Adenovirus-Mediated Gene Transfer of a Secreted Transforming Growth Factor-β Type II Receptor Inhibits Luminal Loss and Constrictive Remodeling After Coronary Angioplasty and Enhances Adventitial Collagen Deposition

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Background—Extracellular matrix (ECM) remodeling is central to the development of restenosis after coronary angioplasty (PTCA). As a regulator of ECM deposition by vascular cells, substantial evidence implicates transforming growth factor-β1 (TGF-β1) in the pathogenesis of restenosis. We investigated the effects of intracoronary expression of a transgenic antagonist of TGF-β1 on luminal loss after PTCA.

Methods and Results—Porcine coronary arteries were randomized to receive a recombinant adenovirus expressing a secreted form of TGF-β type II receptor (Ad5-RIIs), an adenovirus expressing β-galactosidase (Ad5-lacZ), or vehicle only by intramural injection at the site of PTCA. Computerized morphometry 28 days after angioplasty revealed a greater minimum luminal area in Ad5-RIIs–injected arteries (1.71±0.12 mm²) than in the Ad5-lacZ (1.33±0.13 mm²) or vehicle-only (1.08±0.17 mm²; P=0.010 by ANOVA) groups. This was accompanied by greater areas within the internal (P=0.013) and external (P=0.031) elastic lamiae in Ad5-RIIs–treated vessels. Adventitial collagen content at the site of injury was increased in the Ad5-RIIs group, in contrast to decreases in the Ad5-lacZ and vehicle-only groups (P=0.004).

Conclusions—Adenovirus-mediated antagonism of TGF-β1 at the site of PTCA reduces luminal loss after PTCA by inhibiting constrictive remodeling. Antagonism of TGF-β1 stimulates the formation of a dense collagenous adventitia, which prevents constrictive remodeling by acting as an external scaffold. These findings demonstrate the potential of gene therapy–mediated antagonism of TGF-β1 as prophylactic therapy for restenosis. (Circulation. 2001;104:2595-2601.)

Key Words: angioplasty ■ gene therapy ■ restenosis ■ collagen

The long-term success of percutaneous transluminal coronary angioplasty (PTCA) is limited by the high rate of restenosis, which occurs in 30% to 50% of vessels within 6 months of PTCA. Stent implantation is associated with reduced restenosis in vessels >3 mm in diameter; nonetheless, rates of 20% to 30% are observed. Two principal mechanisms contribute to restenosis: neointimal hyperplasia and constrictive remodeling. Extracellular matrix (ECM) composes ᰌ90% of the bulk of neointima, while adventitial accumulation of ECM in injured vessels contributes to the development of constrictive remodeling.

Transforming growth factor-β1 (TGF-β1) regulates ECM synthesis by smooth muscle cells (SMCs) and fibroblasts, increasing synthesis of fibronectin, thrombospondin, fibrillar collagens, elastin, and proteoglycans. All are present in increased quantities in vessels after injury. Accordingly, substantial evidence implicates TGF-β1 in the pathogenesis of restenosis: (1) increased levels of TGF-β1 occur in injured and restenotic vessels, (2) exposure of arteries to TGF-β1 after injury results in increased neointima formation, and (3) TGF-β1 induces the phenotypic modulation of adventitial fibroblasts to myofibroblasts, which are responsible for adventitial ECM deposition.

The secreted exoplasmic domain of the TGF-β type II receptor (TβRII) inhibits the biological effects of TGF-β1 in vitro. Because antagonism of TGF-β1 represents an attractive therapeutic target for the prevention of restenosis, we have undertaken a randomized trial using a recombinant adenovirus (Ad5-RIIs) expressing a secreted form of TβRII to test the hypothesis that localized inhibition of TGF-β1 will inhibit luminal loss after angioplasty. We report a significant reduction in vessel stenosis secondary to the presence of an
ventricular collagen “scaffold” at the site of injury in Ad5-RIIs–treated vessels, which prevents constrictive remodeling.

