Signal Transducer and Activator of Transcription 1 Activation in Endothelial Cells Is a Negative Regulator of Angiogenesis

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Abstract

To determine the role of the transcription factor signal transducer and activator of transcription (STAT) 1 on endothelial cell function, human umbilical vein endothelial cells (HUVEC) were treated with IFN-γ, a potent activator of STAT1. IFN-γ inhibited cell growth and tube formation of HUVECs. Although the potent proangiogenic protein vascular endothelial growth factor (VEGF) stimulated cell growth and tube formation, IFN-γ could suppress these effects of VEGF. Transfection of HUVECs with short interfering RNA targeting STAT1 abrogated IFN-γ–induced inhibition of HUVEC growth and tube formation, and suppressed the inhibition of VEGF-induced tube formation by IFN-γ, indicating that STAT1 is critical for this process. IFN-γ blocks the biological activity of VEGF through inhibition of genes necessary for the VEGF response, including angiopoietin-2, urokinase plasminogen activator, tissue inhibitor of matrix metalloproteinase-1, cyclooxygenase-2, and VEGF receptor 2. To extend these findings *in vivo*, the role of STAT1 in angiogenesis was examined in STAT1-deficient mice using the Matrigel *in vivo* angiogenesis assay. Substantial cellular infiltration and formation of vascular structures occurred in STAT1−/− mice compared with wild-type controls. These data indicate that STAT1 plays a key role in the inhibition of angiogenesis through its action within endothelial cells, and exploiting this process may be useful in treating cancers and vascular tumors. (Cancer Res 2006; 66(7): 3649-57)

Introduction

Knowledge of the signal transduction pathways that control the tumor environment, particularly those that control the induction of the tumor vasculature, is critical to understanding tumor progression. Most tumors cannot grow beyond 2 to 3 mm in diameter without the generation of new blood vessels to ensure an adequate supply of oxygen and nutrients (1). The progressive growth and metastasis of most tumors is angiogenesis dependent, with the balance between proangiogenic molecules and antiangiogenic molecules released by both tumor cells and the surrounding normal cells determining the extent of angiogenesis (2). Antiangiogenic therapy is based on the use of negative regulators of neovascularization and is aimed at suppressing proangiogenic signals or increasing inhibitory signals. Major positive angiogenic molecules include basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), matrix metalloproteinases (MMP), and interleukin (IL)-8 (1, 3, 4), whereas IFNs are among the major endogenous negative regulators of angiogenesis (1, 5).

IFN-γ is a multifunctional cytokine that has antiviral, antiproliferative, and immunomodulatory effects. IFN-γ is an important regulator of $CD4^+$ T helper cells and can increase expression of MHC molecules on cancer and endothelial cells (6, 7). IFNs inhibit the growth of numerous tumor cell types and are used in the clinical management of tumors (8–11). IFN-γ can attenuate nonpathologic angiogenesis during wound healing, as well as tumor angiogenesis (12–15). Although the endogenous antiangiogenic activity of IFNs has been described, the mechanism by which they regulate angiogenesis is not well understood. Although some studies suggest a direct action on the tumor cells, others provide evidence that IFN-γ–mediated antiangiogenesis does not require that the tumor respond to IFN-γ (13, 16–18). Within the tumor stroma, IFN-γ can induce secretion of the angiostatic protein IP-10, as well as other cytokines and chemokines (14). This suggests that nontumor cells in the tumor microenvironment, including endothelial cells, may be responsible for mediating the antiangiogenic activity of IFN-γ.

IFNs are important activators of signal transducers and activators of transcription (STAT) 1 signaling (19–21). STAT1 is required for signaling by IFNs, for innate immunity, and serves as a potent inhibitor of cell growth and as a promoter of apoptosis (22). STAT1 signal transduction is initiated upon IFN binding to its receptor, which activates Janus tyrosine kinases. STAT1 is phosphorylated on a single tyrosine residue by JAKs, which leads to dimerization and translocation to the nucleus where STATs bind to specific sequences within the promoters of target genes to modulate transcription. Tyrosine phosphorylation is necessary for STAT1 dimerization, nuclear translocation, and DNA binding. STAT1 can also be phosphorylated on serine residues by a variety of serine/threonine kinases, an event associated with enhanced transcriptional activity. Changes in gene expression induced by STAT1 signaling ultimately lead to physiologic changes, such as cell growth inhibition, differentiation, and apoptosis. In addition, the STAT1 signaling pathway may be an attractive target for therapeutic intervention in cancer therapy. For example, activation of STAT1 by agents that induce tyrosine phosphorylation is associated with a beneficial therapeutic response in chronic lymphocytic leukemia (23, 24).

Given that the mechanism by which IFNs exert an antiangiogenic effect is unknown and that IFN-γ is a potent activator of STAT signaling, we tested the hypothesis that STAT1 negatively regulates angiogenesis through its action within endothelial cells. We found that activation of STAT1 is necessary for growth inhibition and inhibition of tube formation of human umbilical vein endothelial cells (HUVEC) by IFN-γ and mediates the suppression of the potent proangiogenic activity of VEGF by IFN-γ. We also show that the mechanism by which IFN-γ blocks

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doi:10.1158/0008-5472.CAN-05-3612

the biological activity of VEGF is through inhibiting the transcription of genes necessary for the VEGF response. Reflecting the importance of STAT1 in angiogenesis *in vivo*, STAT1-deficient mice display an increased angiogenic response. Together, these findings indicate that STAT1 is a critical negative regulator of angiogenesis through its activity within endothelial cells.

Materials and Methods

Cell culture. HUVECs (Cambrex Corporation, Walkersville, MD) were maintained in EBM-2 complete endothelial growth medium (Cambrex Corporation) containing 2% fetal bovine serum (FBS), human recombinant VEGF (rhVEGF), bFGF, human epidermal growth factor (hEGF), insulin growth factor (IGF-I), hydrocortisone, ascorbic acid, heparin, and GA-1000 (gentamicin and amphotericin B, 1 μg/mL) according to the instructions of the supplier. HUVECs were used at passage 6 or less. Cells were cultured on 100 mm tissue culture plates and grown under 5% CO_2 at 37°C. Medium was renewed after 3 days, and cells were split after 4 days. When indicated, HUVECs were starved in basal EBM-2 containing no supplements.

Viability assay. A total of 5×10^5 HUVECs were cultured in a six-well tissue culture plate in 2 mL complete EBM-2 overnight. After 16 hours, the medium was exchanged for 1 mL basal EBM-2 medium containing 2% FBS and supplements but lacking VEGF, EGF, and IGF-I. Cells were left untreated or treated with indicated concentrations of human recombinant IFN-γ (R&D Systems, Minneapolis, MN) or human recombinant $VEGF_{165}$ (R&D Systems) or the combination of $VEGF_{165}$ and IFN-γ for 72 hours. Cell viability assays were done in duplicate using CellTiter-Glo (Promega, Madison, WI) to measure the luminescent output from the ATP present in viable cells according to the instructions of the manufacturer. All but 250 μL growth medium was removed from each well, and 250 μL CellTiter-Glo was added. Lysates were incubated 10 minutes with orbital rocking. A total of 100 μL cell lysate was transferred in duplicate to a 96-well opaque plate. The luminescent output was measured on a Luminoskan Ascent luminometer (ThermoLab Systems, Helsinki, Finland).

