEGF-A mRNA knockdown varied from 94% to 99% for the selected clones (Fig. 2B). Similar results for VEGF-A silencing were obtained after cell lines were passaged >25 times (data not shown), confirming that RNAi can persist through multiple cell divisions following stable incorporation of siRNA-expressing plasmids. Finally, to ensure that levels of VEGF-A protein and mRNA were not related to differences in growth rates of selected clones, cellular proliferation was assessed in these clones by [3H]thymidine incorporation and was found to be equivalent in all selected clones (Fig. 2C).

Analysis of hypoxia-responsive genes in sarcoma oligonucleotide microarrays. We next wanted to determine if hypoxia plays a significant role in sarcomas and investigate the effects of hypoxia on RNAi of VEGF-A. We previously analyzed soft tissue sarcoma gene expression using U95A GeneChip microarrays (Affymetrix), which represents about 10,000 human genes, and found that gene expression profiling of sarcomas was useful in classification of histologic subtypes (30, 31). In this study, 38 pathologically confirmed sarcoma samples and 14 normal tissues were analyzed on HG-U133A GeneChip microarrays (Affymetrix), which represent about 14,500 human genes. One-hundred and seven hypoxia-related genes were identified as described in Materials and Methods (Table 1).
genes separated samples into two groups of sarcomas and one group of normal tissues (Fig. 3). Only one normal tissue specimen, skin, clustered with the sarcomas, and only one sarcoma sample, a round cell liposarcoma, clustered with the normal tissues. Certain histologic subtypes (e.g., synovial sarcomas) clustered tightly together based only on hypoxia-related gene expression. These data suggest that sarcomas and normal tissues have distinctly different expression patterns for genes associated with hypoxia.

Expression of 26 of the 107 hypoxia-related genes was significantly higher in sarcomas as compared with normal tissues (Table 2). The six genes most significantly elevated were HIF-1α, procollagen-lysine, 2-oxoglutarate 5-dioxygenase 2 (PLOD2), annexin V, BCL2/adenovirus E1B 19 kDa interacting protein 3-like (BNIP3L), collagen type 5 α1, and vimentin (P < 0.0001). HIF-1α expression was 2.3-fold higher in sarcoma samples than control tissues. VEGF-A expression was also examined and found not to be significantly different in sarcomas and normal tissues. However, the VEGF-A gene on the HG-U133A GeneChip is represented by four probe sets, all of which have sequence homology to genes or sequences other than VEGF-A, and this finding may reflect the lack of a probe set on the HG-U133A GeneChip that is specific for VEGF-A.

Efficacy of vascular endothelial cell growth factor A RNA interference under hypoxic conditions. We next confirmed that HIF-1α induction was intact in HT1080 cells exposed to hypoxia. No HIF-1α protein was detected by Western blot analysis in normoxia, but after exposure to hypoxia, HIF-1α up-regulation was shown in all clones as well as wild-type HT1080 cells (Fig. 4A). To determine if hypoxic up-regulation of VEGF-A would attenuate the efficacy of the VEGF-A siRNAs, VEGF-A secretion was then measured in our clones with stable expression of VEGF-A siRNAs following exposure to hypoxia. In the control NC-2 clone and in wild-type HT1080 cells, VEGF-A secretion was increased by nearly 75% following exposure to 1% hypoxia compared with cells grown in normoxic conditions (Fig. 4B). In contrast, clones expressing VEGF-A siRNAs showed persistent and stable decreases in VEGF-A...

Figure 1. Inhibition of VEGF-A protein secretion in 293HEK and HT1080 cells following transfection with VEGF-A siRNA plasmids. A, the sarcoma cell lines HT1080, ISO-1, T241, 10T1/2, and SVR, and the human embryonic kidney cell line 293HEK were assayed for VEGF-A secretion after 48 hours. B, 293HEK cells were transiently transfected with expression plasmids for VEGF-A siRNAs (V1-V4) and a negative control siRNA (NC). Mock, cells treated with transfection reagent alone. VEGF-A levels in the supernatant were determined after 48 hours. C and D, for transient transfection with selection, 293HEK cells and HT1080 cells were placed under selection with hygromycin following transfection of V1-V4 or NC. Supernatant was harvested after 5 days and assayed for VEGF-A levels. Bars, SD.

