Interferon-γ Induces X-linked Inhibitor of Apoptosis-associated Factor-1 and Noxa Expression and Potentiates Human Vascular Smooth Muscle Cell Apoptosis by STAT3 Activation*5

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Interferon (IFN)-γ actions on the vessel wall play an important role in the pathogenesis of arteriosclerosis, yet the contribution of different IFN-γ signaling pathways to the phenotypic modulation of vascular smooth muscle cells (VSMCs) are poorly understood. We investigated the effects of IFN-γ on VSMCs and arteries through interactions involving signal transducer and activator of transcription (STAT) proteins. In addition to STAT1 activation, IFN-γ consistently phosphorylated STAT3 in human VSMCs but weakly or not at all in human endothelial cells or mouse VSMCs. STAT3 activation resulted in nuclear translocation of this transcription factor. By selectively inhibiting STAT3 and not STAT1 signaling, we identified a number of candidate IFN-γ-inducible, STAT3-dependent gene products by microarray analysis. Results for selected genes, including the pro-apoptotic molecules X-linked inhibitor of apoptosis associated factor-1 (XAF1) and Noxa, were verified by real time quantitative reverse transcription-PCR and immunoblot analyses. IFN-γ-induced STAT3 and STAT1 signaling in VSMCs demonstrated reciprocal inhibition. STAT3 activation by IFN-γ sensitized VSMCs to apoptosis triggered by both death receptor- and mitochondrial-mediated pathways. Knock down of XAF1 and Noxa expression inhibited the priming of VSMCs to apoptotic stimuli by IFN-γ. Finally, we confirmed the in vivo relevance of our observations using a chimeric animal model of immunodeficient mice bearing human coronary artery grafts in which the expression of XAF1 and Noxa as well as the pro-apoptotic effects induced by IFN-γ were dependent on STAT3. The data suggest STAT1-independent signaling by IFN-γ via STAT3 that promotes the death of human VSMCs.

Interferon (IFN-γ), the prototypical T helper 1 cytokine, plays a key effector role in the pathogenesis of inflammatory arteriosclerotic diseases, such as atherosclerosis and graft arteriosclerosis, through its actions on vascular cells (1). Binding of IFN-γ to its cognate receptor leads to activation of receptor-associated Janus kinase 1 (JAK1) and JAK2 and subsequent phosphorylation of the IFN-γ receptor. This results in recruitment, tyrosine phosphorylation, dimerization, nuclear translocation, and induction of gene expression by signal transducer and activator of transcription (STAT1) (2). Full transcriptional activity of STAT1 depends on canonical STAT1 signaling (4, 5), and the transcriptional effects of IFN-γ have been demonstrated in STAT1-deficient mice through undefined alternative pathways (6–8). Non-classical IFN-γ signaling via STAT3 has been described in certain cell types (9–14), including vascular smooth muscle cells (VSMCs) (15), but not in others. For example, IFN-γ-mediated STAT3 activation leads to induction of suppressor of cytokine signaling (SOCS3) in mouse embryonic fibroblasts, but not in human fibrosarcoma or lung adenocarcinoma cells (14), and of secretory group IIA phospholipase A2 in human VSMCs but not in human hepatoma cells (15). The phenotypic effects of STAT1-independent signaling by IFN-γ generally manifest as increased cellular growth and survival (6–8, 16), and in particular STAT3 signaling has been described to inhibit the pro-apoptotic effects of IFN-γ (11, 12, 17).

The responses of VSMCs to immune-mediated injury are central to the development of arteriosclerotic lesions. IFN-γ

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6832

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Table 1.

1 Recipient of a fellowship award from the Thoracic Surgery Foundation for Research and Education.

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4 The abbreviations used are: IFN, interferon; Ad, adenovirus; ASO, antisense oligonucleotide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IAPs, inhibitors of apoptosis; IP-10, IFN-γ-inducible protein of 10 kDa; JAK, Janus kinase; Mig, monokine induced by IFN-γ; MIS, mismatch; qRT-PCR, quantitative RT-PCR; siRNA, small interfering RNA; SOCS, suppressor of cytokine signaling; STAT, signal transducer and activator of transcription; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick end-labeling; VSMC, vascular smooth muscle cell; XAF1, X-linked inhibitor of apoptosis-associated factor-1; EGFP, enhanced green fluorescent protein; FACS, fluorescence-activated cell sorter.
IFN-γ Promotes VSMC Apoptosis by STAT3 Activation

