Interferon-γ Induces Human Vascular Smooth Muscle Cell Proliferation and Intimal Expansion by Phosphatidylinositol 3-Kinase–Dependent Mammalian Target of Rapamycin Raptor Complex 1 Activation

Yinong Wang, Yalai Bai, Lingfeng Qin, Pei Zhang, Tai Yi, Stephanie A. Teesdale, Liping Zhao, Jordan S. Pober, George Tellides

Abstract—Interferon (IFN)-γ, a cytokine characteristically expressed in arteriosclerotic diseases, acts directly on vascular smooth muscle cells to induce cellular proliferation and intimal expansion. Signaling by the mammalian target of rapamycin (mTORC1) raptor complex, known as mTORC1, is associated with cell growth and is active within arteriosclerotic lesions but is not known to be triggered by proinflammatory factors in vascular smooth muscle cells. We investigated the mechanisms for the proarteriosclerotic effects of IFN-γ in the absence of leukocytes by exploiting the species specificity of this cytokine in a chimeric model of immunodeficient mouse recipients bearing human coronary artery grafts and intravenously inoculated with adenovirus encoding a human IFN-γ transgene. We found that IFN-γ–mediated vascular smooth muscle cell proliferation and intimal expansion were associated with phosphorylation of the mTORC1 effector ribosomal protein S6 kinase 1, that the graft morphological changes and S6 kinase 1 activation were inhibited by the mTORC1 inhibitor rapamycin in vivo, and that IFN-γ–induced mTORC1 signaling was dependent on phosphatidylinositol 3-kinase activity under serum-free conditions in vitro. Our work establishes an immunologic stimulus for mTORC1 signaling in vascular smooth muscle cells, emphasizes that mTORC1 activation is critical in immune-mediated vascular remodeling, and provides further mechanistic insight into the successful clinical application of rapamycin therapy for atherosclerosis and graft arteriosclerosis. (Circ Res. 2007;101:560-569.)

Key Words: arteriosclerosis ■ coronary arteries ■ cytokines ■ vascular smooth muscle cells

Atherosclerotic diseases of human coronary arteries, such as atherosclerosis of native vessels, in-stent restenosis, and graft arteriosclerosis, are inflammatory disorders that are characterized by the production of interferon (IFN)-γ by artery-infiltrating T cells and the presence of IFN-γ responses in vascular cells.1–3 A causal role for IFN-γ in the pathogenesis of atherosclerosis and graft arteriosclerosis has been confirmed in experimental studies.4–7 Although it is not the only factor that can cause arteriosclerosis, we view the evidence for a pathogenetic role of IFN-γ to be particularly compelling.8 We initially emphasized the effects of IFN-γ–exposed vascular cells on infiltrating leukocytes.9 Our subsequent work has led to the conclusion that IFN-γ can also act on vessel wall cells to directly promote arteriosclerotic changes.10 The mechanism(s) by which IFN-γ elicits arteriosclerosis in the absence of leukocytes is poorly understood. Although IFN-γ increases the expression of platelet-derived growth factor (PDGF)-BB and PDGF β-receptors in vascular smooth muscle cells (VSMCs) in vitro and in vivo,10 we did not find a proarteriosclerotic effect of PDGF in our chimeric animal model of immunodeficient mice bearing porcine and human artery grafts (A.O. Yakimov, Y.W., G.T. unpublished observations, 2006).

Cellular proliferation is associated with activation of the mammalian target of rapamycin (mTOR) raptor complex (mTORC1) and its downstream effectors, including ribosomal protein S6 kinase 1 (S6K1) (also known as p70 S6K) and ribosomal protein S6, which regulate protein translation and cell growth.11 Phosphatidylinositol 3-kinase (PI3K) is an upstream regulator of this signaling pathway that results in the sequential phosphorylation of mTORC1/S6K1/S6 and may be activated by growth factors binding to their receptors.11 Pharmacological inhibitors of mTORC1, such as rapamycin, have potent antiproliferative effects on growth factor–induced VSMC mitogenesis.12 In clinical trials, rapamycin-coated stents decrease intimal proliferation and restenosis in atherosclerotic coronary arteries,13 and mTORC1 inhibitors reduce the incidence of intimal thickening in coronary arteries of cardiac allografts.14 The beneficial effect of mTORC1 inhibitors on the arteriosclerotic process is...
generally assumed to result from direct inhibition of VSMC proliferation, although a primary immunosuppressive effect may provide a complementary explanation for these observations. Importantly, it is not known whether proinflammatory mediators may directly activate PI3K/mTORC1 signaling in proliferating VSMCs within atherosclerotic lesions or whether this signaling pathway is dependent on growth factor stimulation in this context.

