Overexpression of Soluble Fas Attenuates Transplant Arteriosclerosis in Rat Aortic Allografts

Tao Wang, MD, PhD; Chunming Dong, MD; Susan C. Stevenson, PhD; Edward E. Herderick, BA; Jennifer Marshall-Neff, BA; Sanjay S. Vasudevan, MD; Nicanor I. Moldovan, PhD; Robert E. Michler, MD; N. Rao Movva, PhD; Pascal J. Goldschmidt-Clermont, MD

Background—The killing of vascular cells by activated macrophages is an important step in the process of destabilization of the arterial wall. The death receptor Fas is implicated in vascular cell death. Hence, we extended our studies in a rat aortic allograft model, using adenovirus-mediated overexpression of soluble Fas (sFas) to block Fas binding to Fas ligand (Fas-L). The contribution of Fas to vascular cell injury and consequent transplant arteriosclerosis was investigated.

Methods and Results—Activated monocytes in the presence of macrophage colony-stimulating factor induce endothelial cell apoptosis in vitro, which was significantly inhibited by adenovirus-mediated sFas overexpression. Next, donor rat abdominal aortas were either untreated or transduced with adenoviruses encoding (1) rat soluble Fas (Ad3rsFas), (2) no insert (Ad3Null), and (3) β-galactosidase (Ad3nBgl). A total of 175 aortic grafts were harvested 2 to 90 days after transplantation. Vascular cell apoptosis and CD45+ cell infiltration were significantly reduced in Ad3rsFas-transduced aortas, as compared with control allografts. Moreover, the control allografts developed marked intimal thickening, whereas Ad3rsFas-transduced allografts had significantly less neointima until the 90-day time point.

Conclusions—sFas overexpression protects the integrity of the vessel wall from immune injury and attenuates transplant arteriosclerosis. (Circulation. 2002;106:inin).

Key Words: arteriosclerosis ■ apoptosis ■ endothelium ■ inflammation ■ gene therapy

Cardiac allograft vasculopathy (CAV), characterized by the development of transplant arteriosclerosis, remains the leading cause of late graft failure and patient death in heart transplantation. The archetypal pathological findings of CAV are diffuse concentric intimal proliferation and intense mononuclear cell infiltration, including T cells and macrophages, in most graft vessels. Although the precise molecular and cellular mechanisms are not yet elucidated, endothelial cell (EC) injury and recipient inflammatory response are believed to be crucial in the development of CAV.1,3,4

Increased apoptosis, including vascular EC and smooth muscle cell (SMC), has been observed in acute and chronic rejection; such apoptosis probably is mediated through the Fas/Fas-Ligand (Fas-L) pathway. Fas-L+ T cells and macrophages bind to Fas+ vascular cells, inducing apoptosis. One of earliest features of CAV is the adherence of monocytes to the endothelium, followed by their migration into the intima, which may enhance the development of atherosclerotic lesions. Numerous investigations have focused on the molecular mechanism of monocyte attraction and migration into vascular tissues; little attention, however, has been paid to the actual role of infiltrating macrophages in the mediation of CAV.

Macrophage colony-stimulating factor (MCSF), which is elevated within inflammatory atherosclerotic lesions, was shown to block spontaneous monocyte apoptosis and to stimulate monocyte differentiation, proliferation, and activation. We have previously reported that monocytes, in the presence of MCSF, switched to a phenotype capable of inducing SMC apoptosis.11 In addition, gene transfer of MCSF resulted in monocyte recruitment and induced SMC migration and proliferation in the vessel wall. Furthermore, Boyle et al demonstrated that SMC apoptosis induced by macrophages was dependent on Fas/Fas-L interaction.

We hypothesized that soluble Fas (sFas), a natural apoptosis inhibitor that blocks the binding of Fas to Fas-L, prevents monocyte-induced and CD4+ T cell–induced vascular EC and SMC apoptosis, thus lessening the development of CAV. We constructed an adenovirus encoding rat sFas cDNA and examined its effect on transplant arteriosclerosis in a rat aortic transplant model of chronic rejection. We report here that sFas overexpression protects the integrity of the...
vessel wall and reduces the severity of transplant arteriosclerosis.

