Interferon- γ Induces Fas Trafficking and Sensitization to Apoptosis in Vascular Smooth Muscle Cells via a PI3K- and Akt-Dependent Mechanism

Dalya Rosner,* Victoria Stoneman,*
Trevor Littlewood,* Nicola McCarthy,*
Nichola Figg,* Yinong Wang,[†] George Tellides,[†]
and Martin Bennett*

From the Division of Cardiovascular Medicine,* University of Cambridge Clinical School of Medicine Addenbrooke's Centre for Clinical Investigation, Addenbrooke's Hospital, Cambridge, United Kingdom; and the Interdepartmental Program in Vascular Biology and Transplantation,[†] Boyer Center for Molecular Medicine, Department of Surgery, Yale University School of Medicine, New Haven, Connecticut

Vascular smooth muscle cell (VSMC) apoptosis occurs in advanced atherosclerotic plaques where it may contribute to plaque instability. VSMCs express the death receptor Fas but are relatively resistant to Fas-induced apoptosis due in part to the intracellular sequestration of Fas. Although inflammatory cytokines such as interferon (IFN)- γ present in plaques can prime VSMCs to FasL-induced death, the mechanism of this effect is unclear. We examined Fas expression and FasL-induced apoptosis in human VSMCs in response to IFN- γ . IFN- γ induced Fas trafficking to the cell surface within 24 hours, an effect that required Jak2/Stat1 activity. IFN- γ also stimulated Akt activity, and both Fas trafficking and Stat1 activation were inhibited by blocking PI3K, Akt, or Jak-2. IFN- γ increased Fas-induced apoptosis *in vitro* by $46 \pm 8\%$ (mean $\pm$ SEM, $P = 0.04$), an event that could be abrogated by inhibition of PI3K, Akt, or Jak-2. IFN- γ also increased Fas-induced apoptosis *in vivo* 7.5- to 15-fold ($P < 0.05$) in human arteries transplanted into immunodeficient mice, accompanied by increased Fas and phospho-Ser⁷²⁷-Stat1. We conclude that IFN- γ primes VSMCs to Fas-induced apoptosis, in part by relocation of Fas to the cell surface, a process that involves PI3K, Akt, and Jak-2/Stat1. IFN- γ present in plaques may co-operate with FasL to induce VSMC apoptosis in atherosclerosis. (*Am J Pathol* 2006, 168:2054–2063; DOI: 10.2353/ajpath.2006.050473)

Apoptosis of vascular smooth muscle cells (VSMCs) has been implicated in both normal vascular development and disease states.¹ VSMC apoptosis occurs after vessel injury, in remodeling, and in advanced atherosclerotic plaques.^{2–4} VSMC apoptosis is increased in unstable versus stable angina patients⁵ and is a feature of plaques that have a propensity to rupture. Indeed, direct induction of apoptosis can contribute to rupture of mouse plaques in association with other stimuli such as hemodynamic stress.⁶

Multiple triggers of apoptosis exist in the complex microenvironment of the plaque, and the induction of apoptosis reflects the balance between diverse pro- and anti-apoptotic signaling.⁷ However, increasing evidence implicates the tumor necrosis factor receptor (TNF-R) family of death receptors in plaque VSMC apoptosis.⁷ TNF-R1 (p55), Fas, and death receptors (DR)-3, -4 (TRAIL R1) and -5 (TRAIL R2) all comprise an extracellular domain, a hydrophobic transmembrane domain, and a cytoplasmic tail containing the death domain, a protein motif responsible for protein:protein interactions with adapter molecules.⁸ Ligand binding recruits adapter molecules to the receptor (FADD to Fas, TRADD to TNF-R1, or RIP to both) that then activate the caspase cascade leading to apoptosis.⁸ Although the ligands TNF- α and TRAIL are widely expressed, Fas ligand (FasL) expression is more restricted, particularly to lymphocytes and monocytes/macrophages.

Fas is expressed in human atherosclerotic plaques and co-localizes with regions of apoptosis,⁹ suggesting that VSMC apoptosis is regulated in part through Fas ligation. In contrast to many cell types, Fas/TNF-R1 are

Supported by the British Heart Foundation (grant PG/2001048), the National Institutes of Health (grant PO1 HL70295-01), and by the MRC Cambridge-Yale Program for Cardiovascular Research.

Accepted for publication March 7, 2006.

Address reprint requests to Martin Bennett, Division of Cardiovascular Medicine, University of Cambridge Clinical School of Medicine Addenbrooke's Centre for Clinical Investigation, Box 110, Addenbrooke's Hospital, Cambridge, CB2 2QQ, UK. E-mail: mrb@mole.bio.cam.ac.uk.

sequestered internally in the Golgi in VSMCs,¹⁰ rendering the cells relatively resistant to Fas-induced apoptosis without additional priming.¹¹ VSMC apoptosis consistently localizes to areas of high inflammatory content,¹² suggesting that inflammatory cells (macrophages and T lymphocytes) either directly induce VSMC apoptosis and/or produce cytokines that prime VSMCs for apoptosis by other stimuli. Indeed, monocytes/macrophages can directly induce VSMC apoptosis via Fas or TNF-R1,^{13–15} and nitric oxide,¹⁴ oxidized low-density lipoprotein,¹⁶ free radicals,¹⁷ and cytokines such as interleukin-1 β , TNF- α , and interferon (IFN)- γ ⁹ released from inflammatory cells can sensitize VSMCs to apoptosis. Fas itself can be trafficked to the cell surface after specific stimuli, including FasL binding, p53 activation and/or stabilization,¹⁰ and administration of nitric oxide donors.¹⁴ Trafficking of Fas to the cell surface may thus be an important mechanism in priming VSMCs for death.