Western blot analysis. HUVECs were lysed in radioimmunoprecipitation assay buffer containing 50 mmol/L Tris-HCl (pH 7.4), 1% NP40, 0.25% sodium deoxycholate, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 μg/mL pepstatin, and 1 mmol/L sodium orthovanadate. For Western analysis, 40 μg protein were resolved on 7% SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Blots were incubated with antibodies that recognize the tyrosine phosphorylated form of STAT1 (1:10,000; ref. 25), total STAT1 (1:20,000; Santa Cruz Biotechnology, Santa Cruz, CA), phosphorylated Src, total Src, phosphorylated p38 mitogen-activated protein kinase (MAPK), total p38 MAPK, phosphorylated extracellular signal-related kinase (ERK)-1/2, total ERK-1/2, or β-actin. Blots were incubated with goat anti-rabbit horseradish peroxidase–conjugated secondary antibodies (Calbiochem, La Jolla, CA) and detection was done using the Renaissance chemiluminescent ECL kit (NEN Dupont, Boston, MA).

Nuclear extract preparation and electrophoretic mobility shift assay. Nuclear extracts were prepared by resuspending cells in hypotonic buffer [10 mmol/L Tris (pH 7.4), 10 mmol/L NaCl, 6 mmol/L $MgCl_2$, 1 mmol/L β-mercaptoethanol, 1 mmol/L sodium orthovanadate, 10 μg/mL PMSF, 2 μg/mL pepstatin, 2 μg/mL leupeptin, and 10 mmol/L aprotinin] followed by incubation on ice for 5 minutes. Cells were centrifuged for 10 seconds at 12,000 × *g*, resuspended in hypotonic buffer, and disrupted using a Dounce homogenizer (Type B pestle, 30 strokes). The nuclei were collected by centrifugation for 10 seconds at 12,000 × *g* and washed once with hypotonic buffer. The nuclear pellet was resuspended in 1 pellet volume of high salt buffer [20 mmol/L HEPES (pH 7.9), 420 mmol/L NaCl, 1.5 mmol/L $MgCl_2$, 25% glycerol, 0.2 mmol/L EDTA, 10 μg/mL PMSF, 1 mmol/L sodium orthovanadate, and 1 mmol/L β-mercaptoethanol] and incubated for 30 minutes at 4°C followed by centrifugation for 3 minutes at 12,000 × *g*. Two microliters of nuclear extract were incubated with a double-stranded ^{32}P-labeled oligonucleotide (1 ng) γ-activation sequence derived from the ciliary neurotrophic factor response element (5′-CAGCCTGATTTCCCCGAAATGACGGCG-3′ and its complement) in 10 μL binding buffer [25 mmol/L HEPES (pH 7.9), 100 μmol/L EGTA, 200 μmol/L $MgCl_2$, 500 μmol/L DTT, 1 μg/mL bovine serum albumin, 0.2 μg/μL poly(deoxyinosinic-deoxycytidylic acid), 1% Ficoll, and 0.1 μg/μL salmon sperm DNA] for 15 minutes at room temperature. For supershift analysis, the nuclear extracts were incubated with 1 μg anti-STAT1 antibody (Santa Cruz Biotechnology) for 20 minutes on ice before adding the binding buffer. Protein-DNA complexes were resolved by nondenaturing gel electrophoresis and detected by autoradiography.

Real-time PCR. Total cellular RNA was isolated using the RNEasy Mini kit (Qiagen, Valencia, CA). cDNA was synthesized from 500 ng total RNA using the SuperScript First-Strand Synthesis kit (Invitrogen Life Technologies, Carlsbad, CA). PCR was done in triplicate in 50 μL reaction volumes using 1.5 μL cDNA, 1× SYBR Green master mix (Stratagene, Cedar Creek, TX), 1 μL ROX reference dye (1:400 dilution), and 5 pmol of each primer. PCR was done using primers for STAT1 (5′-TGGTGAAATTGCAAGAGCTG-3′ and 5′-AGACTGCCATTGGTGGACTC-3′), urokinase plasminogen activator (uPA; 5′-TGTGAGATCACTGGCTTTGG-3′ and 5′-GTCAGCAGCACACAGCATTT-3′), angiopoietin 2 (ANG-2; 5′-GCAAGTGCTGGAGAACATCA-3′ and 5′-GTTAACTTCCGCGTTTGCTC-3′), VEGF receptor 2 ($VEGFR_2$; 5′-GCGATGGCCTCTTCTGTAAG-3′ and 5′-CACGACTCCATGTTGGTCAC-3′), cyclooxygenase 2 (COX-2, prostaglandin-endoperoxide synthase 2; 5′-ATTCTTTGCCCAGCACTTCA-3′ and 5′-ATCCTTGAAAAGGCGCAGT-3′), MMP-2 (5′-CGCCCATCATCAAGTTCC-3′ and 5′-TGTCCTTCAGCACAAACAGG-3′), tissue inhibitor of metalloproteinase-1 (TIMP-1; 5′-GCGAGGAGTTTCTCATTGCT-3′ and 5′-GTTTGCAGGGGATGGATAAA-3′), Bcl-2 (5′-GCCCTGTCCATGACTGAGTA-3′ and 5′-AGGGCCAAACTGAGCAGAG-3′), IFN regulatory factor 1 (IRF-1; 5′-AAGGGAAATTACCTGAGGACATCAT-3′) and 5′-CAATTTCTGGCTCCTCCTTACAGCTAA-3′ and β-actin (5′-TCCCTGGAGAAGAGCTACGA-3′ and 5′-AGCACTGTGTTGGCGTACAG-3′). Primer pairs spanned introns. Samples were amplified in a 7500 real-time PCR System (Applied Biosystems, Foster City, CA) for 40 cycles using the following PCR variables: 95°C for 30 seconds, 57°C for 1 minute, and 72°C for 1 minute. Gene expression was quantitated using the comparative C_T method of relative quantification using 7500 System SDS software (Applied Biosystems, Foster City, CA). The mean fold changes ± SE of the three replicates were calculated.

Transfection of small interfering RNA into HUVECs. HUVECs were seeded at a density of 80,000 cells per well in antibiotic-free complete EBM-2 in a 24-well plate. Cells were incubated for 16 hours under CO_2 at 37°C, then the cells were washed and the medium was replaced with Opti-MEM I (Invitrogen Life Technologies). Cells were transfected with indicated concentrations of STAT1 small interfering RNA (siRNA; siGENOME SMARTpool; Dharmacon, Inc., Lafayette, CO) or nontargeted siRNA (siCONTROL Nontargeted siRNA 1; Dharmacon) using LipofectAMINE 2000. After 5.5 hours, the transfection medium was removed and replaced with antibiotic-free complete EBM-2. Inhibition of STAT1 mRNA expression was verified by real-time PCR, and inhibition of STAT1 protein expression was verified by Western blot analysis.