In this study, we find that IFN-γ-mediated VSMC proliferation and intimal expansion in the absence of leukocytes is associated with S6K1 phosphorylation, that these effects are inhibited by rapamycin in vivo, and that IFN-γ-induced mTORC1 signaling is dependent on PI3K activity under serum-free conditions in vitro.

Materials and Methods
Experimental techniques are described in the online data supplement at http://circres.ahajournals.org.

Artery and Cell Culture
Human aorta, denuded of endothelium and adventitia, was punched into 14-mm discs using a custom instrument and cultured in serum-free M199 media supplemented with 2 mmol/L l-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin (Invitrogen). The media was changed after 6 to 8 hours before treating with cytokine.

Human VSMCs were isolated by explant outgrowth from minced aorta or coronary arteries and cultured in supplemented M199 media with 20% FBS. No phenotypic differences were detected between the 2 types of VSMCs, and cultured cells were used at passages 3 to 4. The media was changed to serum-free conditions for 24 hours before treating with cytokine.

Aortic organ cultures or VSMCs were treated with IFN-γ (Upstate) at various doses for different times. In certain experiments, rapamycin or LY294002 (Calbiochem) were added before cytokine treatment. Adherent VSMCs were collected after mobilization with 0.05% trypsin and 0.5 mmol/L EDTA.

Results
Adenoviral Transduction of Immunodeficient Mice
We developed a new animal model to generate sustained, high systemic concentrations of human IFN-γ by modifying a previously described technique for adenoviral transduction of mice. Replication-deficient adenovirus encoding the control transgene LacZ (Ad-LacZ) or the human transgene IFN-γ (Ad-IFN-γ) were injected intravenously into C.B-17 severe combined immunodeficient (SCID)/beige mice 1 week after interposition of human coronary artery segments into the infrarenal aorta. Hepatic infection and liver transgene expression of Ad-IFN-γ resulted in detectable plasma cytokine for VSMCs from our parallel in vitro studies. Human IFN-γ was not detected in untreated or Ad-LacZ–treated SCID/beige mice, and murine IFN-γ or other proinflammatory cytokines, such as tumor necrosis factor, were not detected in the circulation of any of the recipients.

Consistent with our previous observations in untreated hosts, the human artery grafts from Ad-LacZ–treated mice did not express major histocompatibility complex (MHC) antigens except for weak expression by endothelial cells (Figure 1C and 1E). These results indicated that host responses to the vessel surgery or to the viral vector infection were not sufficient to induce interferon-dependent responses in the xenogeneic VSMCs. In contrast, class I and II MHC antigen expression was strongly induced in the human artery grafts by Ad-IFN-γ infection of recipient animals (Figure 1D and 1F). Specifically, all OF the cells in the intima, media, and the inner part of the adventitia expressed murine MHC class I antigens (Figure 1C and 1D and higher-power views in supplemental Figure I), whereas none or only a few cells in the outer adventitia expressed murine MHC class I antigens (Figure 1C and 1D, insets). The level of MHC antigen expression by the graft vascular cells is suprabasal and far greater than that seen with lower levels of circulating IFN-γ secondary to intermittent cytokine administration. We confirmed that the human coronary artery grafts in our model expressed IFN-γ receptors and thus were capable of responding to the circulating ligand (supplemental Figure I). These results establish that the recombinant human IFN-γ synthesized by transduced mouse cells was functional and demonstrated persistent cytokine responsiveness by vascular cells of the artery graft. This in vivo model represents a unique system to test the direct effects of the species-restricted cytokine IFN-γ on human vascular cells in the absence of leukocytes.