The T-lymphocyte-derived cytokine IFN- γ has been reported to traffic Fas to the surface of some cells that contain predominantly intracellular Fas.¹⁸ In atherosclerotic plaques, CD4-positive T lymphocytes express markers of activation *in vivo* and secrete IFN- γ on activation *in vitro*.¹⁹ IFN- γ can increase neointima formation in human arteries,²⁰ and genetic deficiency is associated with reduced levels of atherosclerotic plaque formation in low-density lipoprotein receptor knockout mice.²¹ We therefore examined whether IFN- γ sensitizes VSMCs to Fas-induced apoptosis, focusing on the ability of IFN- γ to traffic Fas, the signaling pathways involved, and the functional outcome *in vitro* and *in vivo*.

Materials and Methods

Cell Culture

Human VSMCs were isolated from aortas of patients undergoing cardiac transplantation, after informed consent and approval from the local ethical review committee. Cells were identified as VSMCs by immunocytochemistry for α -smooth muscle cell actin and calponin. VSMCs were cultured in 20% fetal calf serum or 1% fetal calf serum with penicillin/streptomycin/glutamine (all from Sigma, St. Louis, MO). Exponentially growing, subconfluent VSMCs were stimulated with 500 U/ml human IFN- γ (Sigma) for 24 hours. The following inhibitors were added 30 minutes before IFN- γ stimulation unless otherwise stated: LY-2940002 (Ly, 10 μ M/L), brefeldin A (BFA, 5 μ g/ml), actinomycin D (1 μ g/ml), tyrphostin AG490 (AG490, 10 μ M/L) (all from Sigma); SH5 (20 μ M/L; Calbiochem, La Jolla, CA).

VSMCs Expressing Dominant-Negative Akt

Human VSMCs were infected at 400 multiplicity of infection with purified control Rad60 adenovirus or an adenovirus encoding DN-Akt²² (kindly provided by Dr. Ken Walsh, Boston, MA). Cells were harvested at 48 hours and lysates prepared to determine the expression of

various proteins, Akt kinase activity, or cell surface Fas (see below).

In Vitro Kinase Assay

Determination of Akt kinase activity was performed using a nonradioactive assay kit as described by the manufacturer (Cell Signaling, Beverly, MA). Briefly, Akt was immunoprecipitated from cell lysates and used to phosphorylate a recombinant GSK-3 fusion protein. Phosphorylation of the GSK-3 target protein was determined by Western blotting with a phospho-GSK-3 α/β (S21/9) antibody.

Flow Cytometry

VSMCs were plated in six-well plates for 48 hours before IFN- γ stimulation. Stimulated cells were harvested, washed, and incubated for 1 hour in 3% goat serum/phosphate-buffered saline (PBS) plus 5 μ g/ml of anti-human Fas (CH-11; Upstate Technology, Lake Placid, NY) or mouse IgM isotype control (Sigma). Cells were washed, stained for 30 minutes with 10 μ g/ml of fluorescein isothiocyanate anti-mouse IgM (Sigma) in 3% goat serum/PBS, and then washed again. Events ($n = 5000$ to 10,000) were collected for each sample using a FACS-Calibur flow cytometer (Becton-Dickinson, San Jose, CA) and data analyzed on WinMDI software.

Western Blotting

Lysis of cells in RIPA buffer, electrophoresis on sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels, and Western blotting were as described previously.²³ One μ g/ml of the following primary antibodies were used: rabbit anti-human Fas (Ab-1) (Oncogene, Boston, MA); mouse anti-Stat1 (SM1) and anti-phospho-Ser⁷²⁷ Stat1 (PSM1) (both Abcam); rabbit anti-Akt, anti-phospho-Ser⁴⁷³ Akt, and anti-Stat3; and mouse anti-phospho-Ser⁷²⁷ Stat3 (all Cell Signaling Technology). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies were used at 1:2500 (Amersham, Arlington Heights, IL). ECL or ECL⁺ kits (Amersham) were used for chemiluminescent detection, as per the manufacturer's protocol.

Detection of Cell Surface Fas

Cell surface Fas was detected by biotinylation and purification of cell surface proteins. Human VSMCs were cultured in the absence or presence of 500U/ml human IFN- γ for 24 hours with or without pretreatment for 30 minutes with 5 μ g/ml of BFA. Cells were washed with phosphate-buffered saline and cell surface proteins biotinylated by exposure to sulfo-NHS-SS-biotin for 30 minutes at 4°C as described by the manufacturer (Pierce, Rockford, IL). After quenching the reaction, cells were harvested and lysed, and biotinylated proteins were recovered by incubation with immobilized NeutrAvidin gel (Pierce) for 60 minutes at room temperature. The NeutrA-

vidin column was washed extensively, and biotinylated proteins were eluted with Laemmli sample buffer and analyzed by Western blotting.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) for Fas

Cells were grown in six-well culture plates and washed with Dulbecco's modified Eagle's medium before RNA extraction with RNA-Stat 60 isolation reagent (AMS Biotechnology Ltd., UK), and cDNA was synthesized using SuperRT (HT Biotechnologies, UK). PCR for Fas used the primers 5'-TGGCTTTGTCTTCTTTG-3' (forward) and 5'-TCATCTATTTGGCTTCATTG-3' (reverse), and PCR for β -actin used the primers 5'-GAAACTACCTTCACTC-CATC-3' (forward) and 5'-CGAGGCCAGGATGGAG-CCGCC-3' (reverse).

Time Lapse Microscopy

Apoptosis was scored morphologically throughout time, as described previously.²⁴ Cells were stimulated with IFN- γ $\pm$ AG490, Ly, or SH5 for 24 hours in low (1%) serum media. Membrane-bound FasL (1:2000; Upstate Technology) was then added for a further 24 hours to assess Fas-mediated apoptosis. To control for effects of inhibitors and IFN- γ that were not Fas-specific, pre-stimulated cells were also filmed but without addition of FasL. Results are expressed as the mean $\pm$ SE. Statistical significance was assessed by two-tailed Student's *t*-test.