Methods

Construction of Adenoviral Vectors
Rat soluble Fas (sFas) cDNA, containing bases 56 to 565 of the extracellular Fas domain, was subcloned into phleomycin SK (+) as described previously.16 To generate Ad3sFas, the rat sFas fragment was inserted into the XbaI/EcoRV sites of the backbone shuttle vector pAves6A to create pAvesrsFas and incorporated into an adenoviral vector derived from the “third-generation of adenovirus” with E1, E2a, and E3 deletions (Ad3-, serotype 5). Similar approaches were used to generate Ad3hsFas containing human sFas, Ad3nBgs containing β-galactosidase (β-gal) gene, and Ad3Null containing empty expression cassettes. The average ratio of all virus total particles (particles/mL) to plaque forming units (pfu/mL) was 25.6±2.4:3 (particles/pfu), as determined in AE 1–2a cells derived from the A549 cell line.

Expression of sFas in Cultured Cells
Since there is no antibody available for rat sFas and rat sFas shares little homology with human sFas, we thus used Ad3hsFas (human sFas) to verify sFas protein expression in human aortic endothelial cells (HAECs, Clonetics). HAECs were cultured in EmGm2 medium (Clonetics) containing 5% FBS. HAECs at 60% to 70% confluence were exposed to medium alone or transduced with Ad3Null at multiplicity of infection (MOI) of 50 and 400 or Ad3hsFas at MOI of 10, 50, 200, and 400 in 0.5 mL 0.1%FBS-EmGm2 medium for 6 hours. After washing, cells were further incubated for another 48 hours. Culture supernatants were collected and sFas protein production was detected using an ELISA kit (R&D System).

The efficiency of gene transfer by Ad3sFas was examined in mouse aortic ECs (MAECs, provided by Dr Robert Auerbach, University of Wisconsin) and primary SMC from the aortas of MRL-lpr/lpr mice. MAECs and SMC were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS). Such cells were either exposed to vehicle (PBS) or transduced for 2 hours with either Ad3Null or Ad3nBgs at an MOI of 250 or Ad3sFas at MOIs of 125, 250, or 500 in DMEM with 0.1% FBS. Cells were then cultured for additional 48 hours in DMEM containing 10% FBS. Total RNA was isolated from cells using an RNeasy kit (Qiagen).

Apoptosis Assay
HAECs were cultured on coverslips in 6-well plates and infected with Ad3Null or Ad3sFas (MOIs of 10, 50, 200). Twenty-four hours after transfection, freshly isolated human monocytes were added to HAECs in a ration of 3:1 (monocytes/HAECs) in the presence or absence of MCSF. Forty-eight hours after coculture, HAECs were stained with the wybrant Apoptosis Kit (Molecular Probes). Apoptotic index (=dead cell/alive cell+dead cell) was calculated as described previously.

Gene Transfer and Aortic Transplantation
Male DA (RT1a) and PVG (RT1c) rats (180 to 250 g) were from Harlan Laboratories (Indianapolis). Abdominal aortic allografts (n=175) were orthotopically transplanted from DA to DA (isograft) or PVG rats (allograft) and divided into 5 groups: isografts vehicle (1); isografts vehicle (2); allografts transduced with Ad3nBgs (3), or Ad3Null (4), or Ad3sFas (5). Surgery procedures have been described elsewhere.15 Briefly, rats were anesthetized with methoxyflurane inhalation. DA rat abdominal aorta segments were flushed free of blood and aspirated. A total of 250 to 300 mL of Ad3nBgs, Ad3Null, or Ad3sFas (total 1.5–1.8×10^7 pfu, or 3–3.6×10^7 total particles) or vehicle (saline) was instilled into the aortic lumen and the two ends were clamped. Clamped aortic segments containing viruses were incubated for 40 minutes on ice, washed, and then orthotopically transplanted into DA or PVG rats. All recipients received cyclosporine A (5 mg/kg IM per day for 5 days) to prevent acute rejection.