may cause both proliferation and apoptosis of VSMCs with a net effect of an accumulation of neointimal VSMCs (1). Apoptosis of VSMCs are of particular relevance in vascular remodeling and plaque rupture. Apoptosis is a mode of programmed cell death characterized by morphological changes due to caspase activation. There are two fundamental ways by which caspases are activated during apoptosis; an intrinsically pathway of mitochondrial outer membrane permeabilization, which releases cytochrome c, and an extrinsic pathway involving the stimulation of death receptors (18). In the former case Bcl-2 homology 3-only-containing proteins, e.g. Puma and Noxa, act as mediators for cell stress and DNA damage by initiating mitochondrial outer membrane permeabilization, which results in assembly of the apoptosome and caspase 9 activation (19). Activated caspase 9, in turn, proteolytically activates caspase 3, leading to apoptosis. On the other hand, ligation of death receptors, e.g. Fas and receptors for tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), can activate apoptosis in healthy cells by ligand-induced formation of the death-inducing signaling complex which mediates autocatalytic activation of caspase 8. Active caspase 8 propagates death signals in two ways; that is, by cleavage of the Bcl-2 homology 3-only protein Bid, which leads to mitochondrial outer membrane permeabilization, and by direct proteolytic activation of caspase 3. Counteracting cellular apoptotic processes are factors involved in preventing unregulated cell suicide, the inhibitors of apoptosis (IAPs). IAPs bind directly to caspases and inhibit their function. X-linked inhibitor of apoptosis protein is the best characterized and the most potent member of this family. IAPs are themselves regulated by cellular proteins, including Smac/Diablo and X-linked inhibitor of apoptosis-associated factor-1 (XAF1), which inhibit IAP function and allow caspase-dependent cleavage of substrates (20). Inflammation may cause and/or potentiate cellular apoptosis, and it has been reported that XAF1 and Noxa are also IFN-β-stimulated genes that contribute to viral-induced cell death and type I IFN-dependent sensitization of human malignant cells to pro-apoptotic stimuli (21, 22). Here, we report that the IFN-γ induces expression of XAF1 and Noxa in human VSMCs through STAT3 activation and that these proteins sensitize VSMCs in vitro and in vivo to apoptosis triggered via death receptor or mitochondrial mechanisms.

EXPERIMENTAL PROCEDURES

Cell and Organ Culture—Human cells and tissues were obtained under protocols approved by the Yale Human Investigations Committee and the New England Organ Bank. Human VSMCs were isolated by explant outgrowth from the minced media of coronary arteries or aortae of cadaver organ donors or cardiac transplant recipients after removal of the adventitia and endothelium. The cells were serially cultured on 0.2% gelatin-coated, tissue culture-grade plastic in M199 media supplemented with 20% fetal bovine serum, 2 mmol/liter L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (Invitrogen). Porcine VSMCs were isolated from coronary arteries using the same protocol. Human endothelial cells were isolated by enzymatic harvesting from umbilical cord veins and serially cultured in supplemented M199 media also containing 50 μg/ml fibroblast growth factor-1 (Collaborative Research, Bedford, MA), and 100 μg/ml porcine intestinal heparin (Sigma-Aldrich). Cultured cells were used at passage 3 to 4, and a low concentration (0.5%) of fetal bovine serum-supplemented media was substituted for 48 h before treating with cytokine. Murine aorta from either wild type or STAT1−/−-deficient 129S6/SvEv mice (Taconic Farms, Germantown, NY) were cut into uniform size rings after removing the endothelium and incubated in 0.5% fetal bovine serum-supplemented M199 media. The cells or tissues were treated with human (Upstate Biotechnology, Lake Placid NY), pig (BioSource International, Camarillo, CA), or mouse (R&D Systems, Minneapolis, MN) IFN-γ, oncostatin M (R&D Systems), soluble homotrimeric TRAIL (R&D Systems), and staurosporine (Sigma-Aldrich).

Oligonucleotide Transfection—Antisense oligonucleotide (ASO) to human STAT3 and 5-base mismatch (MIS) control oligonucleotides (oligos) were a gift from ISIS Pharmaceuticals (Carlsbad, CA). The sequence for STAT3 ASO, 5'-GCT CCA TCT GCT GCT TC-3', and for the MIS control oligos, 5'-GCT CCA ATA CCC GTT GCT TC-3', were synthesized using phosphorothioate chemistry. The oligos were synthesized with 2'-O-methoxyethyl modification of the five terminal nucleotides to provide higher hybridization affinity as well as improved nuclease protection. Small interfering RNA (siRNA) for XAF1, Noxa, and a validated negative control without homology to known mammalian genes were from Qiagen (Valencia, CA). The sequence for XAF1 siRNA was: sense, CGG UGU GCA GGA ACU GUA A dTdT; antisense, UUA CAG UUC CUG CAC ACC G dAdG. The sequence for Noxa siRNA was: sense, CGG UCA CUA CAC AAC GUA A dTdT; antisense, UUA CGU UGU GUA GUG ACC G dAdG. Human VSMCs were transfected with ASO or MIS oligos at 1 μM in 1 ml of serum-free media containing 10 μl of FuGENE (Roche Diagnostics) or with siRNA at 0.02–2 μM in 1 ml of serum-free media containing 5 μl of Oligofectamine (Invitrogen) for 6 h and then quenched with serum-supplemented media. Transfection was repeated twice 48 h apart, and knockdown efficacy, cytokine signaling, or pro-apoptotic stimuli were assessed 48 h after the second transfection.