Human/Mouse Transplantation

Human artery segments were interposed into the infrarenal aorta of C.B-17 SCID/beige mice (Taconic Farms, Germantown, NY) using an end-to-end anastomotic technique as previously described²⁰ and approved by the Yale Human Investigation and Animal Care Committees. Human IFN- γ (or saline in controls) was administered subcutaneously at 1 μ g, three times per week, for 4 weeks, starting 1 week postoperatively. Mouse IgM anti-human Fas monoclonal antibody (CH11) or an isotype-matched control antibody was injected once subcutaneously at 125 μ g with the last dose of IFN- γ . The recipients were sacrificed 24 hours after treatment with the antibody and the last dose of cytokine. The grafts were removed and stored at -80°C until analysis.

Frozen sections were cut, fixed with acetone, and blocked in 5% serum. Sections were then incubated with anti-Fas (1:400, CH11; Upstate), anti-cleaved caspase 3 (1:100, ASP175; Cell Signaling Technologies), mouse anti-Stat1 (SM1) and anti-phospho-Ser⁷²⁷ Stat1 (PSM1) (both 1:100, Abcam) or anti- α -smooth muscle actin (1:400, 1A4; DAKO, Carpinteria, CA). After washing, sections were incubated with their appropriate biotinylated secondary antibody. Binding was detected after incubation with ABCComplex (DAKO) and diaminobenzidine solution (DAKO).

Figure 1. IFN- γ increases surface but not total Fas levels in VSMCs. **A:** Flow cytometric analysis of surface Fas in VSMCs either stimulated with IFN- γ for 24 hours or unstimulated (Unstim) were used as a control. **B:** Western blot for Fas in three separate aortic VSMC cultures (2A, 9A, 12A) stimulated with IFN- γ for 24 hours. β -Actin expression is shown as a loading control.

Results

IFN- γ Stimulation Increases Surface Levels of Fas in VSMCs

To examine the effect of IFN- γ on Fas-induced apoptosis in VSMCs, we first examined surface Fas levels in normal human aortic VSMCs after stimulation with IFN- γ . Treatment with 500 IU/ml IFN- γ increased surface Fas expression detected by flow cytometry at 24 hours (Figure 1A) by $103 \pm 6\%$ (mean $\pm$ SEM, $n = 25$, $P = 0.002$). Total Fas expression was unchanged by IFN- γ treatment in whole cell lysates examined by Western blotting (Figure 1B) and in permeabilized cells examined by flow cytometry (data not shown). This suggests that by 24 hours IFN- γ increases surface expression without increasing total Fas expression in VSMCs.

IFN- γ Causes Relocalization of Fas to the Cell Surface

Once on the cell surface, Fas can either be shed (creating soluble Fas) or internalized after FasL binding or during constitutive recycling. The increase in surface but not total Fas suggests that IFN- γ treatment either induces Fas movement from internal stores or reduces internalization. We therefore treated VSMCs with BFA, an agent

Figure 2. Fas is trafficked to the cell surface by IFN- γ . **A:** Flow cytometric analysis of surface Fas in VSMCs stimulated with IFN- γ for 24 hours $\pm$ 30 minutes pretreatment with BFA. Untreated (Unstim) VSMCs were used as controls ($n = 5$). **B:** RT-PCR for Fas or control (β -actin) in VSMCs treated for 24 hours with BFA, IFN- γ , both, or unstimulated. **C:** Western blot for Fas, FADD, caspases 8 and 3, and BCL-X_L in VSMCs treated for 24 hours with BFA, IFN- γ , both, or unstimulated. **D:** Western blot for Fas or control (annexin II) in total, cytoplasmic (flow through) or cell surface fractions of VSMCs treated for 24 hours with BFA, IFN- γ , both, or unstimulated. Note that all cell surface fractions have been concentrated 10-fold compared to total and flow-through fractions.

that blocks protein transport to and from the Golgi. BFA treatment alone slightly reduced surface Fas levels, suggesting that there is continuous movement of Fas from the Golgi to the surface (Figure 2A). However, pretreatment with BFA completely inhibited the IFN- γ -induced up-regulation of surface Fas, suggesting that this effect requires translocation of intracellular Fas from the Golgi to the cell surface. Although BFA altered the subcellular localization of Fas (as demonstrated previously¹⁰), it had no effect on overall cell morphology or arrangement of contractile filaments. BFA treatment did not alter total Fas mRNA expression as assessed by RT-PCR (Figure 2B) or Fas protein expression in VSMCs (Figure 2C), confirming that BFA-induced reduction in surface receptor levels is because of alterations in trafficking rather than protein synthesis or degradation. In addition, neither IFN- γ or BFA affected expression of other components that regulate or mediate Fas signaling, including FADD, caspases 8 and 3, or BCL-X_L (Figure 2C).

To further demonstrate that IFN- γ increases cell surface Fas expression, we treated cells with IFN- γ $\pm$ BFA and analyzed cell surface proteins by biotinylation and purification using an avidin column. We analyzed the total

expression, the cytoplasmic expression (flow through), or surface expression (bound and eluted) of Fas in lysed cells. IFN- γ increased the cell surface expression of Fas, without changing total or cytoplasmic expression. The increase in surface Fas was partially inhibited by BFA (Figure 2D), consistent with its effect on blocking trafficking.

Transcription and Activation of Stat1 Are Required for IFN- γ -Stimulated Fas Trafficking

IFN- γ regulates apoptosis transcriptionally after sequential phosphorylation of the Janus family of tyrosine kinases (Jaks) and Stat family of transcription factors.²⁵ IFN- γ can sensitize cells to Fas-induced apoptosis via activation of Stat1 and its transcriptional target IRF-1.^{26,27} In addition, Stat1 has recently been demonstrated to modulate p53, a molecule we previously showed triggers Fas trafficking in VSMCs.¹⁰ Specifically, Stat1 co-activates p53 and reduces levels of Mdm2, an endogenous inhibitor of p53,²⁸ and both Stat1 and Stat3 have been implicated in control of Fas promoter activity. We therefore investigated the requirement for Stat-mediated transcription in IFN- γ -induced Fas trafficking.