Tube formation assay. A 24-well tissue culture plate was prechilled at −20°C and carefully coated with growth factor–reduced Matrigel (100 μL/well; Becton Dickinson, Bedford, MA) avoiding bubbles. The plate was incubated at 37°C for 30 minutes to allow the Matrigel to solidify. HUVECs (30,000 per well) were suspended in 500 μL EBM-2 containing 2% FBS and gently added to the Matrigel-coated wells. Cells were untreated or treated with 1,000 units/mL IFN-γ, 20 ng/mL VEGF, or the combination of 1,000 units/mL IFN-γ and 20 ng/mL VEGF. After 24 hours, the medium was removed and cells were fixed with cold 70% ethanol and visualized by staining with DiffQuick. Images were captured under phase contrast microscopy at ×20 magnification using a Nikon Coolpix 4500 digital camera and analyzed using Scion Image (http://www.scioncorp.com/). Tube length was assessed by drawing a line along each tubule and measuring the length of the line in pixels then calibrating with a micrometer present in the image. Tube length was measured in five nonoverlapping fields under ×20 magnification.

Migration assay. Nontargeted control siRNA or STAT1 siRNA was introduced into HUVECs by lipid-mediated transfer, and 24 hours later,

transfected HUVECs were resuspended in basal EBM-2 containing no supplements. The assay was done in 96-well MultiScreen-MIC filter plates (Millipore, Billerica, MA). Lower chambers were filled with basal EBM-2 containing no supplements or containing 500 units/mL IFNγ, 10 ng/mL VEGF, or the combination of 500 units/mL IFNγ and 10 ng/mL VEGF. The filter plate containing 8 μm porous membranes was inserted, and the upper wells were filled with 5×10^4 HUVECs in a total volume of 150 μL. The plate was incubated at 37°C under 5% CO_2 for 4 hours. After incubation, nonmigrated cells were removed from the upper well. The cells that migrated to the lower side of the filter were fixed and stained with DiffQuick. Three nonoverlapping fields were counted at ×40 magnification.

***In vivo* angiogenesis assay.** Growth factor–reduced Matrigel (Becton Dickinson) containing HBSS and heparin (20 units/mL) or HBSS and heparin supplemented with VEGF (600 ng/mL) and bFGF (600 ng/mL) in a final volume of 500 μL was injected s.c. into STAT1-deficient mice (generously provided by Dr. David Levy, New York University Medical School, New York, NY) or wild-type C57BL/6 mice. Plugs and surrounding tissues were resected after 6 days, fixed in formalin, embedded in paraffin, and sections were stained with H&E for histologic analysis (Rodent Histopathology Core Facility, Harvard Medical School). Neovascularization in the plug was scored and the surrounding tissue was examined to assess potential inflammation. Experiments were done thrice. These experiments were approved by the institutional review board.

Statistical analysis. The independent Student's *t* test was used for statistical significance between two groups and paired data were evaluated by the paired Student's *t* test. The *P* value was considered significant when it was <0.05.

Results

IFN-γ inhibits endothelial cell proliferation and blocks VEGF-induced proliferation. IFN-γ, which is a potent and selective activator of STAT1, has notable antiangiogenic activity. To determine how STAT1 activation affects endothelial cell biology, *in vitro* angiogenesis assays were used to examine each step in the angiogenic cascade, including proliferation, migration, and differentiation into capillary-like tubes. To determine the effect of IFN-γ on endothelial cell proliferation, HUVECs were incubated with various concentrations of IFN-γ for 72 hours, and their viability was measured using a luminescent-based ATP assay. To confirm that this assay measured proliferation and not simply cell survival, preliminary experiments were done in which cells, excluding trypan blue, were counted on a hemocytometer in parallel to the ATP assay on the day that the culture was initiated and every 24 hours thereafter. Untreated cultures were found to double every ~30 hours, and there was a close concordance between the two methods (data not shown). At concentrations of 500 units/mL or higher, IFN-γ inhibited HUVEC proliferation by ~30% (Fig. 1*A*). VEGF is a potent stimulator of endothelial cell proliferation. To determine whether IFN-γ could antagonize this strong proangiogenic molecule, HUVECs were treated with IFN-γ, VEGF, or the combination of IFN-γ and VEGF, and viability was measured after 72 hours. Again, IFN-γ inhibited proliferation by ~30% whereas VEGF increased HUVEC proliferation by ~100% (Fig. 1*B*). Of note, the simultaneous treatment of HUVECs with IFN-γ and VEGF significantly inhibited VEGF-induced proliferation. These data show that IFN-γ cannot only inhibit endothelial cell proliferation but it can also block VEGF-induced proliferation. This indicates that the negative growth signal stimulated by IFN-γ can override the positive growth signal initiated by VEGF.

STAT1 is necessary for IFN-γ–induced growth inhibition and for inhibition of VEGF activity. To determine whether the negative growth signal initiated by IFN-γ involves activation of STAT1, HUVECs were incubated with 10 to 500 units/mL IFN-γ and then analyzed by Western blotting for the presence of STAT1 tyrosine phosphorylation. Using an antibody specific for the phosphorylation of tyrosine 701, the amino acid residue necessary for activation of STAT1, Western blot analysis revealed that STAT1 was tyrosine phosphorylated within 15 minutes after addition of 100 to 500 units/mL IFN-γ (Fig. 2*A, left*). Importantly, Western analysis also revealed that concentrations of VEGF between 10 and 300 ng/mL do not activate STAT1 in HUVECs. To verify functional activation of STAT1 by IFN-γ, electrophoretic mobility shift assay (EMSA) was used to measure STAT1 DNA-binding activity. In cells treated with IFN-γ or the combination of IFN-γ and VEGF, there is an inducible STAT1 DNA-binding complex that can be disrupted by the addition of a STAT1 antibody (Fig. 2*A, right*). These data suggest that IFN-γ may mediate its

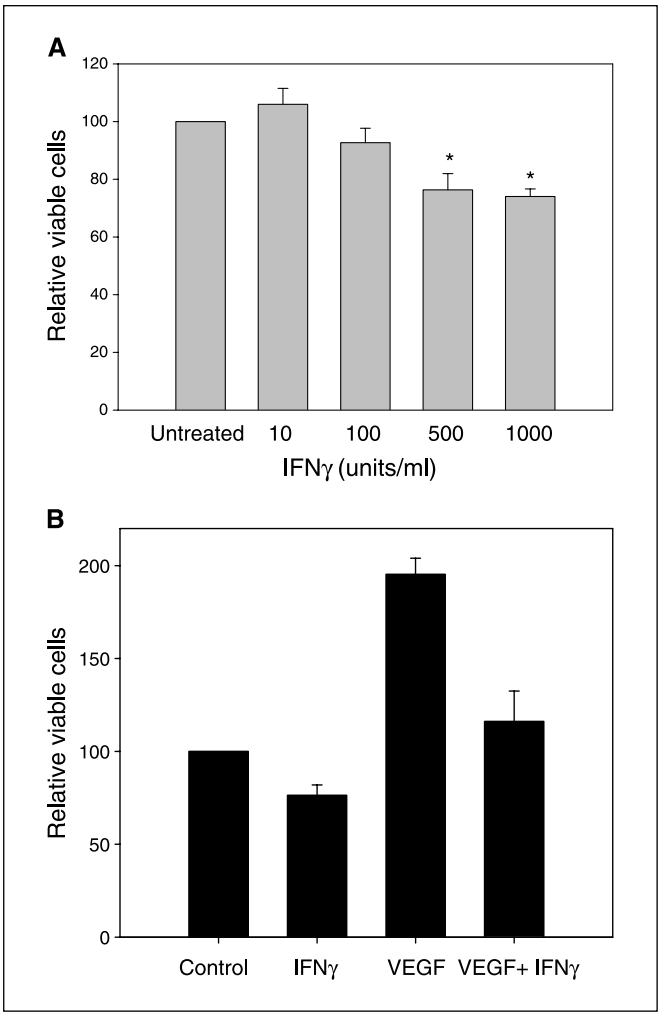

Figure 1. IFN-γ inhibits endothelial cell proliferation and blocks VEGF-induced proliferation. *A,* HUVECs were untreated or treated with indicated concentrations of IFN-γ for 72 hours. Viability was determined by measuring the level of ATP using a luminescence-based assay. The assay was done in duplicate, and each sample was normalized to cells incubated without stimuli to determine the relative number of viable cells. *, $P < 0.05$, when cells incubated with either 500 or 1,000 units/mL IFN-γ were compared with untreated cells. *B,* HUVECs were untreated or treated with IFN-γ (500 units/mL), VEGF (100 ng/mL), or the combination of IFN-γ and VEGF for 72 hours. Viability was measured as described above. Results were normalized to untreated cells. *Columns,* mean; *bars,* SE.