Viral Transduction—A 2.4-kilobase cDNA insert for STAT1β (16) was isolated by PCR from the expression plasmid pRcCMV (provided by Dr. Jacqueline F. Bromberg, Rockefeller University, New York, NY) and subcloned using a zero blunt TOPO PCR cloning kit (Invitrogen). An EcoRI-excisable STAT1β DNA insert was then subcloned into the pBMN-Z-I-Neo retroviral vector (provided by Dr. Garry P. Nolan, Stanford University, Stanford, CA). Retrovirus DNA containing either EGFP or STAT1β was directly transduced into the PA317-packaging cell line using Lipofectamine 2000 (Invitrogen). G418-resistant cells were selected and served as the source of retroviral stocks. VSMCs were transduced with retroviral vectors in the presence of Polybrene at 8 μg/ml for 6 h and then cultured in 20% fetal bovine serum-supplemented media. At 48 h after transduction, the cells were cultured in media containing G418 at 0.8 mg/ml for 2 weeks. In some experiments,
additional transductions were required to increase the efficacy of transgene expression.

**Immunoblotting Analysis**—Protein was extracted from cells or homogenized frozen artery tissue in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM sodium orthovanadate, 0.1% aprotinin, and 1 mM phenylmethylsulfonyl fluoride) and boiled in SDS sample buffer for 5 min. Nuclear extracts were prepared as previously described (23). Equal amounts of protein per sample were separated by SDS-polyacrylamide gel electrophoresis, transferred electrophoretically to a nitrocellulose membrane (Bio-Rad), and blotted with mouse monoclonal antibodies to STAT1 (Cell Signaling Technology, Beverly, MA), STAT3 (BD Biosciences), NOXA (Onco-gene Research Product, San Diego, CA and Abcam, Cambridge, MA), and β-actin (Sigma-Aldrich) with rabbit antibodies to STAT1, phosphotyrosine STAT1, phosphotyrosine STAT3, phosphoserine STAT3 (Cell Signaling Technology), JAK2 (Upstate Biotechnology and Chemicon International, Temecula, CA), SOCS3 (Immu-Biological Laboratories, Gunma, Japan), and XAF1 (Abcam) or with goat antibody to XAF1 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA). Detection of bound antibody was by enhanced chemiluminescence (Pierce).

**Enzyme-linked Immunosorbent Assays**— Supernatant levels of IFN-γ-inducible protein of 10 kDa (IP-10) and plasma levels of INF-γ were measured using enzyme-linked immunosorbent assay kits from R&D Systems (Minneapolis, MN) according to the manufacturer’s instructions.

**Real Time Quantitative RT-PCR**—qRT-PCR was performed as previously described (24). In brief, total RNA was isolated from VSMCs using RNAeasy mini kits with on-column DNase treatment (Qiagen). Real time qRT-PCR reactions were prepared with Taqman reagents (Applied Biosystems, Foster City, CA) for XAF1, Noxa, JAK2, SOC3, IP-10, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). An iCycler and its system interface software (Bio-Rad) were used to run samples and analyze data. All samples were analyzed in triplicate and normalized to GAPDH levels, and the results are expressed as -fold induction compared with untreated controls.

**Microarray Analysis**—Total RNA was isolated from cultured VSMCs under various conditions using RNAeasy mini kits (Qiagen) with on-column DNase treatment. Preparation of cRNA and hybridization to the human genechip set (U133A) was performed as described by the manufacturer (Affymetrix, Santa Clara, CA). The stained chips were read with a GeneChip Scanner 3000 (Affymetrix) and analyzed using Microarray Suite 5.0 image analysis software (Affymetrix). RNA levels were quantified, and gene-specific probe sets categorized as present or marginal were further analyzed, whereas those categorized as absent were excluded. GeneSpring 5.0 data analysis software (Silicon Genetics, Redwood, CA) was used for normalization and -fold change calculations. The microarray analyses were performed three times independently for each experimental condition. For the purposes of this study, genes that were regulated by IFN-γ more than 3-fold relative to untreated controls were also analyzed for the effects of STAT3 knockdown on IFN-γ-induced changes in transcript expression.