Although total levels of Fas did not change after IFN- γ treatment, pretreatment of VSMCs with the transcriptional inhibitor actinomycin D before IFN- γ stimulation blocked surface Fas up-regulation (Figure 3A), despite a small increase in surface Fas with actinomycin D alone. This indicates that transcription of other IFN- γ signaling components is required for trafficking. We used tyrphostin AG490, a specific Jak2 inhibitor to examine Jak/Stat signaling after IFN- γ in VSMCs. Tyrphostin AG490 also blocked the surface Fas increase after IFN- γ stimulation (Figure 3B), without altering total Fas levels (Figure 3C), despite inhibiting both basal and IFN- γ -induced phosphorylation of Jak2 (Figure 3C). The main Stat family members targeted after IFN- γ receptor ligation are Stat1 and Stat3. Western blotting showed no significant changes in protein levels or phosphorylation status of Stat3 after IFN- γ stimulation or AG490 treatment (Figure 3C). However, IFN- γ stimulation increased Stat1 phosphorylation at Ser⁷²⁷ by $120 \pm 8\%$ (mean $\pm$ SEM, $P < 0.003$, $n = 3$) and also increased total Stat1 expression by $90 \pm 5\%$ (mean $\pm$ SEM, $P = 0.02$, $n = 3$), which could be inhibited by AG490 (Figure 3C). Thus, Jak2 activity is required for Fas trafficking, acting via transcription and phosphorylation of Stat1 (but not Stat3).

PI3K and Akt Are Required for IFN- γ -Stimulated Stat1 Phosphorylation and Fas Trafficking

In addition to Jak-Stat signaling, IFN- γ activates a number of other pathways, including PI3K and MAPK signaling. IFN- γ induced phosphorylation of the PI3K target Akt by $190 \pm 12\%$ (mean $\pm$ SEM, $P = 0.002$, $n = 3$) without increasing total Akt (Figure 4A). Inhibition of PI3K with LY294002 (LY) blocked the IFN- γ -induced increase in

Figure 3. IFN- γ -induced Fas relocation is associated with Stat1 activation. **A:** Flow cytometric analysis of surface Fas in VSMCs treated with actinomycin D starting 30 minutes before 24 hours of stimulation with IFN- γ . Untreated (Unstim) VSMCs were used as controls ($n = 4$). **B:** Flow cytometric analysis of surface Fas in VSMCs treated with AG490 starting 30 minutes before 24 hours of stimulation with IFN- γ . Unstimulated (Unstim) VSMCs were used as controls ($n = 6$). **C:** Western blot for Fas, phospho-Jak2, phospho-Ser⁷²⁷ Stat1, Stat1, phospho-Ser⁷²⁷ Stat3, or Stat3 in VSMCs treated for 24 hours with IFN- γ , AG490, both, or unstimulated (Unstim).

phospho-Ser⁴⁷³ Akt, suggesting that IFN- γ regulates Akt phosphorylation through PI3K activation.

We next asked whether PI3K or Akt were involved in Fas trafficking. Pretreatment of VSMCs with inhibitors of PI3K (LY) or Akt (SH5), inhibited up-regulation of surface Fas after IFN- γ stimulation, either completely (LY) or partially (SH5) (Figure 4B). Western blotting showed that treatment with these inhibitors caused no changes in total Fas levels that could account for the reduction in surface Fas (Figure 4C). This suggests that Fas trafficking in response to IFN- γ is at least partially regulated by PI3K through its downstream target Akt.

Because PI3K and Akt have both been implicated in the serine phosphorylation of Stats,^{29–31} we examined their effects on Stat1 activation. Western blotting demonstrated that LY and SH5 reduced both Ser⁷²⁷ phosphorylation of Stat1 and total Stat1 up-regulation after IFN- γ stimulation (Figure 5A), suggesting that the PI3K/Akt pathway may induce Fas trafficking by regulating Stat1. LY and SH5 also reduced basal expression of Ser⁷²⁷ Stat1, but LY and SH5 did not reduce IFN- γ -induced Jak2 phosphorylation (data not shown), suggesting that their effect on Stat 1 does not require Jak2.

Although SH5 inhibits the activation of Akt without decreasing phosphorylation of PDK-1 or other kinases downstream of Ras, such as MAPK, the specificity of this agent is not completely clear, and it may have other

Figure 4. PI3K and Akt regulate Fas trafficking. **A:** Western blots for Akt or phospho-Ser⁴⁷³ Akt in VSMCs treated for 24 hours with IFN- γ ± LY. Unstimulated cells or cells treated with LY alone were used as controls. **B:** Flow cytometric analyses of surface Fas in VSMCs treated with LY ($n = 7$) or SH5 ($n = 6$) starting 30 minutes before 24 hours of stimulation with IFN- γ . Unstimulated (Unstim) VSMCs or cells treated with inhibitor alone were used as controls. **C:** Western blot for Fas or control (β -actin) protein expression in VSMCs treated with LY or SH5 starting 30 minutes before 24 hours of stimulation with IFN- γ .

targets in addition to Akt.³² To further investigate whether Akt is involved in IFN- γ -mediated Fas trafficking, we infected VSMCs with an adenovirus encoding HA-tagged dominant-negative Akt (DN-Akt) or a control adenovirus vector (Rad60) containing no transgene. Akt activation and kinase activity were examined at 48 hours. Expression of DN-Akt inhibited Akt phosphorylation and kinase activity as measured by the ability of immunoprecipitated Akt to phosphorylate GSK-3, a known Akt target (Figure 5B). Phosphorylation of Stat1 in response to IFN- γ was reduced, although IFN- γ still induced total Stat1 in VSMCs expressing DN-Akt (Figure 5B). By densitometry, the ratio of P-Stat1 to total Stat1 was reduced by ~40% in DN-Akt-expressing cells relative to Rad 60-infected cells, suggesting that Akt activation is involved in Stat1 phosphorylation. Consistent with this, DN-Akt inhibited the IFN- γ -mediated increase in surface Fas in VSMCs compared with control virus infection (Figure 5C).

