Adenovirus-Mediated Delivery of Fas Ligand Inhibits Intimal Hyperplasia After Balloon Injury in Immunologically Primed Animals

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Background—Adenoviral constructs have been used for studies of injury-induced vascular hyperplasia in immunologically naive laboratory animals, but their usefulness for intra-arterial gene therapy may be limited by the prevalence of preexisting immunity to adenovirus in the patient population. Here, we explored the efficacy of adenovirus-mediated transfer of Fas ligand, a cytotoxic gene with immunomodulatory properties, in inhibiting injury-induced vascular lesion formation in both naive and immunologically primed animals.

Methods and Results—Lesion formation was evaluated in balloon-injured carotid arteries of naive and adenovirus-immunized rats that were infected with adenoviral constructs expressing Fas ligand (Ad-FasL), the cyclin-dependent kinase inhibitor p21 (Ad-p21), or β-galactosidase (Ad-βgal). In naive rats, Ad-FasL induced apoptosis in medial vascular smooth muscle cells and inhibited intimal hyperplasia by 60% relative to Ad-βgal–treated vessels (P<0.05), whereas the cytostatic agent Ad-p21 decreased lesion size by 58% (P<0.05). In animals preimmunized with an adenoviral vector containing no transgene, Ad-FasL significantly inhibited neointima formation (73% reduction, P<0.05), but Ad-p21 failed to inhibit neointima formation relative to controls. Immunologically primed rats displayed robust T-cell infiltration in Ad-p21– and Ad-βgal–treated vessels, but T-cell infiltration was markedly attenuated in Ad-FasL–treated vessels.

Conclusions—Our data demonstrate that adenovirus-mediated Fas ligand delivery can inhibit intimal hyperplasia in both immunologically primed and naive animals, whereas the efficacy of an adenovirus-mediated p21 delivery is limited to immunologically naive animals. This study documents, for the first time, the therapeutic efficacy of intravascular adenoviral gene transfer in animals with preexisting immunity to adenovirus. (Circulation. 1999;99:1776-1779.)

Key Words: restenosis genes viruses lymphocytes

Adenoviral vectors are useful for studies of injury-induced vascular hyperplasia because of their high transduction efficiency. These properties have resulted in optimistic predictions that adenoviral vectors may be used to deliver therapeutic genes to prevent postangioplasty restenosis.1 However, a limitation for adenovirus-mediated gene therapy in humans is the prevalence of preexisting immunity to adenovirus, which might lead to the destruction of adenovirus-transduced cells.2 Immune responses to adenoviral vectors are typically characterized by mononuclear cell infiltration, transgene elimination, and the inability to readminister the vector.3,4

The Fas-FasL system has been implicated in the regulation of physiological cell turnover, particularly during the down-regulation of an immune response.5 Fas (CD95/Apo-1) is a death receptor expressed on many cell types, but expression of its ligand (FasL) is highly restricted. Fasl expression occurs in immune-privileged tissues, where it is believed to function by killing Fas-bearing inflammatory cells.6 Constitutive FasL expression has also been detected in tumors, where it may function to induce apoptotic cell death in Fas-expressing immune cells when they attempt to enter the tumor.7 Recently, we reported that FasL is also expressed on the vascular endothelium, where it can function as a negative regulator of leukocyte extravasation.8

Potential adenovirus-mediated gene therapy strategies to treat proliferative vessel disorders include delivery of anti-thrombotic, antimigratory, cytostatic, or cytotoxic genes.1 These models of vascular gene therapy have all used immunologically naive laboratory animals. Because a large portion of the human patient population has developed immunity to several adenovirus serotypes, we evaluated the efficacy of adenovirus-mediated gene transfer in the rat carotid model of balloon injury in naive and immunologically primed animals.
Adenovirus-mediated FasL gene transfer was examined because it efficiently induces apoptosis in smooth muscle cells and can function to eliminate peripheral T cells after an immune response. For comparison, adenovirus-mediated gene transfer was performed with cdk inhibitor p21, a negative regulator of smooth muscle cell proliferation that has no known immunomodulatory function. FasL gene delivery was found to inhibit neointima formation in both naive and adenovirus-immunized animals, whereas p21 inhibits neointima formation only in naive animals. These data suggest that FasL gene transfer to the vessel wall can overcome the limitations imposed by host immune responses to repeated adenovirus delivery, leading to an inhibition of pathological cell proliferation in animals with preexisting immunity to adenovirus.