**Fluorescence Microscopy and FACS Analysis**—VSMC nuclear morphology was assessed by 4',6-diamidino-2-phenylindole dihydrochloride staining (Vector Laboratories, Burlingame, CA). Cell surface labeling of VSMCs was performed using a buffer solution and phycoerythrin-conjugated annexin V from BD Pharmingen according to the manufacturer’s instructions. For intracellular staining, VSMCs were fixed with 4% paraformaldehyde, permeabilized with 0.1% saponin, and then labeled with rabbit antibody to human cleaved caspase 3 (Cell Signaling Technology) or control IgG followed by fluorescein isothiocyanate-conjugated goat anti-rabbit IgG. Analysis was performed with a FACSort (BD Biosciences).

**Artery Grafting**—Segments of human epicardial coronary arteries were interposed into the infra-renal aortae of female, 8–12-week-old, “non-leaky” (serum IgG <1 µg/ml) C.B.-17 severe combined immunodeficient/beige mice (Taconic) using an end-to-end microsurgical anastomotic technique as previously described (25). Animal procedure protocols were approved by the Yale Animal Care and Use Committee. At 1 week post-operative, certain recipients were inoculated intravenously with replication-deficient (ΔE1/E3) adenovirus encoding transgenes for human IFN-γ (Ad-IFN-γ) or LacZ (Ad-LacZ) driven by a cytomegalovirus promoter (Qbiogene, Carlsbad, CA) at 1 x 10^9 plaque forming units. Plasma human IFN-γ levels were around 200 ng/ml at 1 and 4 weeks after Ad-IFN-γ transduction and undetectable after Ad-LacZ transduction. Certain recipients were also treated with oligos at 20 mg/kg subcutaneously 3 x per week from post-operative week 1 to 5.

**Graft Analysis**—Immunohistochemistry was performed as previously described (25) using primary antibodies to human major histocompatibility complex class I antigens (Dako, Carpinteria, CA), STAT3 (BD Biosciences), Noxa (Onco-gene Research Product), and XAF1 (Santa Cruz Biotechnology) as well as the 2E1 antibody (26) developed against ASO B (Ad-LacZ) driven by a cytomegalovirus promoter (Qbiogene, Carlsbad, CA) at 1 x 10^9 plaque forming units. Plasma human IFN-γ levels were around 200 ng/ml at 1 and 4 weeks after Ad-IFN-γ transduction and undetectable after Ad-LacZ transduction. Certain recipients were also treated with oligos at 20 mg/kg subcutaneously 3 x per week from post-operative week 1 to 5.

**Statistical Analysis**—Student’s t test and one-way analysis of variance were performed using the Prism software program (GraphPad Software, San Diego, CA). Differences with p < 0.05 were considered to indicate statistical significance.

**RESULTS**

IFN-γ-activated STAT3 in VSMCs—To determine whether STAT1-independent signaling by IFN-γ occurred in VSMCs, we first analyzed IFN-γ-treated cultured human VSMCs for activation of other STAT proteins. We found that IFN-γ-induced phosphorylation of STAT3 in addition to STAT1 in human coronary artery VSMCs cultured in media containing a low concentration (0.5%) of serum in a time- and dose-dependent fashion (Fig. 1, A and B). Tyrosine phosphorylation of
STAT3 showed similar kinetics to that of STAT1. IFN-γ-mediated STAT3 tyrosine phosphorylation was also seen in serum-deprived cultured VSMCs derived from human aortas and umbilical arteries and in human coronary artery and aorta segments maintained in organ culture in the complete absence of serum since procurement from donors (data not shown). These results demonstrated the general applicability of IFN-γ-induced STAT3 activation in human VSMCs and that it was not an artifact of withdrawal from growth factors contained in serum. There was minimal and transient serine phosphorylation of STAT3 in response to IFN-γ treatment of VSMCs (Fig. 1, A and B). STAT3 activation was confirmed by nuclear translocation of this transcription factor as compared with treatment of VSMCs with the cytokine, oncostatin M, which is known to signal via both STAT3 and STAT1 (Fig. 1 C).

STAT3 phosphorylation by IFN-γ was cell type-specific and was either not seen or occasionally weakly detected in human endothelial cells (Fig. 1 D), whereas STAT1 was consistently activated in the same experiments (data not shown). There was no intrinsic defect in STAT3 activation in human endothelial cells, as we have previously shown robust STAT3 phosphorylation in response to oncostatin M in this cell type (23). IFN-γ-mediated STAT3 activation in VSMCs was also species-specific as there was robust signaling in porcine VCMCs (Fig. 1 E), and although there was basal phosphorylation of STAT3 in STAT1-deficient mice, we could not detect further activation by IFN-γ in wild type or STAT-1-deficient mouse aortae (Fig. 1 F).