IFN- γ Increases FasL-Induced Apoptosis in Vitro and in Vivo

Although IFN- γ increases surface Fas by trafficking from internal stores, this does not necessarily mean that Fas-

Figure 5. PI3K and Akt regulate Stat1. **A:** Western blots for phospho-Ser⁷²⁷ Stat1 and Stat1 in VSMCs pretreated with LY or SH5 for 30 minutes before 24 hours of stimulation with IFN- γ . **B:** Western blot for total or phosphorylated Akt (Ser⁴⁷³), total or phosphorylated Stat1 (Ser⁷²⁷) and HA, or *in vitro* Akt kinase assay on the Akt substrate GSK-3 in human VSMCs infected with control adenovirus (Rad60) or adenovirus expressing DN-Akt, in the presence (+) or absence (-) of IFN- γ for 24 hours. **C:** Flow cytometric analysis of surface Fas or IgM isotype control in human VSMCs infected with control adenovirus (Rad60) or adenovirus expressing DN-Akt after IFN- γ treatment ($n = 4$).

induced apoptosis is potentiated by IFN- γ . For example, we have previously demonstrated that Fas-induced apoptosis of human VSMCs is also regulated by differential expression of proteins involved in apoptosis signaling/regulation, including IAPs, Bcl-2 family members, and caspases.¹¹ To determine whether IFN- γ enhances Fas-induced apoptosis *in vitro*, we examined apoptosis morphologically by time lapse videomicroscopy. Unstimulated and IFN- γ -stimulated cells were left untreated or exposed to FasL. Unstimulated cells had a low basal level of apoptosis, which increased twofold when FasL was added ($P = 0.04$). IFN- γ enhanced FasL-induced apoptosis by $46 \pm 8\%$ (mean $\pm$ SEM, $P = 0.04$) (Figure 6A). When IFN- γ -induced surface Fas up-regulation was blocked with AG490, LY, or SH5, enhancement of FasL-induced apoptosis was abrogated ($P = 0.03$, 0.01 , and 0.06 respectively). Treatment with IFN- γ (Figure 6A) or the inhibitors alone (data not shown) did not affect basal apoptosis. This demonstrates that IFN- γ -induced up-regulation of surface Fas potentiates Fas-mediated apoptosis *in vitro*, and this potentiation may be mediated by PI3K, Akt, and Jak/Stat signaling.

To examine whether IFN- γ could also potentiate Fas-mediated apoptosis *in vivo*, we used a previously described xenotransplantation model in which a segment of human artery is transplanted into immunodeficient SCID/beige mice.²⁰ IFN- γ treatment of these mice results in neointima formation after 4 to 6 weeks of treatment in the grafted segment only. This model allows study of the direct effects of human IFN- γ together with FasL on human VSMCs in intact vessels, because both their receptors exhibit a high degree of species specificity, without confounding secondary effects from the mouse immune system. Importantly, the neointima in these grafts consists entirely of VSMCs,²⁰ removing the effects of Fas activation in other cells. Mice were grafted with human mammary artery and treated for 4 weeks with IFN- γ or saline control. IFN- γ levels *in vivo* were measured using enzyme-linked immunosorbent assay. The recombinant human IFN- γ from Upstate Technology has a specific activity of 3.2 U/ng. For most *in vitro* applications, IFN- γ is biologically active from 0.05 to 10 ng/ml. *In vivo*, 1-hour peak levels in serum were 282 to 490 pg/ml, within the biologically active range. Mice then received either 125 μ g of agonistic anti-Fas antibody or control antibody for 24 hours. Expression of Fas, Stat1, phospho-Ser⁷²⁷-P-Stat1, and cleaved caspase 3 (a marker of apoptosis) was examined in grafted segments by immunohistochemistry. Fas expression was detectable in $1.2 \pm 0.2\%$ (mean $\pm$ SEM) of intimal VSMCs by immunohistochemistry in control grafts. In contrast, IFN- γ treatment increased neointima formation as previously described²⁰ and resulted in detectable Fas expression in $12.6 \pm 2.3\%$ (mean $\pm$ SEM, $P < 0.05$) of intimal VSMCs (Figure 7, C and D). Control, IFN- γ alone, or anti-Fas antibody-treated grafts showed $0.1 \pm 0.03\%$, $0.15 \pm 0.1\%$, and $0.2 \pm 0.1\%$ (mean $\pm$ SEM) cleaved caspase 3-positive cells. In contrast, the combination of IFN- γ and agonistic anti-Fas antibody resulted in a 7.5- to 15-fold increase in cleaved caspase 3-positive neointimal cells (Figure 7E) ($1.5 \pm 0.1\%$; mean $\pm$ SEM, $n = 4$, $P < 0.05$). Cleaved caspase 3 was only detected in cells with pyknotic nuclei, confirming that this represented apoptosis (Figure 7E). We also examined serial sections with antibodies to CD68, CD3, and α -smooth muscle actin (SMA); this confirmed that the intima was composed only of SMA-positive cells (Figure 7F). Thus, all apoptotic cells seen in the intima of grafted vessels were VSMCs. Stat1 was expressed uniformly in all cells of the neointima and media; any change in total protein levels with IFN- γ treatment was therefore not detectable (Figure 7, G and H). In contrast, IFN- γ -treated grafts showed an increase in phospho-Ser⁷²⁷-p-Stat1 expression versus control grafts in intimal VSMCs ($23.1 \pm 1.3\%$ versus $3.8 \pm 1.3\%$, mean $\pm$ SEM, $P < 0.05$, $n = 8$) (Figure 7, I and J).