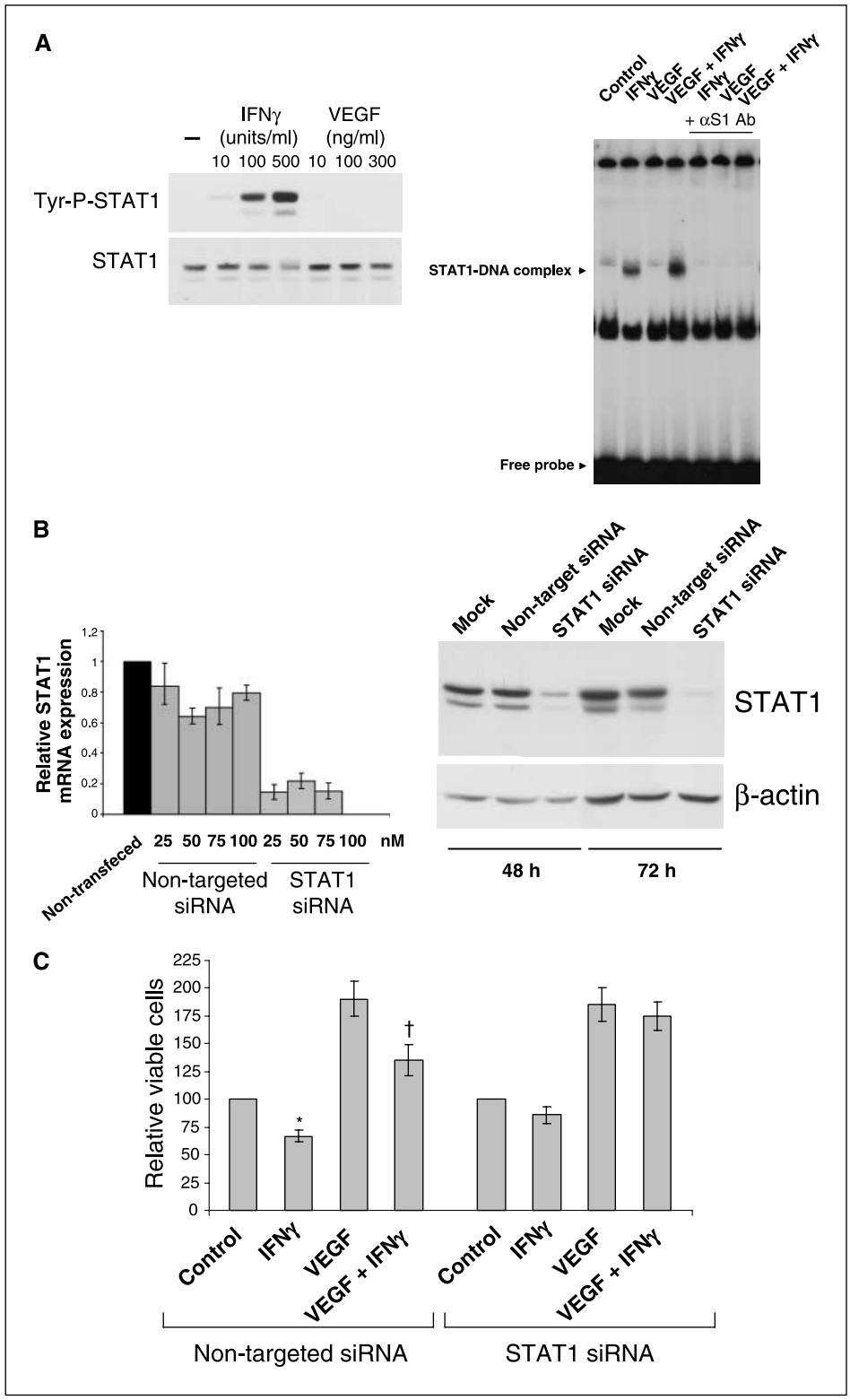

Figure 2. STAT1 is necessary for IFN-γ–induced growth inhibition and for inhibition of VEGF activity. *A,* HUVECs were untreated or treated with indicated concentrations of IFN-γ or VEGF for 15 minutes. Cells were lysed and Western analysis was done using antibodies that recognize the phosphorylated form of STAT1 (*left, top*). Blots were stripped and reprobed for total STAT1 as a loading control (*left, bottom*). DNA-binding activity of STAT1 was examined by EMSA using a radiolabeled probe containing a γ-activation sequence (*right*). Nuclear extracts were prepared from untreated HUVECs or HUVECs treated with IFN-γ (500 units/mL), VEGF (100 ng/mL), or the combination of IFN-γ and VEGF for 15 minutes. Anti-STAT1 antibody was added to the binding reaction mix to verify that the protein-DNA complex contained STAT1. *B,* HUVECs were untransfected or transfected with indicated concentrations of STAT1 siRNA or nontargeted siRNA to specifically inhibit STAT1 mRNA expression. After 48 hours, STAT1 mRNA expression was determined by real-time RT-PCR (*left*). HUVECs were transfected with 25 nmol/L STAT1 siRNA or nontargeted siRNA, and STAT1 protein expression was examined using Western blot analysis. The blot was stripped and reprobed for β-actin as a loading control. *C,* HUVECs were transfected with 25 nmol/L STAT1 siRNA or nontargeted siRNA. After 24 hours, cells were trypsinized, counted, and transferred to six-well plates in growth factor-reduced medium. Cells were treated with IFN-γ (500 units/mL), VEGF (100 ng/mL), or the combination of IFN-γ and VEGF. Viability was determined by measuring the level of ATP using a luminescence-based assay. The assay was done in duplicate, and each sample was normalized to cells incubated without stimuli to determine the relative number of viable cells. *, $P < 0.05$, when cells incubated with VEGF or IFN-γ were compared with untreated cells. †, $P < 0.01$, when cells incubated with VEGF and IFN-γ were compared with VEGF-treated cells.

inhibitory effect on endothelial cell proliferation by activating the STAT1 signaling pathway.