Methods

Generation of Recombinant Adenoviruses
Ad5-RIIs and Ad5-lacZ were generated as described previously. Ad5-RIIs contains a truncated human TβRII cDNA under transcriptional control of the major immediate-early human cytomegalovirus enhancer/promoter (MIEHCMV –700 to +50). Ad5-lacZ contains the cDNA for Escherichia coli β-galactosidase under control of MIEHCMV. Stocks were purified by CsCl gradient centrifugation and titered by serial-dilution end-point assay.

Cell Culture and Infection
Aortic SMCs from Large White pigs were cultured as described previously. SMCs were infected with Ad5-RIIs or Ad5-lacZ at a multiplicity of infection (MOI) of 10. After 48 hours, RIIs expression was confirmed immunocytochemically. β-Galactosidase expression was assessed by X-gal cytochemistry. For preparation of conditioned media, SMCs were infected with Ad5-RIIs or Ad5-lacZ at an MOI of 100. Forty-eight hours after infection, the media were assayed for TGF-β1 antagonist activity.

In Vitro Assay of TGF-β Activity
Mink lung epithelial cells stably transfected with the luciferase cDNA under control of a TGF-β-responsive promoter (MLEC(PAI/L)) were cultured as described previously. Conditioned media were supplemented with human TGF-β1 to concentrations of 0.01, 0.1, and 1.0 ng/mL; then 500 μL of each supplemented medium was added to 24-well plates seeded with MLEC(PAI/L). After overnight incubation, the luciferase activity in each well and the protein content in each cell lysate were assayed.

Porcine PTCA and Adenovirus Injection
Thirty Large White pigs (20 to 28 kg) underwent PTCA. Procedures conformed to the UK Animals (Scientific Procedures) Act 1986 and were authorized by the Home Office. Animals received 150 mg of aspirin 24 hours before and every 48 hours after PTCA. Before each procedure, vessels were randomized to receive 2 x 10^9 infectious particles (IU) of Ad5-RIIs, 2 x 10^9 IU of Ad5-lacZ, or PBS only. Anesthesia was induced by inhalation of 4% halothane. An endotracheal tube was inserted, and anesthesia was maintained with 2% halothane. The left carotid artery was exposed, and an 8F guide catheter was inserted, and anesthesia was maintained with 2% halothane. Heparin 2500 IU was injected before coronary artery cannulation. Glyceryl trinitrate 200 mg was injected into each coronary artery and angiography was performed with a digital image intensifier. Vessel segments in the left anterior descending and/or coronary artery, and angiography was performed with a digital image intensifier. Vessel segments in the left anterior descending and/or right coronary arteries (diameter 2.0 to 2.5 mm) were selected for angioplasty by a blinded operator. A 3.0-mm Inflator catheter (Interventional Technologies) was used for injury (2 x 30 seconds at 8 atm), followed by virus/PBS injection. The Inflator was removed, 200 μg of glycerol trinitrate injected, and a postinjury angiogram performed. The carotid artery was ligated, the neck incision was closed, and the animals were allowed to recover. To assess the efficiency of transgene delivery, host inflammatory responses, and transgene expression in vivo, 2 vessels received nonrandomized injection of 2 x 10^9 IU of Ad5-RIIs, 2 received 2 x 10^9 IU of Ad5-lacZ, and 1 received PBS. These animals were euthanized 3 days after PTCA. Those randomized for morphometric assessment were euthanized 28 days after PTCA.

Efficiency of Transgene Delivery and Inflammatory Response
Infected vessel segments were dissected out, snap-frozen in liquid nitrogen, and embedded in OCT before cryostat sectioning. β-Galactosidase expression was assessed by X-gal cytochemistry. Sections were counterstained with neutral red, and the proportional area of vessel wall X-gal staining was measured with a Leica Quantimet 600S digital analysis system. Inflammatory infiltration was assessed by immunohistochemical staining for CD45 (leukocyte common antigen). Proportional areas of vessel wall staining were calculated as staining area in presence of primary antibody minus staining area in presence of secondary antibody only.