Discussion

The expression of Fas on the surface of cells is a prerequisite for apoptosis signaling through this receptor. The

Figure 6. IFN- γ sensitizes VSMCs to FasL-induced apoptosis. **A:** Time-lapse videomicroscopic quantification of apoptosis in VSMCs stimulated for 24 hours with IFN- γ or control (Unstim). Cells were then additionally treated with FasL or left untreated ($n = 3$). **B–D:** Time-lapse videomicroscopic assessment of apoptosis in VSMCs stimulated for 24 hours with IFN- γ , in the presence or absence of Ly (**B**), SH5 (**C**), or AG490 (**D**). After 24 hours FasL was added ($n = 3$). Data are means, error bars represent SEMs.

majority of Fas in VSMCs is stored in the Golgi apparatus, and cells are refractive to Fas-induced death without presensitization. This phenomenon is observed in a variety of normal and diseased cell types, including melanoma, lymphoma,³³ prostatic³⁴ or breast carcinoma, or normal liver,³⁵ endometrium,³⁶ and endothelial cells,³⁷ and may have evolved as a mechanism for immune evasion. Fas-induced apoptosis may be regulated in VSMCs by a variety of cytokines, many of which are present in the atherosclerotic plaque. For instance, previous reports have shown that surface Fas can be up-regulated by IFN- γ alone or combined with interleukin-1 β and/or TNF- α in pancreatic β cells¹⁸ and VSMCs⁹ *in vitro*. However, the mechanism underlying potentiation of surface Fas expression by IFN- γ is unclear.

We have investigated the ability of IFN- γ to modulate surface levels of Fas in VSMCs. We show that IFN- γ increases surface Fas levels without increasing total Fas expression by trafficking of pre-existent stores to the cell surface. A short, 24-hour exposure to IFN- γ is

sufficient for this process, without new synthesis of Fas. We demonstrate that this effect requires PI3K/Akt, at least in part, and is associated with transcription, and phosphorylation of Stat1. Furthermore, IFN- γ potentiates FasL-induced apoptosis of human VSMCs *in vitro*, an effect that is also inhibited by blocking PI3K, Akt, or Jak/Stat signaling. This signaling pathway can be represented via the speculated model shown in Figure 8. Finally, we show that IFN- γ can increase Fas expression *in vivo*, and enhance Fas-induced apoptosis in an *in vivo* human artery model.

Ligation of the IFN- γ receptor leads to recruitment and activation of Jak kinase and Stat transcription factor family members. Stat phosphorylation occurs on at least two sites within the protein; a tyrosine residue that allows dimerization and nuclear translocation, and a serine residue in the transactivating domain, further increasing transcriptional activity. Although the kinase(s) responsible for serine phosphorylation are debated, PI3K, p38 MAPK, PKC δ , and mTOR kinases have all been reported

Figure 7. IFN- γ sensitizes human arteries to Fas-induced apoptosis *in vivo*. Immunohistochemistry for Fas (blue) in grafted segment of human artery *in vivo* after 4 weeks of control (saline) (**A, B**) or IFN- γ treatment (**C, D**). **B** and **D**: Agonistic anti-Fas antibody was administered for 24 hours before sacrifice additionally in some grafts. **E** and **F**: Sections stained with cleaved caspase 3 (CC3) (**E**) or SMA (**F**), demonstrating apoptotic morphology of cleaved caspase 3-positive cells (arrow), which are SMA-positive on serial sections. Stat1 (**G, H**) or P-Stat1 (**I, J**) expression in grafts treated with saline (control) (**G, I**) or IFN- γ (**H, J**). Arrows indicate CC3-positive cell in **E** and P-Stat1-positive neointimal cells in **J**. Original magnifications: $\times 30$ (**A–D**); $\times 100$ in (**E, F**); $\times 60$ (**G–J**).

to regulate this event, with or without Jak activation as a prerequisite.

We show here that activation of Stat1 is required for Fas trafficking after IFN- γ stimulation because agents that block trafficking (LY, SH-5, DN-Akt, AG490) also reduce Ser⁷²⁷ Stat1 phosphorylation. Neither PKC δ nor mTOR affected Fas relocation (data not shown). Although both LY and AG490 completely blocked the IFN- γ -induced increase in surface Fas, the fact that inhibition of Akt (by either SH-5 or DN-Akt) only partially blocked Fas trafficking suggests that there are pathways downstream from PI3K leading to Stat1 phosphorylation that do not require Akt. Indeed, it is important to note that this model does not exclude a role for other PI3K-inducible kinases involved in Stat activation, because IFN- γ -induced activation of PI3K also activates pathways independent of Stat1.³⁸

The mechanism by which Fas is retained in the Golgi in VSMCs and other cells is unknown, although recent studies have identified a series of Fas-interacting proteins including F1 α and Fas-associated phosphatase-1

Figure 8. Speculated model of IFN- γ -induced Fas trafficking. IFN- γ binds to its receptor, activating Jak2 and PI3K. PI3K in turn phosphorylates Akt and possibly other unknown targets, leading to increased Stat1 expression and activation, and subsequent repression/inhibition of proteins responsible for retention of Fas in the Golgi. Inhibitors of PI3K, Akt, JAK2, or disruption of the Golgi complex (BFA) inhibit the IFN- γ -dependent Fas trafficking as shown.

(Fap-1) that may retain Fas internally. For example, in pancreatic carcinoma, melanoma cells, and normal fibroblasts, Fap-1 co-localizes with Fas in the Golgi and prevents cell surface trafficking of Fas.^{39,40} Inhibition of the phosphatase action of Fap-1, down-regulation of Fap-1 using siRNA, or preventing its interaction with Golgi Fas returned near-normal sensitivity to Fas-induced apoptosis.^{39,40} Although our preliminary experiments do not support a role for either Fap1 or F1 α in the localization of Fas to the Golgi in VSMCs (N.J.M., D.R., M.R.B., unpublished observations), it is possible that other, as yet unidentified, retention proteins are regulated by IFN- γ and are responsible for the intracellular sequestration of Fas in VSMCs.