IFN-γ Induced Intimal Expansion and VSMC Proliferation in Human Coronary Arteries
Human epicardial coronary arteries, 6 to 8 mm in length and 0.5 to 1 mm in diameter, were bisected, and the adjacent segments were grafted to paired SCID/beige mice, 1 of which received Ad-LacZ and the other Ad-IFN-γ, at 1 week postoperatively. In this experimental design, the adjacent human arterial segments were used as matched investigational and control grafts to account for size differences and variable preexistent intimal thickening between individual donor vessels and between different donors. The recipients were euthanized at 5 weeks postoperatively, and the artery grafts were generally snap-frozen for histological and immunohistochemical analyses. The paired artery segments differed considerably in morphology (Figure 2A and 2B). Selected analysis of paraffin-embedded artery grafts confirmed that chronic cytokine exposure resulted in a cellular and matrix-rich expansion of the intima (supplemental Figure II). Morphometric analysis revealed that although Ad-IFN-γ treatment caused significant intimal hypertrophy and a trend toward medial thickening compared with Ad-LacZ controls, lumen area was not compromised by outward vascular

ng/mL after 1 and 4 weeks, respectively, which represent saturating levels of cytokine for VSMCs from our parallel in vitro studies. Human IFN-γ was not detected in untreated or Ad-LacZ–treated SCID/beige mice, and murine IFN-γ or other proinflammatory cytokines, such as tumor necrosis factor, were not detected in the circulation of any of the recipients.
remodeling (Figure 2C through 2F). The results are similar to, but more consistent and much more robust than, those we previously reported with intermittent subcutaneous injection of cytokine.10

The neointima elicited by IFN-γ/H9253 contained smooth muscle actin cells (Figure 3A and 3B and higher-power views in supplemental Figure II) and Ad-IFN-γ resulted in increased cellularity of both the intima and media (Figure 3C and 3D). We confirmed that the IFN-γ–treated VSMCs were proliferating by bromodeoxyuridine (BrdU) incorporation (Figure 3E and 3F), as well as proliferating cell nuclear antigen and Ki-67 immunostaining (data not shown). VSMC proliferation was greater in the intima than in the media (Figure 3G and 3H), whereas a lesser trend toward increased BrdU+ cells in the adventitia of cytokine-exposed grafts did not achieve statistical significance (data not shown).

**IFN-γ–Mediated Vascular Remodeling Is Associated With S6K1 Phosphorylation**

We assessed whether the mTORC1 pathway was activated by IFN-γ in our model by determining the phosphorylation state of a downstream target kinase, S6K1, that plays a key role in regulating protein translation and cell growth. Immunohistochemistry using a phospho-specific monoclonal antibody showed clear-cut phosphorylation of S6K1 (Figure 4A and 4B), and this result was confirmed with a polyclonal antibody to a different phosphorylation site (data not shown). Semi-quantitative scoring by an observer (Y.B.) who was blinded to the experimental protocols confirmed significant phosphorylation of S6K1 by IFN-γ (Figure 4C). Immunohistochemical analysis of flash-frozen artery grafts was performed with monoclonal antibodies to human MHC class I antigens (C and D), mouse MHC class I antigens (C and D, insets), and human MHC class II antigens (E and F). Representative photomicrographs are shown at low magnification. X-Gal staining is colored blue, and positive immunostaining is colored brown.