RT-PCR and Western Blotting
Vessel segments were dissected out and placed in 4 mol/L guanidine isothiocyanate (RNA extraction) or snap-frozen in liquid nitrogen (protein extraction). RNA specimens were homogenized, and total RNA was isolated by acidified phenol/chloroform extraction. Reverse transcription–polymerase chain reaction (RT-PCR) was performed with the following primers: forward, 5'-TTAATAACGAGCATGATAGTCAC-3'; reverse, 5'-TCATTGACCTCATCAGAGC-3'. RT-PCR was also performed with a reverse poly(dT) primer. For protein extraction, equal amounts of protein from both homogenized vessels were separated by PAGE and transferred onto polyvinylidine difluoride membrane. The membrane was probed with polyclonal rabbit antibody to the extracellular domain of TβRII (Upstate Biotechnology). A second blot was probed with rabbit IgG. Secondary anti–rabbit horseradish peroxidase conjugate (Dako) was diluted to 1:2000. Bound antibodies were detected with an enhanced chemiluminescence detection system.

Morphometric Analysis
Arteries were pressure-fixed in situ with 4% formaldehyde. Injured segments were excised with proximal and distal uninjured segments and fixed overnight. Fixed vessels were cut into 3-mm segments, dehydrated, and embedded in parafin. Sections from each segment were stained with Accustain (Sigma Diagnostics) and examined with a Leica Quanmtet 600S digital analysis system. The areas bounded by the luminal surface (lumen area), by the internal elastic lamina (IEL area: at IEL fracture sites, fractured ends were linked by a line describing the boundary between neointima and media), and by the external elastic lamina (EEL area) were measured in the segment with minimum luminal area (MLA) from each vessel with IEL fracture length and injury scores by a blinded operator. Neointimal and medial areas were calculated as IEL area minus lumen area and MLA minus IEL area, respectively.

Quantification of Collagen Content
Collagen content was evaluated in picrosirius red–stained sections from the MLA segment and uninjured proximal segments from each vessel. Sections were examined under identical conditions with circularly polarized light on a Leica Quanmtet 600S digital analysis system. The collagen areas of the total vessel, media plus neointima, and adventitia were determined as the total area of nonzero pixels in each region.

Statistical Analysis
Multiple groups were analyzed by 1-way ANOVA. Comparisons were made between groups by Newman-Keuls post hoc test. Collagen areas from paired proximal and MLA segments were compared by paired t tests.

Results

Expression of RIIs and β-Galactosidase in Cultured SMCs
Two days after Ad5-RIIs infection, RIIs-immunopositive SMCs were observed (Figure 1a). Two days after Ad5-lacZ infection, SMCs showing blue cytoplasmic staining with X-gal were discernible (Figure 1b). No RIIs immunopositivity was observed in Ad5-lacZ–infected SMCs. No X-gal staining was observed in Ad5-RIIs–infected cells.

Antagonism of TGF-β1 by Conditioned Medium From Ad5-RIIs–Infected SMCs
Conditioned medium from Ad5-RIIs–infected SMCs inhibited TGF-β1–mediated induction of luciferase in
MLEC(PAI/L) at all concentrations (Figure 1c; \(P < 0.0005\), \(P < 0.0001\), and \(P < 0.0001\) at 0.01, 0.1, and 1 ng/mL, respectively). Luciferase activity induced by 1 ng/mL TGF-\(\beta\) in the presence of Ad5-RIIs–conditioned medium was significantly lower than that induced by 0.01 ng/mL TGF-\(\beta\) in uninfected conditioned medium (\(P < 0.02\)). RII is thus capable of reducing the biological activity of TGF-\(\beta\) by >100-fold.

Efficiency of Transgene Delivery and Inflammatory Response in Vivo

Assessment of X-gal staining within the coronary artery 72 hours after injection of Ad5-\(\text{lacZ}\) revealed staining in 16.1±2.85% of the vessel wall area (Figure 2a). Immunohistochemical staining for CD45 revealed significantly greater leukocyte infiltration in vessels 72 hours after injection of Ad5-\(\text{lacZ}\) than in Ad5-RIIs– or PBS-injected vessels (Figure 2b through 2e; \(P < 0.0001\)). Infiltration was not significantly greater in Ad5-RIIs–injected vessels than in PBS-injected.