To directly assess the functional role of STAT1 in IFN-γ–induced inhibition of endothelial cell proliferation, STAT1 expression was inhibited by introducing short interfering RNA (siRNA) into HUVECs by lipid-mediated transfection. As a control, a non-targeted siRNA that targets a nonhuman mRNA sequence was introduced into HUVECs as well. STAT1 mRNA expression was measured using quantitative real-time reverse transcription-PCR (RT-PCR). At low concentrations of STAT1 siRNA, there was ~90% inhibition of STAT1 expression 48 hours after transfection (Fig. 2*B, left*). This was followed by almost complete inhibition of STAT1

protein expression at 48 and 72 hours after transfection (Fig. 2*B, right*). A concentration of 25 nmol/L siRNA was chosen for subsequent experiments because this concentration could significantly inhibit STAT1 mRNA expression, whereas the nontargeted siRNA had minimal effect on STAT1 expression. The STAT1 siRNA or nontargeted control siRNA was introduced into HUVECs, and 24 hours later, cells were treated with IFN-γ, VEGF, or the combination of IFN-γ and VEGF (Fig. 2*C*). In cells transfected with nontargeted siRNA, IFN-γ inhibited endothelial cell growth, and the combination of IFN-γ and VEGF inhibited VEGF-induced proliferation of endothelial cells. In contrast, cells in which STAT1 had been depleted grew almost as well in the presence of IFN-γ compared with untreated cells. Moreover, simultaneous exposure of STAT1-depleted HUVECs to IFN-γ and VEGF resulted in cell growth equivalent to that of cells treated with VEGF alone. These data indicate that STAT1 is necessary for IFN-γ–induced inhibition of HUVEC proliferation as well as IFN-γ–induced inhibition of VEGF activity. Thus, STAT1 activity negatively regulates endothelial cell proliferation.

STAT1 is necessary for inhibition of VEGF-induced tube formation by IFN-γ. Endothelial cells plated on Matrigel undergo attachment, migration, and differentiation events to form interconnected networks of endothelial cell-lined tubes, recapitulating the angiogenic process. To determine whether IFN-γ could affect endothelial cell tube formation, HUVECs were cultured on Matrigel-coated tissue culture plates and then incubated with IFN-γ, VEGF, or the combination of IFN-γ and VEGF. Tube-like structures formed after 20 hours in untreated wells (Fig. 3*A*). Cells treated with VEGF formed an extensive tube network. In contrast, treatment with IFN-γ resulted in disrupted, less well connected

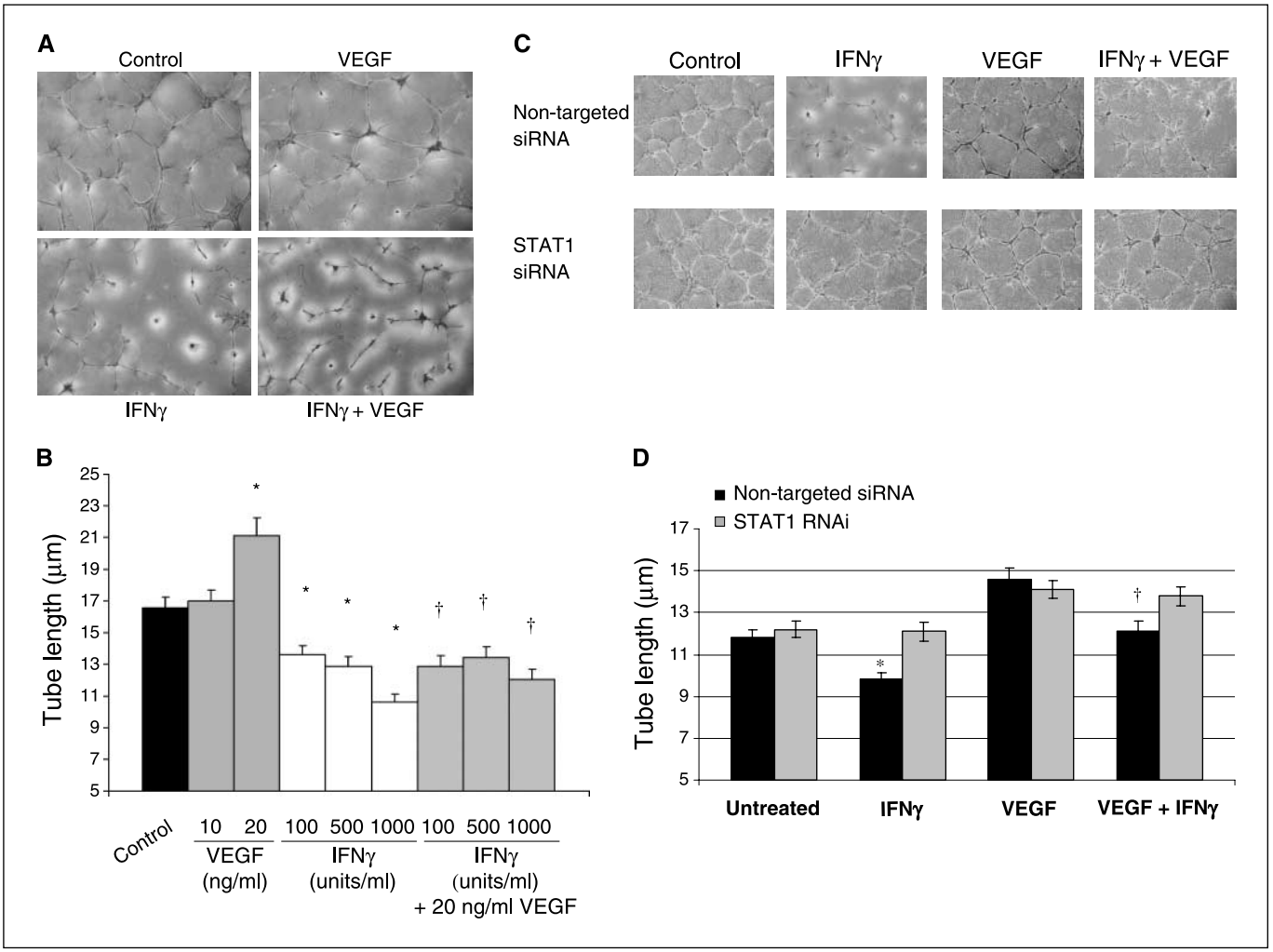

Figure 3. STAT1 is necessary for inhibition of VEGF-induced tube formation by IFN-γ. *A,* each well of a 24-well tissue culture plate was coated with 100 μL growth factor–reduced Matrigel. After the Matrigel solidified at 37°C, HUVECs (30,000 cells in 500 μL) were added to each well. Cells were treated with IFN-γ (1,000 units/mL), VEGF (20 ng/mL), or the combination of IFN-γ and VEGF. After 24 hours, the medium was removed and cells were stained with DiffQuick. Cells were photographed under phase contrast microscopy at ×20 magnification. *B,* tube formation assay was done as described in (*A*) except that HUVECs were treated with 10 to 20 ng/mL VEGF, 100 to 1,000 units/mL IFN-γ, or the combination of 100 to 1,000 units/mL IFN-γ and 20 ng/mL VEGF. Photographs were imported into the ScionImage software program to measure tube length. Tubes were counted only if they were >5 μm in length. The experiment was done four times. *, $P < 0.005$ when cells incubated with VEGF or IFN-γ were compared with untreated cells. †, $P < 0.005$ when cells incubated with the combination of VEGF and IFN-γ were compared with cells treated with 20 ng/mL VEGF. *C,* HUVECs were transfected with 25 nmol/L STAT1 siRNA or nontargeted siRNA. After 24 hours, cells were trypsinized, counted, and transferred to 24-well plates coated with Matrigel. Tube formation assay was done as described in (*A*). After 24 hours, the medium was removed and cells were stained with DiffQuick. Cells were photographed under phase contrast microscopy at ×20 magnification. *D,* photographs were imported into the ScionImage software program to measure tube length. Tubes were counted only if they were >5 μm in length. The experiment was done twice. *, $P < 0.005$ when cells incubated with IFN-γ were compared with untreated cells. †, $P < 0.005$ when cells incubated with the combination of VEGF and IFN-γ were compared with cells treated with VEGF.