**Rapamycin Inhibits IFN-γ–Induced Intimal Expansion, VSMC Proliferation, and S6K1 Phosphorylation**

We investigated if mTORC1 activation by IFN-γ was necessary for its proarteriosclerotic effects. Administration of the mTORC1 inhibitor, rapamycin at a dose of 0.5 mg/kg per day IP for 4 weeks, which inhibited allogeneic T-cell–mediated vascular injury in the same immunodeficient mouse model, failed to prevent IFN-γ–induced vascular remodeling and mTORC1 activation (data not shown). However, rapamycin at a higher dose of 1.5 mg/kg per day IP for 4 weeks effectively prevented cytokine-mediated intimal expansion without reducing outward vascular remodeling, thus resulting in luminal enlargement (Figure 5A through 5F and higher-power views in supplemental Figure III). This dose of...
rapamycin also diminished IFN-γ-mediated increases in BrdU labeling of intimal and medial cells (Figure 6A through 6D) and phosphorylation of S6K1 within the artery grafts (Figure 6E through 6G). The therapeutic benefit of mTORC1 inhibition was not an indirect result of decreasing adenoviral production of human cytokine, as plasma levels of IFN-γ were similar with or without rapamycin treatment at 1 week (Figure 6H) and at 4 weeks (202 ± 17 versus 194 ± 21 ng/mL, respectively).

Activation of mTORC1 by IFN-γ is PI3K-Dependent

The limited size and availability of our human artery grafts prevented further biochemical studies in vivo. We tested whether IFN-γ could activate mTORC1 in cultured tissues and cells to confirm that VSMCs were relevant targets in arterial cytokine signaling, to gain insight into the regulation of the signaling pathway and to determine whether IFN-γ-mediated mTORC1 activation was dependent on the induction of growth factors. Because mTORC1 signaling may be affected by growth factors derived from platelet degranulation during serum production and by cellular changes induced by serum withdrawal, we initially used an organ culture system with serum-free media in which human arteries cultured in the complete absence of serum because procurement from the donor may remain viable for weeks (D. Tereb, G.T. unpublished observations, 2000). Cytokine treatment of aorta denuded of endothelium and adventitia confirmed that IFN-γ activated mTORC1 in the arterial media and resulted in phosphorylation of its target, S6K1, and the S6K1 target, S6, in a time-dependent and dose-dependent fashion (Figure 7A and 7B). Similarly, IFN-γ resulted in phosphorylation of mTOR (Figure 7C and 7D), S6K1, and S6 (Figure 8A and 8B) in VSMCs cultured under serum-free conditions after initial explant growth and passaging of cells in media containing optimal concentrations of serum. Similar results were obtained in cultured VSMCs in the presence of low serum concentration (0.5%) but with higher basal mTORC1 signaling in cells not treated with cytokine (data not shown). Thus, the signaling effects of IFN-γ in VSMCs under serum-free conditions in vitro were comparable to those in vivo, and we did not find evidence that IFN-γ was influenced...
or bound to serum, beyond the confounding effects of serum-induced phosphorylation of mTOR/S6K1/S6.

We further investigated the activation of mTORC1 by IFN-γ in serum-deprived, cultured VSMCs. Rapamycin decreased IFN-γ-mediated, mTORC1-dependent activation of S6K1 and S6 but not mTOR phosphorylation (Figure 8A). Furthermore, phosphorylation of mTOR, S6K1, and S6 by IFN-γ was inhibited by a PI3K inhibitor, LY294002. Rapamycin and LY294002 did not diminish IFN-γ-induced signal transducer and activator of transcription (STAT1) tyrosine phosphorylation, whereas the Janus kinase 2 inhibitor AG490 abolished both mTORC1 and STAT1 signaling by IFN-γ (data not shown). Finally, cycloheximide did not prevent IFN-γ-induced mTORC1 signaling (data not shown). These results establish that the activation of mTORC1/S6K1/S6 by IFN-γ in VSMCs is Janus kinase 2– and PI3K-dependent and does not require new protein synthesis or induction of growth factors.