**Methods**

**Rat Carotid Artery Balloon-Injury Model, Histology, and Immunohistochemistry**

The left carotid arteries of male Sprague-Dawley rats (400 to 500 g) were injured with 2F embolectomy catheters (Baxter Edwards Healthcare) as described. Vessels were treated with the indicated viral solution (1×10^8 pfu) or saline for 15 minutes. Serotype 5 adenoviral vectors expressing murine FasL, human p21, bacterial β-galactosidase, or no transgene are deleted in E1 and E3 regions and express transgenes under the control of cytomegalovirus promoter. Rats were immunized by the intravenous administration of 1×10^8 pfu of the transgene-null adenoviral vector via the penile vein 10 days before balloon injury. Vessels harvested 1, 3, or 14 days after balloon injury were typically fixed in 100% methanol and embedded in paraffin. Sections were stained with hematoxylin and eosin, Richardson’s combination elastic tissue trichrome stain, Hoescht 33258, or TUNEL. Intimal and medial areas were measured with a computerized sketching program. For immunohistochemistry, sections were rehydrated, blocked with 5% goat serum and 0.01% Triton X-100 in PBS, and incubated with the T-cell marker antibodies anti-CD3 (Sigma) or anti-CD8 (Serotech) or the monocytic/macrophage marker anti-CD68 (Servec) for 1 hour. After they had been rinsed with PBS, biotinylated antibody to rabbit IgG or mouse IgG was applied for 20 minutes, followed by alkaline phosphatase–conjugated streptavidin. Antibody complexes were detected by the Fast Red system (BioGenex) followed by counterstaining with Mayer’s hematoxylin.

**Adenovirus Serotype 5–Specific Antibody Measurement**

Sera collected from naive and immunized rats at different time points were analyzed by ELISA essentially as described. Briefly, serial 2-fold dilutions of rat sera were added to wells coated with wild-type adenovirus 5. Bound virus-specific antibodies were detected by the addition of rabbit anti-rat IgG (Jackson ImmunoResearch Laboratories), followed by horseradish peroxidase–conjugated goat anti-rabbit IgG (Cappel-Organon Teknika) used as the secondary antibody. The serum titer was defined as the reciprocal of the highest dilution that produced an optical density at 490 nm ≤0.1.

**Data and Statistical Analysis**

All data were evaluated with 2-tailed, unpaired Student’s t test or compared by 1-way ANOVA followed by Fisher’s t test and were expressed as the mean±SEM. A value of P<0.05 was considered statistically significant.

**Results**

**Animal Model**

Two experimental groups of rats were analyzed in the study. In group 1, carotid arteries of immunologically naive rats (n=30) underwent balloon injury, immediately followed by the localized delivery of either saline (vehicle), Ad-β-gal (1×10^8 pfu), Ad-FasL (1×10^8 pfu), or Ad-p21 (1×10^8 pfu). In group 2, 38 rats were immunized against human adenovirus by intravenous injection of replication-defective adenoviral vector containing no insert (1×10^8 pfu). Animals were uniformly seronegative for adenovirus 5–specific antibodies at the time of immunization, and antibody titers of 955±437 (range, 400 to 1600) developed 2 weeks after immunizations when balloon injury was performed. Two weeks after immunization, left common carotid arteries of 20 immunized rats were balloon-injured and immediately treated with either saline (vehicle), Ad-β-gal, Ad-FasL, or Ad-p21 at the same viral titers as used in group 1. Most animals were euthanatized at 14 days after injury to assess intimal hyperplasia. In group 1, 10 animals were euthanatized at 24 hours after injury to assess apoptosis and cell density. At the time of harvest, all vessels were patent, and the medial layers appeared normal with regard to size and cellularity (Figure 1).