Figure 2. Selection and analysis of HT1080 clones stably transfected with VEGF-A siRNA plasmids. A, VEGF-A siRNA expression plasmids pV2 and pV3 and the negative control plasmid pNC were stably transfected into HT1080 cells, and 10 clones were analyzed for VEGF-A secretion by ELISA in comparison to wild-type HT1080 cells. B, one negative control clone, NC-2, and three clones with stable VEGF-A siRNA expression, V2-3, V2-4, and V3-4, were then assayed for VEGF-A RNA levels by quantitative real-time PCR. Relative VEGF-A RNA amounts are given compared with VEGF-A RNA levels in HT1080 wild-type cells. C, proliferation rates of selected clones were determined by [3H]thymidine incorporation in cpm and were found to be equivalent for all clones. Bars, SD.
expression, ranging from 5% to 20% of levels seen with controls. When VEGF-A protein secretion from VEGF-A siRNA-producing clones was expressed as a percentage compared with secretion from wild-type cells, suppression of VEGF-A was not significantly different in normoxia and hypoxia (Fig. 4C). These results were confirmed at the mRNA level by quantitative real-time PCR (Fig. 4D). These in vitro results suggest that tumor hypoxia would not be a significant barrier for targeting VEGF-induced angiogenesis by RNAi.

RNA interference of vascular endothelial cell growth factor A inhibits angiogenesis and tumor growth. To evaluate the effects of VEGF-A siRNAs in vivo, clones of HT1080 were injected s.c. into the flanks of athymic nude mice, and tumor volumes were measured thrice a week (Fig. 5A). Eleven days after tumor inoculation, there was a noticeable difference between the sizes of tumors in mice injected with the control cells versus those injected with cells stably expressing VEGF-A siRNAs. At 18 days, the average size of the negative control tumors was 250 mm³ compared with 70 to 107 mm³ for clones with VEGF-A silencing. Tumors were harvested after 18 days, and vessels visualized by immunostaining for CD31. Microvessel density was significantly decreased in tumors expressing VEGF-A siRNAs as compared with controls (Fig. 5B and C).

Tumor sections were also analyzed by H&E staining and by immunofluorescence and immunohistochemistry for HIF-1α, VEGF, cellular proliferation, and apoptosis. Control tumors were

---

**Figure 3.** Hierarchical clustering analysis and heat map. Hierarchical clustering analysis of 38 human sarcomas and 14 normal tissues divides samples into two clusters of sarcomas and one cluster of normal tissues. Only one normal tissue (skin, red arrow) clustered in a sarcoma group, and only one sarcoma (round cell liposarcoma 2, blue arrow) clustered with the normal tissues. The heat map shows expression of the 26 genes/33 probe sets most highly up-regulated in sarcomas as listed in Table 2. Red, high expression; black, moderate expression; and green, low expression.
increased apoptosis as measured by TUNEL staining (Fig. 6). Function as measured by immunohistochemistry for PCNA (Fig. 6). Silencing of VEGF-A expression showed decreased proliferation (Fig. 6B). Tumors with VEGF-A siRNA expression grew significantly more slowly in an orthotopic mouse tumor model targeting VEGF-A. Tumors with VEGF siRNA expression grew significantly more slowly in an orthotopic mouse tumor model targeting VEGF-A. Tumors with VEGF siRNA expression grew significantly more slowly in an orthotopic mouse tumor model targeting VEGF-A. Tumors with VEGF siRNA expression grew significantly more slowly in an orthotopic mouse tumor model targeting VEGF-A. Tumors with VEGF siRNA expression grew significantly more slowly in an orthotopic mouse tumor model targeting VEGF-A. Hypoxia-inducible factor 1α expression (Fig. 6B). VEGF-A staining was significantly more abundant in control tumors than VEGF-A siRNA tumors, and interestingly, hypoxic up-regulation of hypoxia-related gene expression with up-regulation of several genes including HIF-1α. Importantly, hypoxic up-regulation of VEGF-A in sarcoma cells did not attenuate the efficacy of siRNAs targeting VEGF-A. Tumors with VEGF siRNA expression grew significantly more slowly in an orthotopic mouse tumor model and showed decreased VEGF expression, decreased blood vessel density, and increased apoptosis. These findings suggest that RNAi of VEGF can be an effective antiangiogenic strategy for sarcomas.