Discussion

In this study, we found that IFN-γ–induced VSMC proliferation and intimal expansion of human coronary arteries are dependent on mTOR signaling via the mTORC1 pathway. We have previously described the chimeric animal model of
SCID/beige mice bearing pig coronary arteries or human internal mammary arteries. Administration of donortype IFN-γ by daily subcutaneous injections resulted in arteriosclerosis of the xenogeneic artery grafts in the absence of leukocytes without affecting the murine aorta, because interactions of IFN-γ with its cognate receptor are species-restricted. In this first-generation model with relatively low levels of circulating IFN-γ, the porcine coronary and human somatic arteries did not have preexistent intimal thickening, and modest arteriosclerotic changes were only noted in artery grafts from IFN-γ-treated recipients. However, this model was not sufficiently robust to demonstrate significant arteriosclerotic changes of human coronary arteries which display variable preexistent intimal thickening (A.O. Yakimov, Y.W., G.T. unpublished observations, 2006). We have modified our experimental system in 2 ways to successfully extend our model to clinically relevant arteries. First, the strategy of grafting adjacent artery segments to paired hosts minimizes bias resulting from intra- and interdonor differences in vessel diameter and vessel wall thickness. Second, adenoviral delivery of human IFN-γ results in sustained, saturating systemic levels of cytokine. The duration of transgene expression in SCID/beige mice is greater than that previously reported for immunocompetent animals and may reflect ineffective clearance of virus. Histological examination of livers from SCID/beige mice treated with Ad-LacZ or Ad-IFN-γ viruses did not reveal an inflammatory infiltrate (Y.W., G.T. unpublished observations, 2006). The use of immunodeficient recipients may also explain the absence of circulating mouse proinflammatory cytokines, including tumor necrosis factor, the action of which is not highly species-restricted. Our results indicate that any undetected inflammatory responses of SCID/beige mice to replication-deficient adenovirus are insufficient to induce MHC antigen expression by human artery grafts.

The immunohistochemistry findings with species-specific reagents suggest that all of the vascular cells in artery grafts from animals treated with Ad-IFN-γ are of human origin. These results differ from reports that murine bone marrow–derived stem cells contribute to neointima formation after mechanical injury or allograft rejection and destruction of native vascular cells. Our findings in human arteries are in agreement with other recent murine studies using more sophisticated analytic techniques and more subtle models of vascular injury, including syngeneic grafts between sex-mismatched apolipoprotein E–deficient mice, that found that all VSMCs in the neointima originate from the local vessel wall and not from circulating progenitor cells. We cannot exclude a role for human precursor vascular cells resident within the adventitia, such as those described in the proximal murine aorta, although we found 5-fold fewer BrdU+ cells in the adventitia compared with the intima of human coronary artery grafts in IFN-γ–treated recipients. It is important to note that although our experimental system involves arterial grafting, our conclusions are not based on rejection responses and thus are not limited to graft arteriosclerosis that occurs after organ transplantation. Our reductionist approach to examining the interactions between a single cytokine and vascular cells has implications for all arteriosclerotic diseases characterized by the expression of IFN-γ, including atherosclerosis.

Canonical signaling by ligand-bound IFN-γ receptors is via activation, dimerization, and nuclear translocation of STAT1. Full transcriptional activity of STAT1 depends on phosphorylation of a serine residue by a pathway dependent on IFN-γ–mediated activation of PI3K. However, transcrip-
tional effects of IFN-γ have also been convincingly demonstrated in STAT1-deficient mice. Noncanonical signaling by IFN-γ has been described, including mitogen-activated protein kinase-, Src-, CRKL-, and PI3K-dependent mTORC1 activation. Cytokine-mediated activation of mTORC1 has not been described in vascular cells, although IFN-γ is known to signal via PI3K in VSMCs and endothelial cells. The precise mechanism of PI3K activation by IFN-γ is not known and may be STAT-dependent or alternatively may involve nonclassical IFN-γ–signaling intermediates.

mTORC1 includes the adaptor protein raptor and regulates temporal aspects of cell growth that couples growth cues to the accumulation of mass. In contrast, mTORC2 contains rictor (instead of raptor) and regulates spatial aspects of cell growth, such as the organization of the actin skeleton. S6K1 is an effector of mTORC1 but not of mTORC2. Furthermore, mTORC1, but not mTORC2, is rapamycin-sensitive, although chronic treatment with rapamycin may indirectly inhibit TORC2 activity by sequestering newly synthesized mTOR protein and suppressing de novo assembly of this multiprotein complex. We interpret our results as IFN-γ activating mTORC1 in VSMCs via PI3K and that this signaling may occur in the absence of growth factors.