Expression of RII in Porcine Coronary Arteries After Ad5-RIIs Infection

RT-PCR revealed RII mRNA within the coronary artery 72 hours after injection of Ad5-RIIs (Figure 3a). No band was obtained from the Ad5-\(\text{lacZ}\)–injected artery. No band was obtained by PCR without reverse transcription. Western blotting of protein extracts from the Ad5-RIIs–infected vessel revealed a band of \(\approx 15\) kDa, which was absent from the Ad5-\(\text{lacZ}\)–infected vessel and undetected in the absence of anti-RII (Figure 3b). The predicted mass of RII is 14.6 kDa.

Effect of Ad5-RIIs on Luminal Loss and Constrictive Remodeling After PTCA

Twelve arteries each from the Ad5-RIIs–infected and Ad5-\(\text{lacZ}\)–infected groups and 9 from the PBS group were suitable for analysis (Figure 4a through 4c). No differences in angiographic diameters before and immediately after PTCA, lumen area and collagen content of the proximal reference segments, injury score, or IEL fracture length at the site of MLA were observed between groups (data not shown).

Mean MLA was larger in the Ad5-RIIs group than in either control group (Figures 4a through 4c and 5a; \(P < 0.01\)). The IEL (Figure 5b) and EEL (Figure 5c) areas at the site of MLA were also larger in the Ad5-RIIs group (\(P < 0.01\) and \(P < 0.03\), respectively). Luminal loss at the site of MLA compared with the proximal reference segment was also significantly reduced in the Ad5-RIIs group (Figure 5d; \(P < 0.0026\)). No differences in neointimal area, medial area, or intima:media ratio were observed, although comparison of

Figure 1. Transgene expression in SMCs after infection with Ad5-RIIs and Ad5-\(\text{lacZ}\) in vitro. a, RII immunostaining in SMCs 2 days after infection with Ad5-RIIs. Brown-stained SMCs are immunopositive for extracellular domain of human type II TGF-\(\beta\) receptor. b, Cytoplasmic staining with X-gal in SMCs 2 days after Ad5-\(\text{lacZ}\) infection. Blue SMCs express \(\beta\)-galactosidase. Bars=10 \(\mu\)m. c, Inhibition of TGF-\(\beta\) activity in vitro by conditioned medium from Ad5-RIIs–infected SMCs. Induction of luciferase expression in MLEC(PAI/L) by TGF-\(\beta\)–supplemented conditioned medium is significantly lower in cells exposed to medium from Ad5-RIIs–infected SMCs than in cells exposed to medium from Ad5-\(\text{lacZ}\)–infected or uninfected SMCs across a 100-fold range of concentrations of TGF-\(\beta\). No significant difference between Ad5-\(\text{lacZ}\)–infected and uninfected conditioned medium was observed.
the Ad5-RIIs group with the pooled Ad5-lacZ and PBS group data revealed a reduced intima:media ratio in the Ad5-RIIs group (1.09 ± 0.18 versus 1.83 ± 0.29; P = 0.038), suggesting a minor effect on neointima formation.

**Effect of Ad5-RIIs on Collagen Content After PTCA**

Collagen area at the site of MLA in the Ad5-RIIs group was greater than in the Ad5-lacZ or PBS groups (Figures 4d through 4f and 6a; P = 0.02). The collagen areas within the adventitia and media plus neointima were also greater in the Ad5-RIIs group (P = 0.028 and P = 0.0001, respectively). Comparison of collagen area at the site of MLA with that of the proximal reference segment revealed no change in the Ad5-lacZ group, a decrease (P = 0.016) in the PBS group, and an increase (P = 0.032) in the Ad5-RIIs group (Figure 6b; P = 0.004 by ANOVA). Increased collagen area in the Ad5-RIIs group was attributable to increased adventitial collagen (P = 0.019), in contrast to unchanged adventitial collagen in the Ad5-lacZ group and a decrease (P = 0.026) in the PBS group (Figure 6b; P = 0.003 by ANOVA). Reduced medial plus neointimal collagen was seen in all groups. This was significantly less marked in the Ad5-RIIs group (Figure 6b; P = 0.013 by ANOVA).