### Table 2. Expression levels of hypoxia-related genes significantly up-regulated in sarcomas

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Probe set</th>
<th>Normal tissues (n = 14)</th>
<th>Sarcoma samples (n = 38)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (SE)</td>
<td>Mean (SE)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypoxia-inducible factor 1α</td>
<td>200989_at</td>
<td>1,836.41 (365.21)</td>
<td>4,156.08 (300.10)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PLOD2</td>
<td>202620_s_at</td>
<td>331.79 (78.89)</td>
<td>1,370.68 (150.04)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Annexin V</td>
<td>200782_at</td>
<td>3,108.48 (361.10)</td>
<td>6,549.06 (462.77)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>BNIP3L</td>
<td>221478_at</td>
<td>743.54 (132.63)</td>
<td>1,771.35 (122.75)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Collagen 5 α1</td>
<td>212489_at</td>
<td>362.28 (86.24)</td>
<td>3,071.91 (478.59)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Vimentin</td>
<td>201426_s_at</td>
<td>10,351.16 (1944.74)</td>
<td>24,602.41 (1,579.80)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Collagen 5 α1</td>
<td>212488_at</td>
<td>737.84 (188.65)</td>
<td>5,286.67 (925.23)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PLOD2</td>
<td>202619_s_at</td>
<td>393.69 (88.29)</td>
<td>1,407.84 (203.68)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Integrin 5α</td>
<td>201389_at</td>
<td>651.86 (74.55)</td>
<td>1,662.48 (228.98)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Transforming growth factor β1</td>
<td>201506_at</td>
<td>1,946.11 (414.57)</td>
<td>7,796.07 (1,471.60)</td>
<td>0.0004</td>
</tr>
<tr>
<td>Solute carrier family 2 member 3</td>
<td>202497_x_at</td>
<td>204.72 (64.07)</td>
<td>596.73 (99.83)</td>
<td>0.0018</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 3</td>
<td>210095_s_at</td>
<td>4,269.93 (632.27)</td>
<td>9,521.79 (725.67)</td>
<td>0.0025</td>
</tr>
<tr>
<td>Lysyl oxidase</td>
<td>213640_s_at</td>
<td>643.23 (24.92)</td>
<td>200.35 (34.97)</td>
<td>0.0026</td>
</tr>
<tr>
<td>CD99</td>
<td>201029_s_at</td>
<td>2,888.61 (362.11)</td>
<td>4,630.09 (426.87)</td>
<td>0.0028</td>
</tr>
<tr>
<td>Serpin E2</td>
<td>212190_at</td>
<td>1,115.58 (288.29)</td>
<td>4,084.52 (937.73)</td>
<td>0.0042</td>
</tr>
<tr>
<td>Placental growth factor</td>
<td>200652_s_at</td>
<td>122.10 (25.82)</td>
<td>308.27 (56.46)</td>
<td>0.0043</td>
</tr>
<tr>
<td>Angiopoietin 2</td>
<td>205572_at</td>
<td>69.36 (20.86)</td>
<td>361.47 (96.21)</td>
<td>0.0050</td>
</tr>
<tr>
<td>Serpin E1</td>
<td>202627_s_at</td>
<td>216.61 (43.90)</td>
<td>1,056.07 (292.69)</td>
<td>0.0072</td>
</tr>
<tr>
<td>Insulin-like growth factor 2</td>
<td>202410_x_at</td>
<td>128.86 (45.99)</td>
<td>2,242.93 (769.49)</td>
<td>0.0093</td>
</tr>
<tr>
<td>Adrenomedullin</td>
<td>202912_at</td>
<td>725.49 (98.20)</td>
<td>1,365.18 (217.37)</td>
<td>0.0100</td>
</tr>
<tr>
<td>Enolase 1</td>
<td>217294_s_at</td>
<td>1,947.34 (246.63)</td>
<td>3,290.06 (439.80)</td>
<td>0.0104</td>
</tr>
<tr>
<td>Insulin-like growth factor binding protein 3</td>
<td>212143_s_at</td>
<td>1,027.89 (230.20)</td>
<td>1,939.46 (254.37)</td>
<td>0.0111</td>
</tr>
<tr>
<td>Solute carrier family 2, member 3</td>
<td>202499_x_at</td>
<td>324.21 (70.52)</td>
<td>672.62 (113.23)</td>
<td>0.0119</td>
</tr>
<tr>
<td>Thioredoxin</td>
<td>208864_s_at</td>
<td>1,860.60 (281.47)</td>
<td>2,834.88 (248.00)</td>
<td>0.0138</td>
</tr>
<tr>
<td>Lactate dehydrogenase B</td>
<td>201030_x_at</td>
<td>4,954.59 (1,261.36)</td>
<td>8,579.08 (648.90)</td>
<td>0.0187</td>
</tr>
<tr>
<td>Lipocortin 1 (annexin 1)</td>
<td>201012_at</td>
<td>1,758.76 (930.27)</td>
<td>4,566.52 (672.38)</td>
<td>0.0211</td>
</tr>
<tr>
<td>Insulin-like growth factor 2</td>
<td>210881_s_at</td>
<td>83.61 (21.92)</td>
<td>1,533.64 (610.10)</td>
<td>0.0211</td>
</tr>
<tr>
<td>Lysyl oxidase</td>
<td>204298_s_at</td>
<td>216.29 (137.41)</td>
<td>654.38 (119.32)</td>
<td>0.0218</td>
</tr>
<tr>
<td>Phosphoglycerate kinase 1</td>
<td>200737_at</td>
<td>1,133.17 (121.11)</td>
<td>1,777.04 (246.73)</td>
<td>0.0233</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>200946_at</td>
<td>218.49 (20.00)</td>
<td>3,317.66 (45.69)</td>
<td>0.0240</td>
</tr>
<tr>
<td>Thrombospondin 1</td>
<td>201109_s_at</td>
<td>269.66 (49.74)</td>
<td>681.56 (171.80)</td>
<td>0.0262</td>
</tr>
<tr>
<td>Transforming growth factor β3</td>
<td>200944_at</td>
<td>599.51 (123.44)</td>
<td>1,822.97 (223.68)</td>
<td>0.0267</td>
</tr>
<tr>
<td>Thrombospondin 1</td>
<td>201107_s_at</td>
<td>27.80 (7.22)</td>
<td>74.88 (19.40)</td>
<td>0.0277</td>
</tr>
</tbody>
</table>