Reverse Transcriptase–Polymerase Chain Reaction
Total RNA was isolated from cells and aortic grafts at 2, 5, 15, and 30 days after transplantation (Tx). RNA was reverse-transcribed with Superscript II reverse transcriptase (GIBCO). The cDNA was amplified by using the rat sFas-specific primers, which overlap the promoter region of the Ad3sFas insert and do not hybridize with endogenous full-length Fas, sense 5’-CTGTTGATCATGGCTGTCCTG-3’ and antisense 5’-TTTGAACATTTAAGCTGAAT-3’, amplifying a fragment of 566 base pairs. As a control, mouse endogenous Fas were also detected in cells by using following primers: sense 5’-CTGGGATCATGCTGTCCTG-3’ and antisense 5’-CTCCA-GACTTITGCTCCTAC-3’, amplifying a fragment of 969 base pairs.

β-Galactosidase (gal) Staining
Twenty aortic allografts, harvested at 3 to 7 days after Tx, were either en face fixed or cut into 7- to 14-μm sections and fixed with 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 30 minutes. These sections were then incubated with X-gal reagents (Life Technologies) for 3 to 12 hours at 37°C, counterstained with eosin, and visualized for blue positivity by light microscopy.

Immunohistochemistry
Paraffin sections of aortic allografts harvested at 2, 15, 30, 45, and 60 days after Tx were stained with antibodies against Fas or Fas-L, the leukocyte common antigen CD45 and Microphase (CD11b/c) (Pharmingen), smooth muscle α-actin (Sigma), and proliferating cell nuclear antigen (PCNA, Vector Laboratories). In situ terminal dUTP nick end label (TUNEL) was also performed in these samples to detect cell apoptosis with the use of the ApopTag In Situ Apoptosis Detection Kit (Oncor) or the ApoTACS in situ TACS Blue, a TUNEL-based apoptosis detection kit (R&D System).

Apoptosis and inflammatory cell infiltration in allografts harvested at 45 days were quantitatively analyzed (5 grafts per group). The apoptotic index and number of CD45+ cells/mm2 was calculated for all grafts.

Morphometric Analysis
Six representative sections per graft and 5 to 7 aortic grafts per group at 45, 60, and 90 days after Tx, stained with Van Gieson elasin, were selected for quantitative measurement for the presence and severity of CAV. The thickness and surface area of the neointima and the media of aorta were measured with the use of a customized software-drawing package. Average intimal and medial thickness was calculated by dividing the entire internal elastic lamina (IEL) length into 100 equally spaced segments. The average of 100 measurements was used to define the average intimal and medial thickness for each section.

Statistical Analysis
All data are expressed as mean±SEM. The morphological measurements were analyzed with the use of StatView and the Statistical Analysis System software (SAS). Dunnett’s 2-tailed Student’s t test was used to compare treatment groups with the control allograft group. A probability value ≤0.05 was considered statistically significant.

Results

Ad3-sFas Induces sFas Overexpression in Cultured Cells and Aortic Allografts
We studied sFas protein productions by using cultured HAECs. With the use of an ELISA assay, the release of sFas was found to be significant at MOI 10, reaching its peak at 200 MOI (Figure 1A) in the supernatant of Av3hsFas-transduced HAECs. With RT-PCR, rat sFas expression was confirmed in Ad3sFas-transduced MAECs and SMC, whereas no transcript for sFas was detected in vehicle control,
Ad3Null-transduced or Ad3nBg-transduced MAECs and SMCs. Endogenous Fas mRNA was detected in MAECs but not in explanted SMCs from Fas-null mouse aortas (MRL-lpr/lpr mice) (data not shown).

In rat aortic allografts, sFas mRNA was detected exclusively in Ad3rsFas-transduced allografts at 2 and 5 days, reduced at 15 days by approximately two thirds (data not shown) and undetectable at 30 days (Figure 1B). In Ad3nBg-transduced aortic allografts harvested at 5 days after transplantation, β-gal expression was detected selectively in luminal ECs (15% to 40%) (Figure 1C, D), whereas such β-gal expression was not observed in nontransduced control allografts (Figure 1E). In Ad3rsFas-transduced allografts, Fas positivity was strongest at the level of the endothelium at 3 to 30 days after transplantation, which is consistent with β-gal expression in Ad3nBg-transduced aortas (Figure 2, C and D). In addition, elevated Fas staining was also detected in the media of Ad3rsFas-transduced aortic allografts. The source for such Fas expression in the media, however, remains to be determined. As β-gal was not detectable in the media of Ad3nBg-transduced aortas (Figure 1D), the increased Fas positivity may be due to paracrine sFas secretion by Ad3rsFas-transduced ECs.