We cannot exclude that IFN-γ also activates the mTORC2 complex through the adaptor protein SIN1 to further promote cell growth, although our preliminary studies show that IFN-γ treatment of human VSMCs does not result in phosphorylated Akt at Ser473 (supplemental Figure IV), which is specific for mTORC2 signaling and unnecessary for S6K1 activation.

Activation of mTORC1 signaling has been documented in neointimal VSMCs of atherosclerotic human coronary arteries. In animal models, increased activity of PI3K/mTOR/S6K1 has been described in balloon-injured rat arteries and administration of rapamycin (at 6 mg/kg per d) inhibits lesion formation. Similarly, rapamycin (at 1 to 8 mg/kg every other day) reduces the size of atherosclerotic aortic lesions and also decreases the expression of IFN-γ mRNA in the spleens of apolipoprotein E-deficient mice, despite increasing serum lipids. In parallel studies in SCID/beige mice bearing human somatic artery grafts and reconstituted with allogeneic human peripheral blood mononuclear cells (PBMCs), we recently reported that rapamycin prevents alloimmune-mediated arterial injury and is associated with decreased production of IFN-γ by graft infiltrating T cells. Although the systemic levels of IFN-γ differ significantly between our 2 models, the local concentration of cytokine within the grafts...
may not be dissimilar, as IFN-γ is produced in the vessel wall after PBMC reconstitution versus in the host liver after adenoviral infection. This notion is supported by real-time RT-PCR studies that show overlapping expression levels of IFN-γ-inducible chemokine transcripts within the artery grafts after PBMC versus Ad-IFN-γ treatment (P.C.Y. Tang, Y.W., G.T. unpublished observations, 2006). One significant difference between the 2 models is that rapamycin was modestly effective at 0.1 mg/kg per day and completely effective at 0.5 mg/kg per day in preventing intimal inflammation and expansion after PBMC reconstitution, whereas rapamycin was ineffective at 0.5 mg/kg per day and required higher dose of 1.5 mg/kg per day to inhibit intimal thickening secondary to Ad-IFN-γ. The results from these analogous experimental systems suggest that rapamycin has a lower therapeutic threshold in preventing IFN-γ production by T cells versus IFN-γ responses by vascular cells. We speculate that higher levels of rapamycin may be required for direct vascular effects than for primary immunosuppressive effects in patients.

Interestingly, rapamycin decreases intimal expansion but not outward vascular remodeling, resulting in net enlargement of the lumen and suggesting mTORC1-independent mechanisms for IFN-γ-induced vessel expansion. These data argue for a direct effect of IFN-γ on outward vascular remodeling and against a mechanotransduction-mediated compensatory process to maintain lumen size in response to altered blood flow. Our experimental results mirror those of a clinical trial using intravascular ultrasound to assess coronary artery morphology in cardiac allografts, which found less

Figure 6. Rapamycin inhibited IFN-γ-induced VSMC proliferation and S6K1 phosphorylation. Paired SCID/beige mouse recipients of human coronary artery grafts were euthanized 4 weeks after intravenous inoculation with Ad-IFN-γ at 10^9 pfu and 24 hours after BrdU administration and treated with either vehicle or rapamycin at 1.5 mg/kg per day IP for 4 weeks. Immunohistochemical analysis of flash-frozen artery grafts was performed with monoclonal antibodies to BrdU (A and B) and phospho–Thr389-S6K1 (E and F) and an irrelevant, isotype-matched control antibody (E and F, insets). Representative photomicrographs are shown at high magnification, and positive immunostaining is colored brown. Arrows mark the internal elastic lamina to delineate the intima from media. BrdU^+ cells were counted in the intima and media and averaged from 5 cross-sections (x-sec) for each artery graft (C and D). The intensity of phospho-S6K1 immunostaining was graded according to an arbitrary 0 to 3 scale (G). IFN-γ plasma levels were determined by ELISA 1 week after inoculation with Ad-IFN-γ (H). Comparisons between treatment groups was by paired t test; n=5 per group for immunostaining analysis and n=6 for IFN-γ levels. *P<0.05.
intimal expansion and greater lumen area but similar vessel area with rapamycin therapy compared with conventional immunosuppression.38