The IFN- γ -induced increase in surface Fas expression has functional relevance. Prestimulation with IFN- γ significantly enhanced FasL-induced apoptosis, but this increase was reduced when surface Fas up-regulation was inhibited *in vitro* by PI3K, Akt, or Stat1 inhibition. Furthermore, IFN- γ could enhance sensitivity to an agonistic antibody to Fas in intact human arteries because grafted human arteries demonstrated increased VSMC apoptosis only in the presence of both IFN- γ treatment and agonistic antibodies to Fas. Neither IFN- γ nor agonistic Fas antibodies alone induced apoptosis. Previous *in vivo* studies of the role of IFN- γ in atherosclerosis have used IFN- γ knockout mice. Interpretation of these studies is

difficult because IFN- γ loss is systemic, occurs throughout the life of the animal, and can act on a variety of cell types and proinflammatory pathways. In our xenotransplantation system, IFN- γ , which has no species cross-specificity, can only act on cells of human origin in the transplanted artery. Stimulation occurs at a defined time point and its effect is localized to the human artery.²⁰ Recipient mice do not contain lymphocytes or natural killer cells nor do they accumulate lipid in response to IFN- γ treatment, further reducing the complexity of interactions in our system. Thus, our results describe the direct effect of IFN- γ on arterial VSMCs. Because neointima formation *in vivo* requires several weeks of IFN- γ treatment, we cannot elucidate the role of immediate, early Fas trafficking versus increased total Fas expression *in vivo*, and changes in subcellular localization of Fas cannot be studied *in vivo*. However, increased Fas expression and Stat1 phosphorylation after IFN- γ treatment was observed. The requirement for IFN- γ priming as a prerequisite for apoptosis *in vivo* may be essential to maintain the integrity of the vasculature. VSMCs and endothelial cells within the vessel wall express FasL, and monocytes/macrophages can induce VSMC apoptosis using Fas/FasL interactions.¹³ Sequestration of Fas internally may therefore protect VSMCs from apoptosis in the normal vessel, with apoptosis only occurring after cytokine priming.

In conclusion, we demonstrate that IFN- γ acts directly on VSMCs to prime them for apoptosis *in vitro* and *in vivo*. IFN- γ causes rapid Fas trafficking to the VSMC surface, and a 24-hour exposure alone is sufficient for this process. Trafficking requires Stat1 activation and is mediated through PI3K and partly by its downstream effector Akt. In this way, IFN- γ present in atherosclerotic plaques may promote FasL-induced apoptosis of VSMCs.

References

- McCarthy N, Bennett M: The regulation of vascular smooth muscle cell apoptosis. *Cardiovasc Res* 2000, 45:747–755
- Han D, Haudenschild C, Hong M, Tinkle B, Leon M, Liao G: Evidence for apoptosis in human atherosclerosis and in a rat vascular injury model. *Am J Pathol* 1995, 147:267–277
- Geng Y, Libby P: Evidence for apoptosis in advanced human atheroma: colocalization with interleukin-1 β converting enzyme. *Am J Pathol* 1995, 147:251–266
- Isner J, Kearney M, Bortman S, Passeri J: Apoptosis in human atherosclerosis and restenosis. *Circulation* 1995, 91:2703–2711
- Bauriedel G, Hutter R, Welsch U, Bach R, Sievert H, Luderitz B: Role of smooth muscle cell death in advanced coronary primary lesions: implications for plaque instability. *Cardiovasc Res* 1999, 41:480–488
- von der Thüsen JH, van Vlijmen BJ, Hoeben RC, Kockx MM, Havekes LM, van Berkel TJ, Biessen EA: Induction of atherosclerotic plaque rupture in apolipoprotein E^{-/-} mice after adenovirus-mediated transfer of p53. *Circulation* 2002, 105:2064–2070
- Littlewood TD, Bennett MR: Apoptotic cell death in atherosclerosis. *Curr Opin Lipidol* 2003, 14:469–475
- Ashkenazi A, Dixit VM: Apoptosis control by death and decoy receptors. *Curr Opin Cell Biol* 1999, 11:255–260
- Geng YJ, Henderson LE, Levesque EB, Muszynski M, Libby P: Fas is expressed in human atherosclerotic intima and promotes apoptosis of cytokine-primed human vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol* 1997, 17:2200–2208
- Bennett M, Macdonald K, Chan S-W, Simari R, Luzio J, Weissberg P: Cell surface trafficking of Fas: a rapid mechanism of p53-mediated apoptosis. *Science* 1998, 282:290–293
- Chan S-W, Hegyi L, Scott S, Cary N, Weissberg P, Bennett M: Sensitivity to Fas-mediated apoptosis is determined below receptor level in vascular smooth muscle cells. *Circ Res* 2000, 86:1038–1046
- Kockx MM: Apoptosis in the atherosclerotic plaque—quantitative and qualitative aspects. *Arterioscler Thromb Vasc Biol* 1998, 18:1519–1522
- Boyle J, Bowyer D, Weissberg P, Bennett M: Human blood-derived macrophages induce apoptosis in human plaque-derived vascular smooth muscle cells by Fas ligand/Fas interactions. *Arterioscler Thromb Vasc Biol* 2001, 21:1402–1407
- Boyle JJ, Weissberg PL, Bennett MR: Human macrophage-induced vascular smooth muscle cell apoptosis requires NO enhancement of Fas/Fas-L interactions. *Arterioscler Thromb Vasc Biol* 2002, 22:1624–1630
- Imanishi T, Han DK, Hofstra L, Hano T, Nishio I, Conrad Liles W, Gown AM, Schwartz SM, Gordon AM: Apoptosis of vascular smooth muscle cells is induced by Fas ligand derived from monocytes/macrophage. *Atherosclerosis* 2002, 161:143–151
- Lee T, Chau L: Fas/Fas ligand-mediated death pathway is involved in oxLDL-induced apoptosis in vascular smooth muscle cells. *Am J Physiol* 2001, 280:C709–C718
- Li PF, Dietz R, vonHarsdorf R: Reactive oxygen species induce apoptosis of vascular smooth muscle cell. *FEBS Lett* 1997, 404:249–252
- Augstein P, Dunger A, Salzsieder C, Heinke P, Kubernath R, Bahr J, Fischer U, Rettig R, Salzsieder E: Cell surface trafficking of Fas in NIT-1 cells and dissection of surface and total Fas expression. *Biochem Biophys Res Commun* 2002, 290:443–451
- Stemme S, Faber B, Holm J, Wiklund O, Witztum J, Hansson G: T-lymphocytes from human atherosclerotic plaques recognize oxidized low-density-lipoprotein. *Proc Natl Acad Sci USA* 1995, 92:3893–3897
- Tellides G, Tereb DA, Kirkiles-Smith NC, Kim RW, Wilson JH, Schechner JS, Lorber MI, Pober JS: Interferon-gamma elicits arteriosclerosis in the absence of leukocytes. *Nature* 2000, 403:207–211
- Buono C, Come CE, Stavrakis G, Maguire GF, Connelly PW, Lichtman AH: Influence of interferon-gamma on the extent and phenotype of diet-induced atherosclerosis in the LDLR-deficient mouse. *Arterioscler Thromb Vasc Biol* 2003, 23:454–460
- Fujio Y, Nguyen T, Wencker D, Kitsis RN, Walsh K: Akt promotes survival of cardiomyocytes in vitro and protects against ischemia-reperfusion injury in mouse heart. *Circulation* 2000, 101:660–667
- Bennett MR, Evan GI, Schwartz SM: Apoptosis of rat vascular smooth muscle cells is regulated by p53-dependent and -independent pathways. *Circ Res* 1995, 77:266–273
- Bennett MR, Evan GI, Schwartz SM: Apoptosis of human vascular smooth muscle cells derived from normal vessels and coronary atherosclerotic plaques. *J Clin Invest* 1995, 95:2266–2274
- Battle TE, Frank DA: The role of STATs in apoptosis. *Curr Mol Med* 2002, 2:381–392
- Sato T, Selleri C, Young NS, Maciejewski JP: Inhibition of interferon regulatory factor-1 expression results in predominance of cell growth stimulatory effects of interferon- γ due to phosphorylation of Stat1 and Stat3. *Blood* 1997, 90:4749–4758
- Tomita Y, Bilim V, Hara N, Kasahara T, Takahashi K: Role of IRF-1 and caspase-7 in IFN- γ enhancement of Fas-mediated apoptosis in ACHN renal cell carcinoma cells. *Int J Cancer* 2003, 104:400–408
- Townsend PA, Scarabelli TM, Davidson SM, Knight RA, Latchman DS, Stephanou A: STAT-1 interacts with p53 to enhance DNA damage-induced apoptosis. *J Biol Chem* 2004, 279:5811–5820
- Stephanou A, Scarabelli TM, Brar BK, Nakanishi Y, Matsumura M, Knight RA, Latchman DS: Induction of apoptosis and Fas receptor/Fas ligand expression by ischemia/reperfusion in cardiac myocytes requires serine 727 of the STAT-1 transcription factor but not tyrosine 701. *J Biol Chem* 2001, 276:28340–28347
- Dalpe AH, Eckerle S, Frey M, Heeg K: Triggering of Toll-like receptors modulates IFN- γ signaling: involvement of serine 727 STAT1 phosphorylation and suppressors of cytokine signaling. *Eur J Immunol* 2003, 33:1776–1787
- Nguyen H, Ramana CV, Bayes J, Stark GR: Roles of phosphatidylinositol 3-kinase in interferon- γ -dependent phosphorylation of STAT1