Collectively, these results indicate that Ad3hsFas/Ad3rsFas could effectively transduce ECs in vitro and in the vessel wall in vivo and induce overexpression of sFas.

sFas Overexpression Inhibits Activated Monocyte-Induced EC Apoptosis In Vitro

We have shown that monocytes activated with MCSF induce SMC apoptosis, providing direct contact between the two types of cells, a process that was efficiently inhibited by blocking antibodies targeted at the Mac-1 receptor on macrophages (CD11b/c-CD18).11 We have repeated these experiments, but this time using HAECs as target vascular cells for MCSF-activated macrophages. The apoptotic index was 16.0 ± 4.6% for control HAECs, 15.8 ± 4.8% for HAECs cultured with MCSF (100ng/mL) but without monocytes, and 17.7 ± 7.1% for HAECs cocultured with monocytes without MCSF. In contrast, HAECs cultured with both MCSF and monocytes displayed significantly increased apoptotic index (54.8 ± 12.6%, P < 0.0001) (Figure 3, bar 1 to 4). These results indicate that monocytes can induce EC apoptosis in the presence of MCSF, as is the case in SMCs.11 Ad3hsFas significantly inhibits MCSF-activated monocyte-induced HAEC apoptosis (9.2 ± 5.5%, 11.5 ± 3.7%, 11.45 ± 6.3% at MOIs of 10, 50, and 200, respectively, P < 0.0001 versus vehicle HAECs + Monocyte+MCSF). In contrast, Ad3Null-transduced cells had apoptotic indexes similar to that of controls. Except for the fact that at 50 MOI of Ad3Null, HAEC apoptosis was increased in the presence of monocytes even without MCSF (Figure 3), perhaps adenoviral antigens may activate monocytes or ECs, leading to the destruction of the transduced cells.17 The inhibitory effect of Ad3hsFas on apoptosis was associated with increased sFas secre-
tion as detected by ELISA (range, 4.2–19.8 ng/mL versus vehicle-control at 0.142–0.176 ng/mL, \(P<0.0001\)). Hence, overexpression of sFas protects ECs from apoptosis induced by MCSF-activated monocytes, probably by blocking Fas/Fas-L interaction.

**Overexpression of sFas Inhibits Leukocyte Infiltration and Apoptosis**

Representative sections taken at 45 days after Tx from each group stained for anti–Fas-L, CD45 (leukocytes), and CD11b/c (macrophage). In vehicle controls (Figure 4A, C, and E) and Ad3Null or Ad3nBg-transduced aortas (not shown), most of infiltrating cells, located in neointima and adventitia, stained Fas-L and/or CD45, or CD11b/C positive. In contrast, in Ad3rsFas-transduced aortas, strong FasL staining was detected in the endothelium and cells that had adhered to it (Figure 4B).

At 3 to 45 days after Tx, apoptosis was detected in all layers of the vessel wall and in particular the media of control allografts, Ad3Null- and Ad3nBg-transduced allografts. In contrast, TUNEL positivity was significantly less in Ad3rsFas-transduced allografts. At 45 days after Tx, the number of apoptotic cells and CD45\(^+\) cells (T cells and macrophages) in neointima and media in Ad3rsFas-transduced allografts were markedly decreased compared with vehicle-treated or Ad3Null-transduced aortas (Figure 5, A and B). Moreover, Ad3rsFas-transduced grafts displayed
well-preserved architecture of the media with intact elastin layers, whereas the media and elastin layers were substantially disrupted in vehicle controls and Ad3Null-transduced allografts. Furthermore, intimal proliferation and media SMC loss, demonstrated by α-actin staining, were decreased in Ad3rsFas-transduced grafts compared with controls (Figure 6). These findings suggest that overexpression of sFas by Ad3rsFas inhibits both apoptosis of vascular cells and leukocyte infiltration in the early period following transplantation.