We have previously reported a “proof-of-concept” experimental study that neutralization of IFN-γ prevents the intimal expansion and outward vascular remodeling of human coronary arteries induced by allogeneic T cells in SCID/beige mice.6 In investigating the translational relevance of our work, we have noted phospho-S6K1 expression by VSMCs in our limited patient samples of cardiac graft arteriosclerosis (Y.W., G.T. unpublished observations, 2006). An important consideration is that activation of PI3K-mTOR is certainly a common (and perhaps an invariable) pathway of VSMC activation by diverse mechanical and inflammatory stimuli,34–37 including alloantibodies.39 Finally, although we have demonstrated that mTORC1 activation is necessary for the proatherosclerotic effects of IFN-γ, our observations do not imply that it is sufficient. mTORC1 signaling may play a nonredundant permissive role, such as increasing protein translation, but other consequences of IFN-γ signaling may also be required in the pathogenesis of arteriosclerosis.

In conclusion, our work establishes an immunologic stimulus for mTORC1 signaling in VSMCs, emphasizes that mTORC1 activation is critical in immune-mediated vascular remodeling, and provides mechanistic insight into the successful clinical application of rapamycin therapy for atherosclerosis and graft arteriosclerosis.

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**Disclosures**

None.

**References**


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Supplementary Information

Expanded Materials and Methods

Artery grafting

Segments of human epicardial coronary arteries from explanted hearts of cadaveric organ donors or cardiac transplant recipients were interposed into the infrarenal aortae of female, 8-12 week old, ‘non-leaky’ (serum IgG < 1 µg/ml) SCID/beige mice (Taconic) using an end-to-end microsurgical anastomotic technique. Human subject protocols were approved by the Yale Human Investigations Committee and the New England Organ Bank, and animal procedure protocols were approved by the Yale Animal Care and Use Committee. At 1 week post-operatively, the animals were inoculated i.v. with Ad5.CMV-human IFN-γ or Ad5.CMV-LacZ (Qbiogene) at 1 x 10^9 PFU (approximately 1.3 x 10^10 viral particles). Plasma human IFN-γ levels were measured by ELISA (R&D Systems) at 1 and 4 weeks after adenovirus administration. Certain animals were treated with vehicle (4% ethyl alcohol/Cremaphor) or rapamycin (Calbiochem) at 1.5 mg/kg/day i.p. for 4 weeks after viral infection, and certain animals received BrdU (Sigma-Aldrich) at 100 mg/kg s.c. and 30 mg/kg i.p. 24 hours before and again at 30 mg/kg i.p. 12 hours before sacrifice.

Graft analysis

Artery grafts were procured at 5 weeks post-operatively (4 weeks after viral infection) and analyzed by standard histological techniques for Elastica-van Gieson (EVG) and X-gal staining and by immunohistochemistry using mouse monoclonal antibodies to human α-SMA, human HLA-A/B/C, human HLA-DR, (Dako), mouse H-2D^d, BrdU (BD Biosciences-Pharmingen), and human phospho-Thr389 S6K1 (Cell Signaling Technology) or rabbit polyclonal antibodies to human IFN-γ receptor-α (Santa Cruz Biotechnology). Binding of
secondary antibody (Jackson ImmunoResearch) was detected with peroxidase/3-amino-ethyl carbazole kits (Vector Laboratories). Cell counting of nuclei surrounded by positive immunostaining was performed under high magnification and averaged from 5 cross-sections for each graft. The intensity of phospho-S6K1 immunostaining was semiquantitatively graded by an observer blinded to the experimental protocols according to an arbitrary scale (0-absent, 1-weak, 2-moderate, and 3-strong staining). The perimeters of the endothelium, internal elastic lamina (IEL), and external elastic lamina (EEL) were assessed and averaged from 5 serial cross-sections, 150 μm apart, for each artery graft and area measurements of the lumen (within the endothelium), intima (between the endothelium and IEL), media (between the IEL and EEL), and whole vessel (within the EEL) were calculated using computer-assisted image analysis and a software program (ImageJ, http://rsb.info.nih.gov/ij/).