STAT3-dependent, IFN-γ-inducible Molecules in Human VSMCs—To investigate if STAT3 activation by IFN-γ resulted in transcription, we selectively inhibited STAT3 responses. Transduction of VSMCs with retroviral vectors encoding dominant negative constructs of STAT3Y705-F and STAT3Δβ (27, 28) were unsuccessful for selectivity or efficacy reasons (data not shown). We used an alternative approach to knock down STAT3 expression by ASO, which inhibited STAT3, but not STAT1, phosphorylation in response to IFN-γ (Fig. 2 A). This strategy was not completely effective, as further exposure of the immunoblots revealed low levels of STAT3 and phospho-STAT3 (data not shown).

We initially examined for transcriptional effects of STAT3 signaling by IFN-γ in human coronary artery VSMCs using microarray analysis. We identified 15 of 73 transcripts with >3-fold up-regulation or down-regulation by IFN-γ whose expression was partially repressed by ASO to STAT3 as compared with MIS control oligos (supplemental Table 1). These included the proapoptotic molecules XAF1 and Noxa and the signaling molecules JAK2 and SOCS3. In contrast, the great majority of IFN-γ-inducible transcripts, including the known STAT1-dependent molecule, IP-10, showed enhanced expression in the presence of STAT3 ASO versus MIS control oligos, suggesting competitive interactions between STAT3 and canonical STAT1 signaling by IFN-γ in VSMCs.

We verified selected microarray results of STAT3-dependent signaling by IFN-γ through further mRNA and protein analysis. Pretreatment of VSMCs with STAT3 ASO inhibited the IFN-γ-induced expression of XAF1, Noxa, JAK2, and SOCS3 but enhanced that of IP-10 (Fig. 2 B–F). The immunoblots for XAF1 and Noxa were of suboptimal quality due to reagent limitations, although similar results were obtained using two different antibodies in more than six independent experiments. Knockdown of STAT3 also increased the induction of other known STAT1-dependent molecules by IFN-γ, such as IFN regulatory factor-1, IFN-inducible T cell α chemoattractant, and monokine-induced by IFN-γ (Mig) (data not shown). These results further supported cross-regulation of STAT3 and STAT1 signaling by IFN-γ in VSMCs.
inhibitor that lacks the transactivation domain of functional STAT1α (16). STAT1β, thus, competes with STAT1α for phosphorylation in response to IFN-γ but does not result in IFN-γ transcriptional responses. We confirmed that STAT1β overexpression resulted in increased phosphorylation of STAT1 (in particular the lower molecular weight STAT1α component) by IFN-γ (Fig. 3B). STAT3 expression and phosphorylation of STAT3 by IFN-γ were minimally affected by STAT1β transduction (Fig. 3B). We verified the dominant negative effects of our construct in VSMCs as the IFN-γ-mediated up-regulation in transcript and protein expression for known STAT1-dependent molecules, such as IP-10 (Fig. 3, C and E), IFN regulatory factor-1, IFN-inducible T cell α chemoattractant, and Mig (data not shown), was inhibited by STAT1β overexpression. In contrast, the IFN-γ-mediated up-regulation of XAF1, Noxa, JAK2, and SOCS3 mRNA and protein expression was not diminished and was often enhanced in STAT1β-transduced versus EGFP-transduced VSMCs (Fig. 3, D and F). These results confirmed STAT1-independent signaling of IFN-γ in VSMCs.

STAT3 Signaling by IFN-γ Sensitized VSMCs to Death Receptor- or Mitochondrial-triggered Apoptosis—Because XAF1 and Noxa are known to be pro-apoptotic molecules (19, 20), we investigated whether STAT3 signaling by IFN-γ sensitized VSMCs to apoptotic stimuli. We assessed for apoptosis of low passage human coronary artery VSMCs by binding of phycoerythrin-conjugated annexin V to phosphatidylserine exposed on the outer leaflet of the plasma membrane early in programmed cell death. We verified cell death under our experimental conditions by propidium iodide uptake, a marker of membrane integrity that is a late-stage event common to both apoptosis and necrosis (data not shown).
IFN-γ Promotes VSMC Apoptosis by STAT3 Activation

Pilot experiments showed that STAT1 activation by IFN-γ similarly induced a pro-apoptotic phenotype of VSMCs, as transduction with STAT1β also inhibited the IFN-γ-mediated sensitization of VSMCs to TRAIL-induced apoptosis (Fig. 5F). Because the pro-apoptotic role of STAT1 signaling by IFN-γ has been previously well described (17), we focused the rest of our studies on STAT3-dependent pathways of apoptosis.