*Some genes are represented by more than one probe set on the HG-U133A GeneChip.

### Discussion

This study establishes for the first time that RNAi of VEGF-A using a plasmid-based strategy can stably silence VEGF-A expression in cancer cells. We examined oligonucleotide microarrays from 38 human sarcoma tumors and 14 normal tissues and found that sarcomas have a distinctly different pattern of hypoxia-related gene expression with up-regulation of several genes including HIF-1α. Importantly, hypoxic up-regulation of VEGF-A in sarcoma cells did not attenuate the efficacy of siRNAs targeting VEGF-A. Tumors with VEGF siRNA expression grew significantly more slowly in an orthotopic mouse tumor model and showed decreased VEGF expression, decreased blood vessel density, and increased apoptosis. These findings suggest that RNAi of VEGF can be an effective antiangiogenic strategy for sarcomas.
The efficacy and mode of delivery of siRNAs vary considerably (33). Using previously published selection criteria for siRNAs, four siRNA sequences were chosen, and two were found to be highly effective in knockdown of VEGF-A gene expression. Investigators continue to publish additional criteria that can be used in algorithms to select siRNA sequences and to increase the efficiency of RNAi (34, 35). We chose to incorporate the selected siRNA sequences into expression vectors as opposed to using chemically synthesized siRNAs. Synthesized siRNAs are readily available and have high transfection efficiencies, but their effects are transient (2). Vector-based siRNA delivery requires an initial period of plasmid construction but allows for large-scale production and sustained gene silencing. The VEGF-A siRNA vectors studied here included a hygromycin resistance gene, which allowed for selection of cells with stable VEGF-A siRNA expression. Stable VEGF-A siRNA clones were analyzed after >25 passages, and VEGF-A inhibition was found to be persistent and stable. In addition, the results of our in vivo studies showed persistent VEGF-A silencing in tumors grown for almost 3 weeks.

The three primary VEGF-A isoforms (VEGF121, VEGF165, and VEGF189) have varied expression in different tissues as well as varied biological functions (36). Zhang et al. were able to specifically target mouse VEGF188 or both VEGF164 and VEGF188 with isoform-specific siRNAs, but could only decrease levels of these isoforms by 20% (16). In this study, the two VEGF siRNAs (V2 and V3) used for stable transfections targeted all VEGF isoforms. V4 targeted only VEGF189, and in general, had less efficacy in VEGF silencing after transient transfection.