**Western blotting**

Protein was extracted from homogenized frozen grafts and cultured artery tissue or cells in lysis buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 5mM EDTA, 1mM sodium orthovanadate, 0.1% aprotinin, and 1 mM PMSF), and boiled in SDS sample buffer for 5 minutes. Equal amounts of protein per sample were separated by SDS-PAGE, transferred electrophoretically to a nitrocellulose membrane (Bio-Rad Laboratories), and immunoblotted with rabbit antibodies to phospho-Ser2448 mTOR, mTOR, phospho-Thr389 S6K1, phospho-Thr421/Ser424 S6K1, S6K1, phospho-Ser235/236 S6, phospho-Ser240/244 S6, S6, phospho-Ser473 Akt, and Akt (Cell Signaling Technology), or mouse monoclonal antibody to β-actin (Sigma-Aldrich), followed by horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch). Detection of bound antibody by enhanced chemiluminescence (Pierce Biotechnology) was performed according to the manufacturer's instructions.
Statistical analysis

Two-tailed, paired t tests were performed using the Prism software program (GraphPad Software). Differences with $P<0.05$ were considered to indicate statistical significance.

Supplementary Figure Legends

Supplemental Figure I. Artery grafts express IFN-γ-inducible human MHC antigens and constitutive IFN-γ receptors. Paired SCID/beige mouse recipients of human coronary artery grafts were sacrificed 4 weeks after i.v. inoculation with either Ad-LacZ or Ad-IFN-γ at $10^9$ PFU. Immunohistochemical analysis of flash-frozen artery grafts was performed with monoclonal antibodies to human MHC class I antigens (A, B), an irrelevant, isotype-matched control antibody (A, B insets), a polyclonal antibody to the human IFN-γ receptor (C, D), and non-immune rabbit serum (C, D insets). Representative photomicrographs are shown at high magnification. Positive immunostaining is a brown color. Arrows mark the internal elastic lamina to delineate the intima from media.

Supplemental Figure II. IFN-γ induced a cellular and matrix-rich intimal expansion. Paired SCID/beige mouse recipients of human coronary artery grafts were sacrificed 4 weeks after i.v. inoculation with either Ad-LacZ or Ad-IFN-γ at $10^9$ PFU. Histological analysis of paraffin-embedded artery grafts was performed by H&E (A, B) and EVG staining (C, D). Immunohistochemical analysis of paraffin-embedded artery grafts was performed with a monoclonal antibody to human α-SMA (E, F). Representative photomicrographs are shown at high magnification. Positive immunostaining is a brown color. Arrows mark the internal elastic lamina to delineate the intima from media.
Supplemental Figure III. Rapamycin inhibited IFN-γ-induced intimal expansion. Paired SCID/beige mouse recipients of human coronary artery grafts were sacrificed 4 weeks after i.v. inoculation with Ad-IFN-γ at 10⁹ PFU and treated with either vehicle or rapamycin at 1.5 mg/kg/day i.p. x 4 weeks. Histological analysis of flash-frozen artery grafts was performed by H&E (A, B) and EVG staining (C, D). Immunohistochemical analysis of flash-frozen artery grafts was performed with a monoclonal antibody to human α-SMA (E, F). Representative photomicrographs are shown at high magnification. Positive immunostaining is a brown color. Arrows mark the internal elastic lamina to delineate the intima from media.

Supplemental Figure IV. Akt was not phosphorylated at Ser473 by IFN-γ in vitro. Human VSMCs cultured in serum-free media for 24 hours were treated with IFN-γ at 100 ng/ml or 10% serum for different times as indicated. Western blotting was performed using polyclonal antibodies to phospho-Ser473- and total Akt.
Supplemental Figure II

LacZ

IFN-γ

A

B

C

D

E

F

H&E

EVG

α-SMA
Supplemental Figure III

A: IFN-γ H&E
B: IFN-γ + Rapa H&E
C: IFN-γ EVG
D: IFN-γ + Rapa EVG
E: IFN-γ α-SMA
F: IFN-γ + Rapa α-SMA
Supplemental Figure IV

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