Induction of XAF1 and Noxa by IFN-γ Potentiated VSMC Apoptosis—To determine whether the induction of XAF1 and Noxa by IFN-γ played a role in the sensitization of VSMCs to pro-apoptotic stimuli, we used a siRNA approach. XAF1 and Noxa transcript and protein expression in IFN-γ-treated VSMCs were effectively knocked down using specific, but not control, siRNA at 0.2 μM (Fig. 6, A–D), and this dose was used for subsequent experiments. Similar to our experience with ASO knockdown of target molecules, the effects of siRNA were also only partially effective.

Inhibition of XAF1 expression prevented the priming of VSMCs to TRAIL-triggered apoptosis by IFN-γ (Fig. 6, E and G). Similarly, inhibition of Noxa expression diminished the sensitization of VSMCs to staurosporine-induced apoptosis by IFN-γ (Fig. 6, F and H). In Noxa, but not XAF1, knockdown experiments, IFN-γ induced a modest rate of apoptosis in VSMCs from 2 of 5 donors, and this direct cytokine effect was also inhibited by siRNA to Noxa. We confirmed our findings that IFN-γ augments death receptor- and mitochondrial-triggered apoptosis of VSMCs by induction of XAF1 and Noxa with additional siRNA sequences to these molecules (data not shown).

IFN-γ-induced XAF1 and Noxa Expression and Apoptosis of VSMCs in Vivo Was STAT3-dependent—We tested if our observations in human primary cells were relevant in vivo using a chimeric model of immunodeficient mice bearing human coronary artery grafts. To directly confirm the uptake of oligos by human artery grafts in this model, we administered an unrelated human ASO, 13920, for which an antibody, 2E1, has been developed. We found intense and specific staining within the arterial wall of the graft (Fig. 7A).

The recipients were transduced with replication-deficient adenovirus encoding transgenes for human IFN-γ or LacZ, and because the interactions of IFN-γ with its cognate recep-
IFN-γ Promotes VSMC Apoptosis by STAT3 Activation

FIGURE 4. IFN-γ sensitizes VSMCs to death receptor- or mitochondrial-triggered apoptosis. Human coronary artery VSMCs were treated with TRAIL at 40 ng/ml for 19 h (A) or staurosporine (Stau) at 0.5 μM for 19 h (B) either with or without cotreatment of IFN-γ at 100 ng/ml for 19 h or pretreatment or not with IFN-γ at 100 ng/ml for 72 h. Cellular apoptosis was assessed by annexin V binding by FACS analysis. The pooled results of % annexin V+ cells under the different conditions using VSMCs from multiple donors are shown (C). Data represent the mean ± S.E., n = 9, *p < 0.05 (analysis of variance). Apoptosis of VSMCs pretreated or not with IFN-γ at 100 ng/ml for 72 h and treated with or without TRAIL at 40 ng/ml for 19 h was also assessed by cleaved caspase 3 expression of permeabilized cells by FACS analysis (D). Ctrl, control.

We found unambiguous activation of STAT3 despite predominant STAT1 activation by IFN-γ in human VSMCs. Certain (30–33), but not all (34) studies of STAT signaling in murine cells have reported evidence for IFN-γ-induced STAT3 activation using electrophoretic mobility shift assays. More detailed studies of mouse embryonic fibroblasts by immunoblotting documented weak and transient IFN-γ-mediated tyrosine phosphorylation of STAT3 (compared with STAT1) in wild type mice that was much stronger and more prolonged in STAT1-deficient animals (12–14). In contrast, less STAT3 activation by IFN-γ was found in macrophages from STAT1-deficient versus wild type mice (8). Convincing evidence of STAT3 phosphorylation and binding to promoter elements in the presence of IFN-γ-induced STAT1 activation has been observed in certain human cell types, including VSMCs (15), hematopoietic progenitors (9, 10), neutrophils (10, 11), myelocytic leukemia cells (9, 11), adipocytes (35), hepatocytes (36), hepatoma cells (37), neuroblastoma cells (38), and synovial cells (39) but not endothelial cells (23), lymphocytes (11), or monocytes and eosinophils (10). However, IFN-γ has also been described to dephosphorylate constitutively activated STAT3 in tibular sclerosis complex-deficient mouse embryonic fibroblasts and human prostate cancer cells (40, 41). Part of the discrepancy in the literature may be explained by the cell type and species specificity of STAT3 activation by IFN-γ, as we found for vascular cells. The molecular basis for IFN-γ-mediated STAT3 signaling is poorly understood, although binding to the STAT1 docking site of the IFN-γ receptor and activation by Src non-receptor tyrosine kinases play a role (13, 14). Possible reasons for differential STAT3 signaling include the relative abundance of STAT proteins in different cells under various conditions combined with competition for limiting activation factors, such as binding to the IFN-γ receptor docking site. This mechanism may explain the aforementioned increased phosphorylation of STAT3 by IFN-γ in STAT1-deficient mice (12–14) and, conversely, the prolonged activation of STAT1 by interleukin-6 in STAT3-deficient animals (33).