tube networks. Simultaneous treatment with IFN-γ and VEGF resulted in disrupted networks similar to IFN-γ-treated cells. To quantify the effects of IFN-γ on tube formation, the total length of tubes was measured using image analysis software. A concentration of 20 ng/mL VEGF significantly increased tube length ($P < 0.005$) by 5 μm compared with untreated cells (Fig. 3*B*). In contrast, concentrations of 100 to 1,000 units/mL IFN-γ significantly decreased tube length ($P < 0.005$). Cells treated with 20 ng/mL VEGF in combination with increasing amounts of IFN-γ had shorter tubes, closely resembling cells treated with IFN-γ alone. These results indicate that IFN-γ inhibits tube formation even in the presence of the potent endothelial cell differentiation factor, VEGF.

To determine whether STAT1 is necessary for IFN-γ–induced disruption of endothelial tube formation, STAT1 expression was inhibited by introducing STAT1 siRNA or control nontargeted siRNA into HUVECs and 24 hours later, cells were treated with IFN-γ, VEGF, or the combination of IFN-γ and VEGF. In cells transfected with nontargeted siRNA, IFN-γ or the combination of IFN-γ and VEGF inhibited tube formation (Fig. 3*C*) and significantly decreased tube length (Fig. 3*D*). When HUVECs in which STAT1 expression was inhibited were treated with IFN-γ or the combination of IFN-γ and VEGF, IFN-γ did not inhibit tube formation or prevent VEGF from stimulating an extensive tube network (Fig. 3*C*). Likewise, tube length in STAT1 siRNA cells was similar in IFN-γ-treated cells compared with untreated cells, and tube length in cells treated with the combination of IFN-γ and VEGF was similar to the length of tubes formed by cells treated with VEGF alone (Fig. 3*D*). These data indicate that STAT1 is necessary for IFN-γ–induced disruption of endothelial tube formation.

STAT1 does not inhibit endothelial cell migration. To determine whether STAT1 activation was important for endothelial cell migration, HUVECs were transfected with nontargeted siRNA or STAT1 siRNA, and 24 hours later, were treated with IFN-γ, VEGF, or the combination of IFN-γ and VEGF in a migration assay. Cell migration was measured by counting the number of cells that migrated through a porous membrane after 4 hours. IFN-γ had no effect on endothelial cell migration in HUVECs transfected with nontargeted siRNA nor did IFN-γ inhibit VEGF-induced migration. Consistent with these findings, inhibition of STAT1 expression using STAT1 siRNA had no effect on endothelial cell migration (data not shown). Altogether, the *in vitro* angiogenesis assays indicate that STAT1 negatively regulates endothelial cell proliferation and endothelial cell differentiation into tube-like structures, although not migration.

IFN-γ blocks genes necessary for the VEGF response. To better understand how STAT1 negatively regulates endothelial cell function, we next addressed the mechanism by which IFN-γ blocks the biological effects of VEGF. The activation of endothelial cells by $VEGF_{165}$ leads to the autophosphorylation of $VEGFR_2$ and the subsequent tyrosine phosphorylation of numerous downstream targets, including enzymes that generate second messengers, such as phospholipase Cγ and phosphatidylinositol 3-kinase, and protein kinases, such as Src and PKC, among others (26). Activation of these signaling proteins generates further downstream signaling events, such as activation of the kinases AKT, p38 MAPK, and ERK1/2, which, in turn, promote the various biological functions of VEGF. One possible mechanism by which IFN-γ could inhibit VEGF activity is through inhibition of proximal signaling events initiated by $VEGFR_2$. To test this possibility, HUVECs were treated with IFN-γ, VEGF, or the combination of IFN-γ and VEGF, and cells were examined for the phosphorylation of key signaling proteins, which are known targets of $VEGFR_2$. VEGF induced prominent phosphorylation of Src, p38 MAPK, and ERK1/2, and this was not attenuated by the addition of IFN-γ (Fig. 4*A*). These data indicate that both $VEGFR_2$ activity and the proximal signaling events initiated by $VEGFR_2$ remain functional in the presence of IFN-γ.

Because STAT1 and STAT3 proteins can oppose each other in function when they are activated in the same cell, we considered the possibility that activated STAT1 inhibited VEGF-induced STAT3 activity. Although VEGF has been reported to induce STAT3 phosphorylation in some systems, we found no evidence of STAT3 activation in HUVECs in response to VEGF by Western blot or EMSA (data not shown). Therefore, the ability of STAT1 to inhibit VEGF function does not involve STAT1 blocking STAT3 activity.

VEGF exerts its effects on endothelial cells through activation of key target genes. Therefore, we examined whether IFN-γ could inhibit gene expression induced by VEGF. HUVECs were treated for 3.5 hours with IFN-γ, VEGF, or the combination of IFN-γ and VEGF, and the expression of a number of candidate genes known to be induced by VEGF and representative of the various biological functions induced by VEGF were examined by RT-PCR. VEGF induced prominent expression of a number of target genes, including *uPA, ANG-2, TIMP-1, COX-2, MMP-2*, and *$VEGFR_2$* (Fig. 4*B, left*). The expression of a subset of these genes, *uPA, ANG-2*, and *TIMP-1*, was completely suppressed by IFN-γ. The expression of *COX-2* and *MMP-2* were partially inhibited by IFN-γ. *$VEGFR_2$* was induced by VEGF and, surprisingly, by IFN-γ. *$VEGFR_2$* expression in the presence of IFN-γ and VEGF was similar to the level of expression induced by IFN-γ, indicating that the presence of IFN-γ prevents further induction by VEGF. Although Bcl-2 expression was not induced within 3.5 hours of VEGF stimulation, IFN-γ seemed to suppress basal expression of *Bcl-2* even in the presence of VEGF. Importantly, IFN-γ-induced expression of a well-characterized STAT1 target gene, *IRF-1*, whose expression further increased in the presence of VEGF (Fig. 4*B, right*). These results indicate that IFN-γ both positively and negatively regulates gene expression in HUVECs and can block expression of genes involved in the VEGF response.