**Efficacy of Adenovirus-Mediated Gene Transfer**

Intimal hyperplasia in the naive rats (group 1) was greatest in vessels treated with saline or Ad-β-gal with intima/media ratios of 1.3 (Figure 1B through D). In this group, both Ad-FasL and Ad-p21 inhibited intimal hyperplasia by 60% and 58%, respectively (Figure 1K). No difference in medial area was detected in the Ad-FasL– or Ad-p21–treated vessels compared with saline- or Ad-β-gal–treated vessels. Ad-FasL–treated vessels harvested at 24 hours after injury revealed 5-fold more TUNEL-positive nuclei than in the Ad-β-gal control vessels (P<0.01). Many TUNEL-positive nuclei appeared pyknotic (Figure 1A). At this time point, medial cellularity was reduced by 41% (P<0.05) in the Ad-FasL–treated vessels relative to the Ad-β-gal control vessels, but medial cellularity returned to normal by 14 days.

Intimal hyperplasia in immunized animals (group 2) treated with saline or Ad-β-gal was similar to that in vessels observed in naive animals (Figure 1E through J). In this group, Ad-FasL treatment resulted in an intima/media ratio of 0.34±0.11, a 72% reduction compared with the Ad-β-gal control group (P<0.05) (Figure 1L). However, Ad-p21 was ineffective at inhibiting intimal hyperplasia in immunized rats.

**Inflammation in Vessels of Immunized Animals**

To test whether ectopic FasL expression can modulate the cellular immune response to adenoviral antigen, we performed immunohistochemical staining for CD3-positive T cells on sections of arteries harvested from immunized rats 3 or 14 days after injury and local gene delivery. Robust T-cell infiltration was observed in Ad-p21– and Ad-β-gal–treated vessels, but T-cell infiltration was markedly attenuated in Ad-FasL–treated vessels (Figure 2A through D). Surprisingly, T-cell accumulation occurred predominantly in the medial layers at the 14-day time point (Figure 2E). Ad-FasL–treated vessels had 76% (P<0.05) and 73% (P<0.05) fewer T cells than Ad-β-gal– and Ad-p21–treated vessels, respectively. T-cell infiltration in the Ad-FasL–treated vessels was not significantly different from that in the saline-treated
control vessels. Staining for CD8-positive T cells also revealed marked infiltration in the media of Ad-p21– and Ad-βgal–treated vessels, but CD8-positive cell infiltration was markedly reduced in Ad-FasL–treated vessels. Monocytes/macrophages were rarely observed in any vessels.

Discussion

A limitation of adenovirus-mediated gene therapy is the immune response that induces the destruction of genetically altered cells.3,4 Repeat administration of recombinant adenoviral vectors can be associated with confounding immune responses and low gene transfer efficiency, and high doses can promote an inflammatory response and intimal hyperplasia when administered locally to normal vessels of seronegative laboratory animals.2,12

In this study, we compared the efficacy of 2 genes, FasL and p21, for their ability to inhibit intimal hyperplasia in balloon-injured carotid arteries of rats that were either naive or immunized with an empty adenoviral vector. Both the cytostatic agent Ad-p21 and the cytotoxic agent Ad-FasL were effective at inhibiting intimal hyperplasia in naive rats, consistent with previous reports.9,10 However, only Ad-FasL inhibited neointima...
formation in immunized rats (Figure 1). Administration of recombinant adenovirus constructs elicits cellular and humoral responses to viral and foreign transgene products. In our study, animals were immunized with an adenovirus construct expressing no transgene to eliminate the immune response to the transgene-encoded proteins after the second adenovirus administration. Prior immunization resulted in the production of anti-adenovirus antibodies, and robust T-cell infiltration was noted in arteries that were subsequently reinfected with Ad-βgal or Ad-p21. In contrast, T-cell infiltration was markedly reduced in the immunized rats that received Ad-FasL (Figure 2), whereas levels of anti-adenovirus antibodies were unaffected (unpublished data). These data suggest that local delivery of FasL has 2 effects on the vessel wall: it induces apoptosis in Fas-bearing smooth muscle cells, and it suppresses the destructive response of T lymphocytes toward cells expressing viral proteins.

FasL is naturally expressed on the vascular endothelium, where it appears to function as an inhibitor of inflammatory cell infiltration. The data presented here suggest that local delivery of FasL to the denuded vessel wall essentially confers this anti-inflammatory property of the endothelium to the smooth muscle cells of the media. Therefore, the abilities of FasL to inhibit intimal hyperplasia and augment the endogenous immunosuppressive properties of the vessel wall make it uniquely suited for gene therapy of inflammatory-fibroproliferative disorders.

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References
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