We also identified a number of candidate IFN-γ-inducible, STAT3-dependent gene products in human VSMCs and verified the results for selected genes, notably the pro-apoptotic molecules XAF1 and Noxa and the signaling molecules JAK2 and SOCS3 by further transcript and protein analysis. The validity of our experimental approach is supported by other reports documenting SOCS3 as an IFN-γ-inducible, STAT3-dependent molecule (and IP-10 as a STAT1-regulated molecule) in STAT1-deficient mouse embryonic fibroblasts (13, 14). A similar IFN-γ/STAT3 regulatory pathway was found for C/EBPδ (13) and secretory group IIA phospholipase A2 (15), although XAF1, Noxa, and JAK2 have not been previously identified as IFN-γ-inducible, STAT1-independent genes in microarray analyses of murine cells (7, 8, 13). We did not identify C/EBPδ or secretory group IIA phospholipase A2, as IFN-γ-
inducible molecules in human VSMCs under our experimental conditions.

The concept of negative cross-regulation between STAT3 versus STAT1 signaling that we found in human VSMCs is well established in other cell types (42). For example, IFN-γ stimulation results in greater induction of STAT1-dependent transcripts, such as IP-10, Mig, and IFN regulatory factor (IRF1) in STAT3-deficient versus wild type mouse embryonic fibroblasts (33), whereas STAT3 overexpression leads to reduced IP-10, Mig, and IRF1 but increased SOCS3, mRNA in IFN-α-treated human monocytic cells (43). However, the induction of SOCS3 by IFN-γ is not consistently greater in STAT1-deficient mouse embryonic fibroblasts, as SOCS3 is regulated through both STAT1 and STAT3 signaling (13, 14). Although our experiments did not directly address the question, we did not observe significantly altered STAT1 phosphorylation after STAT3 knockdown at limited dose and time responses of IFN-γ.

Instead, our results are supportive of a model for diminished formation or function of transcriptionally active STAT1 homodimers in the presence of activated STAT3 (and vice versa), possibly by sequestration of STAT1 in STAT1:STAT3 heterodimers (43) and/or competition between STAT1 and STAT3 homodimers for binding to promoter elements or limiting transcription cofactors.

We describe XAF1 and Noxa as mediators of pro-apoptotic effects of IFN-γ in addition to several other reported IFN-γ-inducible, pro-apoptotic molecules (44). XAF1 and Noxa have been previously described as type I IFN-inducible molecules that promote apoptosis (21, 22). The modest, but statistically significant effects of Noxa siRNA on the IFN-γ + staurosporine-triggered intrinsic pathway of apoptosis may be due to incomplete knock down of Noxa or the redundant pro-apoptotic effects of other STAT3-dependent, IFN-γ-inducible molecules, such as XAF1. In contrast, XAF1 siRNA was more effective in preventing the IFN-γ + TRAIL-triggered extrinsic pathway of apoptosis, although we did not perform a direct comparison to Noxa in preventing apoptosis from mitochondrial-dependent mechanisms. Our finding that IFN-γ-mediated STAT3 signaling potentiates VSMC apoptosis is unexpected as STAT3 is usually regarded as an oncogene that enhances cellular proliferation and survival (45). Specifically, STAT3 activation inhibits IFN-γ-induced apoptosis in human neutrophils and in human cervical carcinoma cells that is associated with up-regulation of the anti-apoptotic molecules, Bel-xL, survivin, and cellular IAP2 as well as down-regulation of the pro-apoptotic molecule, Bax (12, 17). However, we did not detect regulation of these molecules by IFN-γ in human VSMCs in our microarray analysis. Similar to our findings, other investigators have also reported pro-apoptotic effects of STAT3 signaling in certain cells, including involving mouse mammary epithelial cells (46), SOCS3-deficient mouse embryonic fibroblasts (47), and wild type or Tyk2-deficient mouse B cells (48). The reasons for disparate effects of STAT3 on cell death are poorly understood as direct comparative studies have not been performed between cells that are sensitive versus resistant to dying in response to STAT3 signaling.
Generally, cell type-specific responses have been invoked as the explanation for different outcomes, although one group has proposed an “orchestrating model” in which contradictory signals elicited by cytokines are regulated by STAT3 to produce a specific effect in a target cell (49).