- on serine 727 and activation of gene expression. *J Biol Chem* 2001, 276:33361–33368
32. Kozikowski AP, Sun H, Brognard J, Dennis PA: Novel PI analogues selectively block activation of the pro-survival serine/threonine kinase Akt. *J Am Chem Soc* 2003, 125:1144–1145
33. Haynes A, Daniels I, Abdulmunem A, Carter G, Metheringham R, Gregory C, Thomson B: CD95 (Fas) expression is regulated by sequestration in the Golgi complex in B-cell lymphoma. *Br J Haematol* 2002, 118:488–494
34. Hyer ML, Voelkel-Johnson C, Rubinchik S, Dong J, Norris JS: Intracellular Fas ligand expression causes Fas-mediated apoptosis in human prostate cancer cells resistant to monoclonal antibody-induced apoptosis. *Mol Ther* 2000, 2:348–358
35. Sodeman T, Bronk SF, Roberts PJ, Miyoshi H, Gores GJ: Bile salts mediate hepatocyte apoptosis by increasing cell surface trafficking of Fas. *Am J Physiol* 2000, 278:G992–G999
36. Yamashita H, Otsuki Y, Matsumoto K, Ueki K, Ueki M: Fas ligand, Fas antigen and Bcl-2 expression in human endometrium during the menstrual cycle. *Mol Hum Reprod* 1999, 5:358–364
37. Li W, Dalen H, Eaton JW, Yuan XM: Apoptotic death of inflammatory cells in human atheroma. *Arterioscler Thromb Vasc Biol* 2001, 21:1124–1130
38. Navarro A, Anand-Apte B, Tanabe Y, Feldman G, Larner AC: A PI-3 kinase-dependent, Stat1-independent signaling pathway regulates interferon-stimulated monocyte adhesion. *J Leukoc Biol* 2003, 73:540–545
39. Ungefroren H, Kruse ML, Trauzold A, Roeschmann S, Roeder C, Arlt A, Henne-Bruns D, Kalthoff H: FAP-1 in pancreatic cancer cells: functional and mechanistic studies on its inhibitory role in CD95-mediated apoptosis. *J Cell Sci* 2001, 114:2735–2746
40. Ivanov VN, Lopez Bergami P, Maulit G, Sato TA, Sassoon D, Ronai Z: FAP-1 association with Fas (Apo-1) inhibits Fas expression on the cell surface. *Mol Cell Biol* 2003, 23:3623–3635