Absence of STAT1 results in increased blood vessel growth. If STAT1 negatively regulates angiogenesis, then mice that lack STAT1 should have enhanced angiogenic responses. The role of STAT1 in angiogenesis *in vivo* was examined by measuring blood vessel growth in Matrigel plugs in STAT1-deficient mice. Matrigel alone or Matrigel supplemented with the angiogenic factors VEGF and bFGF was injected s.c. into STAT1-deficient or wild-type mice. New blood vessel growth was evaluated after 6 days by histologic examination of Matrigel plug sections. Injection of Matrigel-containing angiogenic factors induced partial angiogenic responses in wild-type mice (Fig. 5*A*). In contrast, substantial cellular infiltration and formation of cords, tubes, and blood-containing vessels occurred in STAT1-deficient mice injected with Matrigel-containing angiogenic factors. Because inflammation can cause cellular infiltration and angiogenic responses, we also examined the surrounding skin and muscle layers for the presence of inflammation. Notably, there was minimal inflammation present in the surrounding tissues (data not shown). The extent of neovascularization within the histologic sections was scored blindly based on the extent of cell infiltration and the presence of vascular structures (Fig. 5*B*). Examination of at least eight animals in each group

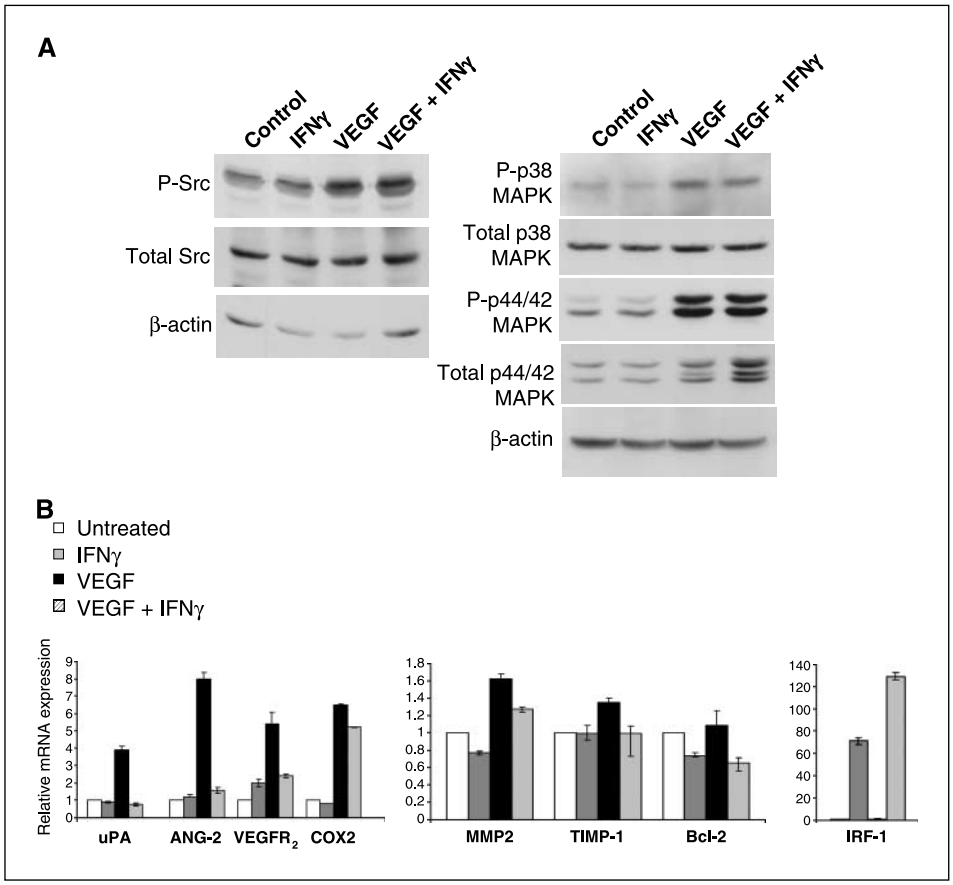

Figure 4. IFN-γ blocks transcription of genes necessary for the VEGF response. *A,* HUVECs were starved for 16 hours and then stimulated with IFN-γ (500 units/mL), VEGF (100 ng/mL), or the combination of IFN-γ and VEGF for 15 minutes. Cells were lysed and Western analysis was done using antibodies that recognize the phosphorylated form of Src, p38 MAPK, or ERK1/2. Blots were stripped and reprobed with antibodies that recognize total Src, p38 MAPK, ERK1/2, or β-actin. *B,* HUVECs were starved for 16 hours and stimulated for 3.5 hours as described in (*A*). RNA was isolated and real-time PCR using SYBR green technology was done to analyze mRNA expression of indicated VEGF target genes, *uPA, ANG-2, VEGFR$_2$, COX-2, MMP2, TIMP-1*, and *B-cell chronic lymphocytic leukemia/lymphoma 2* (*Bcl-2*). IRF-1 was amplified as a positive control for IFN-γ-induced gene expression. mRNA levels were normalized to β-actin. The assay was done in triplicate. Representative of three experiments. *Columns,* mean of three replicates; *bars,* SE.

revealed higher scores for angiogenesis in the STAT1-deficient mice injected with Matrigel-containing angiogenic factors compared with wild-type animals. The *in vivo* angiogenesis assays indicate that the lack of STAT1 results in increased new blood vessel formation and supports the hypothesis that STAT1 negatively regulates angiogenesis.

Discussion

The present study provides evidence that STAT1 negatively regulates angiogenesis through its action within endothelial cells. IFN-γ causes growth inhibition and disrupts tube formation of endothelial cells; however, in the absence of STAT1, these effects are lost. Importantly, IFN-γ can antagonize VEGF activity within endothelial cells, a process also found to be dependent on STAT1. Furthermore, STAT1-deficient mice have increased angiogenic responses in the Matrigel angiogenesis assay, indicating that STAT1 exerts negative regulation on the angiogenic process *in vivo*. Altogether, these findings suggest that STAT1 is a prominent negative regulator of angiogenesis.

The critical steps in endothelial cell activation that occur during angiogenesis can be isolated and studied *in vitro* to provide insight into the specific process affected by a given factor. Of these steps, STAT1 negatively regulates endothelial cell proliferation and tube formation. In contrast to type I IFNs, IFN-α and IFN-β, IFN-γ does not inhibit endothelial cell migration (27, 28). Endothelial cell proliferation and tube formation was inhibited by IFN-γ even in the presence of the potent endothelial cell mitogen and differentiation factor, VEGF, in a STAT1-dependent manner. These findings imply that the antiangiogenic effect of IFN-γ is due to a direct effect on endothelial cells through the inhibition of endothelial cell proliferation and tube formation mediated by the activity of STAT1. Given that previous studies showed that IFN-γ can inhibit bFGF-induced endothelial cell proliferation, it is likely that IFN-γ can suppress the mitogenic activity of a broad array of angiogenic factors (29). The Matrigel *in vivo* assay provided further examination of the role of STAT1 in angiogenesis. Neovascularization increased under proangiogenic conditions in STAT1-deficient mice, further supporting the idea that STAT1 inhibits endothelial cell activation during angiogenesis *in vivo*.