Our results also suggest a pro-apoptotic role for STAT1 signaling by IFN-\(\gamma\)/H9253 in human VSMCs similar to that described in other cell types (17, 44, 45). Although we demonstrate that STAT3 and STAT1 signaling reciprocally inhibit each other, there is no reason a priori why both signaling pathways cannot result in analogous cellular phenotypes through the regulation of different genes with similar functional effects. Our microarray data is consistent with this hypothesis as several genes with known pro-apoptotic effects (e.g. TRAIL, phospholipid scramblase, and oligoadenylate synthetases) were IFN-\(\gamma\)-inducible and STAT3-independent but likely STAT1-dependent (i.e. displayed increased expression in the presence of STAT3 ASO). In contrast, there were no apparent candidate anti-apoptotic genes that were identified as IFN-\(\gamma\)-inducible. Because STAT3 and STAT1 both have a pro-apoptotic phenotype, it is unlikely that STAT3 knockdown prevents IFN-\(\gamma\)-mediated sensitization to apoptotic agents in VSMCs by increasing STAT1 signaling. This suggests that the IFN-\(\gamma\)-mediated, STAT3-dependent pro-apoptotic effects were independent of STAT1 signaling. A limitation of our work is that we did

**FIGURE 6.** Induction of XAF1 and Noxa contributes to IFN-\(\gamma\)-mediated potentiation of VSMC apoptosis. Human coronary artery VSMCs were transfected with control (Ctrl) siRNA (open bars) or siRNA to XAF1 or Noxa (filled bars). Transfected VSMCs with different concentrations of siRNA were treated with or without IFN-\(\gamma\) at 100 ng/ml for 6 h, and XAF1/GAPDH mRNA or Noxa/GAPDH mRNA were determined by qRT-PCR and normalized to untreated control cells (A and B). Alternatively, transfected VSMCs were treated with IFN-\(\gamma\) at 100 ng/ml for 72 h, and the expression of XAF1 and Noxa was determined by immunoblotting (C and D). Apoptosis of VSMCs transfected with 0.2 \(\mu\)M siRNA was assessed by annexin V binding by FACS analysis (E and F), and pooled results of % annexin V+ cells from multiple donors are shown (G and H). Data represent the mean ± S.E., \(n = 4\) for XAF1 siRNA and \(n = 6\) for Noxa siRNA; *, \(p < 0.05\) treated versus untreated; **, \(p < 0.05\) XAF1 or Noxa siRNA versus Ctrl siRNA (analysis of variance).
not directly contrast the roles of STAT1 and STAT3 signaling in our systems using analogous loss-of-function strategies; rather, we focused on the novel observations regarding STAT3-dependent pro-apoptotic effects of IFN-γ/H9253 in human VSMCs.

We find that IFN-γ has inconsistent effects on cultured VSMC apoptosis when used as a single agent but reproducibly potentiates death induced by other agents. Similar effects have been described for IFN-γ sensitization of human VSMCs to apoptosis triggered by Fas ligand (50) and for IFN-β-induced cell death through XAF1 and Noxa (21, 22).

Variable rates of apoptosis of primary cells from different human donors add to the complexity of the results (51). The in vivo relevance of our findings of VSMC apoptosis are limited by relatively small effects, and this is partly a technical issue in that apoptotic cells disappear, unlike dividing cells, which accumulate. Although differences in the rate of cellular proliferation or apoptosis can be biologically significant, there are a number of caveats in interpreting the studies of STAT3 activation by IFN-γ. These include the analysis of growth-dysregulated cells, such as malignant cell lines or mouse embryonic fibroblasts, the use of undefined growth factors contained in serum, the impact of serum withdrawal on cultured cells, the cell-specific responses, and the species-restricted effects. We address some of these concerns by utilizing an in vivo humanized animal model, although new variables of chronic IFN-γ treatment are introduced, e.g. autocrine cytokine loops (2) and up-regulation of STAT3 (13, 52).

In conclusion, our findings indicate that although STAT1 is predominantly activated by IFN-γ/H9253 in human VSMCs, simultaneous activation of STAT3 occurs and is biologically significant since it inhibits STAT1 signaling, results in transcription of multiple genes, including pro-apoptotic molecules, and leads to sensitization of VSMCs to death receptor- and mitochondrial-mediated apoptosis. The counter-regulation of STAT1/STAT3 signaling by IFN-γ/H9253 in VSMCs has important implications for the pathogenesis and complications of arteriosclerosis, a disease process in which this cytokine and cell type are of great relevance.

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IFN-γ Promotes VSMC Apoptosis by STAT3 Activation

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