Overexpression of sFas Attenuates Transplant Arteriosclerosis

Because SMC proliferation in the neointima of arterial allografts represents a “response to injury,” we expected that by preserving the endothelium and media of the grafts, Ad3rsFas transduction could reduce neointima expansion. At 45 days after Tx, marked intimal thickening was observed in vehicle-treated (57.81 ± 13.5 μm) and Ad3Null-transduced allografts (61.5 ± 13.8 μm). In contrast, in Ad3rsFas-transduced allografts, such intimal formation was significantly reduced (26.28 ± 3.4 μm, P < 0.05 versus allograft controls). The benefit of Ad3rsFas transduction was maintained until 60 days but started to taper by 90 days after Tx, perhaps as a consequence of the progressive loss of expression of sFas. All isografts displayed essentially normal aortic structure at all time points (Figure 7 and Table).

Discussion

The destabilization of the vessel wall resulting from vascular cell injury leads to both thromboembolic and proliferative complications. After mechanical (percutaneous coronary intervention) or alloimmune injury (transplant) to vascular...
cells, inflammatory cells bind to and induce the apoptosis of ECs and SMCs. Apoptosis of vascular cells triggered by activated inflammatory cells can be reconstituted in vitro by coinubcation of monocytes, ECs (this study), or SMCs, providing exposure of monocytes to MCSF.13 Previous inhibitors of the Fas pathway have been shown to prevent apoptosis of SMCs induced by macrophages. In this report, we show that a replication-incompetent adenovirus that codes for the sFas (Ad3rsFas) efficiently blocks the killing of ECs by MCSF-activated macrophages. sFas overexpression in allograft endothelium reduced vascular cells apoptosis, infiltration of the arterial wall by leukocytes, and disruption of the media layer in a rat aortic allograft model of chronic rejection. Since the transgene was expressed mainly by ECs, providing for a paracrine effect of secreted sFas, we postulate that CD45+ cells and monocytes/macrophages.

Our approach to control the Fas pathway is fundamentally different from that used by others.25 Previous attempts to protect “endothelial injury” by antagonizing Fas-mediated apoptosis in transplantation have focused mainly on the overexpression of recombinant Fas-ligand.26,27 The anticipated mechanism of such a Fas-L approach is that overexpressed Fas-L in the endothelium binds to Fas on infiltrating T cells and monocytes and induces T-cell and monocyte apoptosis. Although some studies showed that Fas-L gene transfer could prolong allograft survival, others found that ectopic Fas-L expression offered no beneficial effects.28 Instead, it was associated with certain degrees of toxicity, leading to cytokine release, infiltration of granulocytic cells, formation of pyogenic lesions, and destruction of the transplanted tissues.25 Furthermore, Takeuchi and colleagues29 showed that Fas-L overexpression in cardiac allografts (transgenic mice) induced accelerated rejection.

Our sFas strategy mimics the natural way the immune system limits the activity of cytolytic T-cells after infection.14 The protective role of soluble Fas has been implicated in liver transplantation.30 The anticipated mechanism for the sFas approach is that overexpressed sFas by endothelium acts as a decoy,14 neutralizing Fas-L on infiltrating T cells and monocytes, and prevents the killing of ECs by these immune cells. Because Fas-L binding to sFas does not trigger apoptosis, sFas overexpression in the endothelium protects ECs from being damaged/killed by inflammatory cells. Unlike overexpressed Fas-L, sFas does not stimulate the release of cytokines and other toxins such as metalloproteinases due to inflammatory cells apoptosis.3 Hence, our approach may offer certain advantages over the Fas-L strategy.

In conclusion, our study supports that blocking Fas/Fas-L interaction by overexpression of sFas in graft endothelium inhibits vascular cell apoptosis, CD45+ mononuclear cell infiltration, and significantly attenuates the disruption of the arterial wall and transplant arteriosclerosis. These data are consistent with our earlier observation obtained in Fas-deficient mice indicating that absence of Fas reduces damage to the media of coronary arteries in cardiac allografts.31 These two lines of evidence point to the Fas pathway as a suitable target for intervention and prevention of the development of CAV.

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