Hypoxia leads to significant increases in VEGF-A expression primarily through HIF-1α-mediated increase in VEGF-A transcription (20). Interestingly, despite significant increases in VEGF-A secretion induced by hypoxia in wild-type cells, cells with
VEGF-A siRNAs maintained relatively constant levels of VEGF-A silencing. RNAi of VEGF-A could accommodate a dramatic increase in VEGF-A gene expression and maintain up to 95% gene silencing. Despite the robustness of this gene silencing mechanism, we could never attain complete inhibition of VEGF-A expression even under normoxic conditions.

Even with near complete abrogation of VEGF-A protein secretion, HT1080 cells still formed tumors in the flanks of mice, albeit at a significantly slower rate. Several mechanisms exist by which VEGF-silenced tumors might stimulate angiogenesis. For instance, tumors may induce surrounding stromal cells to secrete VEGF. It has been shown in tumors derived from VEGF-null embryonic stem cells that VEGF-A from tumor stroma can support tumor vascularization (37). Immunofluorescence localization of VEGF-A in the VEGF-suppressed tumors did indeed show low levels of VEGF-A expression in sporadic cells. HT1080 cells also secrete other proangiogenic factors including basic fibroblast growth factor 2 and angiopoietin 2 (data not shown), which can support tumor angiogenesis. Perhaps RNAi interference of multiple angiogenic factors could completely abrogate new blood vessel formation and tumor growth. Finally, tumors can grow along preexisting blood vessels rather than forming new blood vessels (38).

Hierarchical clustering analysis using expression data from >100 hypoxia-related genes on oligonucleotide microarrays of sarcomas and normal tissues revealed that sarcomas and normal tissues have distinctly different expression patterns for these genes. Furthermore, certain histologic subtypes of sarcoma have unique expression patterns, such as synovial sarcomas and gastrointestinal stromal tumors. Numerous hypoxia-responsive genes were up-regulated in sarcomas including HIF-1α, and these data support prior studies demonstrating areas of hypoxia within soft tissue sarcomas (24, 25).

There was no significant elevation in VEGF-A expression in our microarray data from human sarcomas, yet we found elevated

Figure 6. Central necrosis and expression of HIF-1α, VEGF-A, PCNA, and TUNEL in HT1080 tumors. A, H&E sections of tumor expressing VEGF-A siRNA (V2-3) and control tumor (NC-2). Arrows, area of central necrosis. Black bars, 200 µm. B, HIF-1α immunofluorescence (red) and Hoechst nuclear stain (blue) reveal prominent HIF-1α nuclear staining near central, necrotic regions of both tumors. C, faint cytoplasmic HIF-1α staining is shown in more peripheral portions of tumors. D, VEGF-A immunofluorescence (red) and Hoechst nuclear stain (blue) show reduced VEGF-A in tumors expressing VEGF-A siRNA. E, PCNA immunohistochemistry (brown) and F, TUNEL immunofluorescence (red) show decreased proliferation and increased apoptosis in VEGF-A siRNA tumors. White bars, 20 µm.
VEGF secretion in the five sarcoma cell lines tested in vitro including HT1080 tumors. The likely reason for this discrepancy is that the four VEGF-A probes present on the HG U133A GeneChip share homology with sequences other than VEGF-A and so recorded expression levels may not be completely specific for VEGF-A. In addition, only small portions of these generally large tumors were used for microarray analysis, and sampling error is possible. Finally, the microarray analysis involved comparison of a heterogeneous group of sarcomas and normal tissues, and large variations in VEGF-A between sarcoma histologic subtypes and different normal tissues likely exist.

In conclusion, this study suggests that hypoxia plays an important role in human sarcomas based on hypoxia-related gene expression patterns of these tumors compared with normal tissues. RNAi of VEGF is an effective strategy in inhibiting tumor angiogenesis, and hypoxic up-regulation of VEGF-A expression does not attenuate the efficacy of VEGF-A gene silencing.

Acknowledgments


Grant support: NIH grant 5 K12 CA 87723-03 and the Massachusetts General Hospital Surgical Research Council Junior Faculty Award.

The authors wish to thank Dr. Othon Iliopoulos for assistance with experiments done under hypoxia and for helpful discussion.

References


Analysis of Hypoxia-Related Gene Expression in Sarcomas and Effect of Hypoxia on RNA Interference of Vascular Endothelial Cell Growth Factor A


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/65/13/5881

Cited articles
This article cites 35 articles, 10 of which you can access for free at:
http://cancerres.aacrjournals.org/content/65/13/5881.full.html#ref-list-1

Citing articles
This article has been cited by 17 HighWire-hosted articles. Access the articles at:
/content/65/13/5881.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.