IFN-γ blocks or attenuates gene expression of several known regulators of endothelial cell activation and angiogenesis. Suppression of these genes by IFN-γ clearly could influence the balance of angiogenic molecules in favor of the nonangiogenic state. IFN-γ completely suppressed VEGF-induced up-regulation of *ANG-2, uPA,* and *TIMP-1*. Expression of ANG-2 is up-regulated at sites of vascular remodeling and neovascularization and is itself a target for antiangiogenic cancer therapy (30). uPA is involved in the degradation of the extracellular matrix leading to tumor cell migration (31). Although TIMP-1 is a well-characterized inhibitor of MMPs, other biological functions of TIMP-1, including anti-apoptotic, proangiogenic, and growth-stimulatory effects, have also been described (32–36). The magnitude of *TIMP-1* induction by VEGF was small, but its induction was completely inhibited by the presence of IFN-γ. IFN-γ suppressed the VEGF-induced expression of *VEGFR$_2$, COX-2,* and *MMP-2*. Surprisingly, IFN-γ induced expression of *VEGFR$_2$*, which was up-regulated to a greater extent by VEGF; however, the combination of IFN-γ and VEGF led to

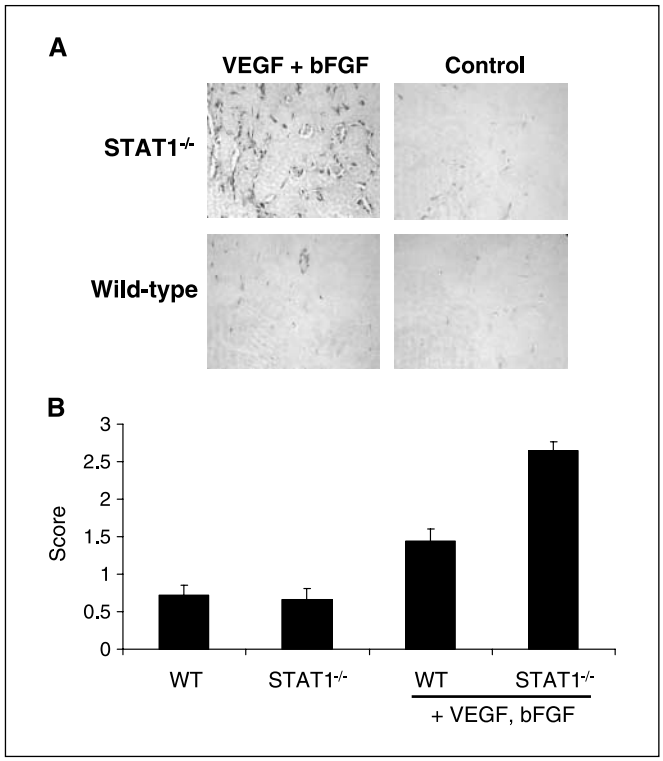

Figure 5. Absence of STAT1 results in increased blood vessel growth. *A,* Matrigel alone or Matrigel supplemented with 600 ng/mL VEGF and 600 ng/mL bFGF was injected s.c. into STAT1-deficient (STAT1−/−) and wild-type mice. Angiogenesis was evaluated after 6 days by histologic analysis of Matrigel plug sections. *B,* Matrigel plugs were scored for extent of cell infiltration and presence of blood vessels. Score: 0, no cell infiltration, no vessels; 1, disorganized cell infiltration, some vessels at edge; 2, marked cell infiltration, some vessels at edge and center; 3, deep cell infiltration and vessels at edge and center.

induction of $VEGFR_2$ only to the level achieved with IFN-γ alone, indicating that IFN-γ prevents further gene induction in the presence of VEGF. The synthesis of prostaglandins, which are inflammatory mediators that promote angiogenesis, is initiated by the cyclooxygenase enzymes, including COX-2 (37). Although IFN-γ suppression of *COX-2* induction by VEGF was not complete, it is possible that partial inhibition of *COX-2* expression could impede prostaglandin synthesis. Likewise, partial suppression of MMP-2 expression by IFN-γ could prevent migration and invasiveness of tumor cells (38).

There are several possible mechanisms to explain how IFN-γ blocks VEGF transcriptional responses. IFN-γ activation of STAT1 may sequester coactivators, such as cAMP-responsive element binding protein–binding protein (CBP)/p300, similar to the mechanism by which IFN-γ suppresses *MMP-9* gene expression (39). IFN-γ induces the *in vivo* association of STAT1 and CBP and decreases the association of CBP to the *MMP-9* promoter. Another possibility is that STAT1 associates with corepressors to suppress the induction of VEGF target genes. Proteins, such as SMRT and PIASx, are known to act as corepressors upon association with STAT proteins and down-regulate STAT target gene expression (40, 41). This raises the possibility that STAT proteins can associate with corepressors to down-regulate expression of genes regulated by transcription factors that control VEGF target gene expression. Indeed, examination of the *ANG-2* upstream regulatory sequences reveals potential STAT binding sites overlapping or adjacent to binding sites for the RBPJK and BCL-6 transcriptional repressors. A third possibility is that STAT1 competes for binding sites with other positively acting transcription factors in the promoters of VEGF target genes. STAT1 binding to these sites could block access to the positive regulatory elements in VEGF target genes. IFN-γ suppression of VEGF transcriptional responses may occur indirectly through STAT1-mediated induction of other transcription factors, such as IRF-1, as well. IFNs can inhibit tumor necrosis factor-α induction of *MMP-9* expression by a mechanism involving competitive inhibition of nuclear factor-κB (NF-κB) binding by IRF-1 (42). IRF-1 binds to the *MMP-9* promoter at a position that overlaps with the NF-κB binding site and prevents NF-κB from gaining access to the promoter. It is likely that IFN-γ operates through multiple mechanisms to inhibit expression of VEGF target genes.

Many tumors secrete VEGF in high concentrations, and inhibition of the VEGF signaling pathway is an intensely explored approach to cancer therapy. Early clinical studies with IFN-γ, based on doses that approached the maximal tolerated dose, resulted in very low response rates (43–45). Subsequent clinical studies suggested that optimal biological responses could be induced by doses below the maximum tolerated dose (46, 47). Clinical trials in which low-dose, less-frequent administration of IFN-γ was delivered resulted in higher response rates (10, 11). More recently, preclinical studies in which IFN-γ was delivered directly to endothelial cells makes targeted delivery of IFN-γ to the tumor vasculature an attractive approach (48). Our findings support further efforts to include IFNs or other agents that specifically activate STAT1 as a strategy to target the tumor vasculature and inhibit tumor growth.

Activation of STAT1 signaling enhances the inhibitory signal in the angiogenic balance between proangiogenic and antiangiogenic molecules. STAT1 negatively regulates angiogenesis by inhibiting activation of endothelial cells through a mechanism that involves blocking potent proangiogenic signal transduction and downstream biological effects. It is also likely that STAT1 induces expression of antiangiogenic molecules, such as IP-10 or IFI16, as well (14, 49). Previous studies ascribed a role for STAT1 in tumor angiogenesis through its signaling activity within tumor cells (50). For example, reconstitution of STAT1 in STAT1-null fibrosaracoma cells suppressed the metastatic potential of the cells and decreased the expression of the proangiogenic genes, *bFGF, MMP-2*, and *MMP-9*. The present study complements these findings by focusing on the function of STAT1 in endothelial cells. Taken together, STAT1 activation can regulate angiogenesis both within tumor cells and within cells located in the tumor environment. STAT1 inhibits tumor angiogenesis by suppressing the production of angiogenic factors by both tumor cells and endothelial cells. Agents that activate STAT1 could potentially target both the tumor cell as well as the tumor endothelium. Thus, enhancing the antiangiogenic signal through activation of STAT1 may represent a novel approach to cancer therapy.

Acknowledgments

Received 10/6/2005; revised 12/21/2005; accepted 2/2/2006.

Grant support: Claudia Adams Barr grant from the Dana-Farber Cancer Institute and the Brent Leahey Fund.

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We thank Dr. Roderick Bronson (Rodent Histopathology Core Facility, Harvard Medical School) for expert help with the histologic analysis and scoring of Matrigel plugs.

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