**Discussion**

We have investigated the potential for localized antagonism of TGF-β1 to inhibit (re)stenosis after porcine coronary angioplasty.
angioplasty and have shown that injection of Ad5-RIIs at the time of PTCA results in a 40% reduction in luminal loss 28 days after injury. Increased MLA is due to reduced constrictive remodeling, determined by significant increases in IEL and EEL areas. These areas (rather than perimeters) were used as indices of remodeling because they are the parameters proven to correlate with luminal loss in the first descriptions of constrictive remodeling. Surprisingly, we observed an increase in collagen content in Ad5-RIIs–treated vessels due to increased adventitial collagen content at the site of injury, in contrast to decreased collagen in control groups. This suggests that the principal effect of TGF-β1 on coronary ECM in our control groups was stimulation of collagen degradation. This seems contrary to the effects of TGF-β1 on ECM synthesis by SMCs in vitro and to previous studies of modulation of TGF-β1 activity after rat carotid artery injury, which reported decreased collagen content in association with luminal preservation in vessels exposed to TGF-β1 antagonists. Our findings, however, are in keeping with previous observations that collagen content is significantly lower in restenotic vessels than in nonrestenotic.

Figure 5. Morphometric assessment of lumen loss and constrictive remodeling at site of MLA 28 days after PTCA. MLA (a), IEL area (b), and EEL area (c) are significantly greater in Ad5-RIIs group. Percent luminal loss at site of MLA vs proximal uninjured segment is significantly reduced (d). No significant difference was detected between Ad5-lacZ and PBS groups for any parameter. Percent luminal loss = 100 × [1 − (MLA/lumen area proximal reference segment)].

Figure 6. Collagen content at site of MLA 28 days after PTCA. Collagen content in all layers of vessel wall is greater in Ad5-RIIs group (a). Comparison of collagen content at site of MLA with proximal reference segment shows increased total vessel and adventitial collagen in Ad5-RIIs group and decreased collagen in control groups (b). Collagen is decreased in media plus neointima in all groups. This is significantly less marked in Ad5-RIIs group. No significant difference was detected between Ad5-lacZ and PBS groups for any parameter.
It is unlikely that virus-induced inflammatory responses within the vessel wall are responsible for increased collagen content in the Ad5-RIIs group, because collagen content is significantly lower in the Ad5-lacZ group despite Ad5-lacZ eliciting significantly greater leukocyte infiltration 72 hours after intracoronary injection. It is possible that RIIs may act as a local enhancer of TGF-β1, increasing ECM deposition and reducing collagen degradation within the vessel. Potentiation of the biological activity of TGF-β1 was not observed in this study in vitro, however, and has not been observed previously with virtually identical molecules.\textsuperscript{13,20} It seems more likely that differences in the responses of cells from different vessels to different mechanisms of injury might account for our observations.

All previous studies of TGF-β antagonism after vascular injury have been performed in noncoronary vessels. The responses of porcine coronary SMCs to TGF-β1 in vitro differ from those of rat arterial SMCs,\textsuperscript{23} and coronary SMCs display distinct behavior from noncoronary SMCs in vitro, leading to the suggestion that repair mechanisms may differ between coronary and noncoronary vessels.\textsuperscript{24} The stimulatory effect of TGF-β1 on collagen synthesis by porcine SMCs in vitro is significantly less marked in cells cultured on collagen matrices than on monolayers.\textsuperscript{25} This also appears to be true for porcine fibroblasts in vivo,\textsuperscript{26} suggesting that the fibrogenic effects of TGF-β1 on coronary SMCs in vivo, where cells are surrounded by basement membrane within a 3D collagen matrix, may differ from those observed in noncoronary SMC monolayers.

The mechanism of injury used in noncoronary angioplasty is distinct from that in PTCA. Fogarty balloon injury results in low-pressure, high-stretch injury, whereas PTCA involves a high-pressure, low-stretch injury. Differences in matrix metalloproteinase (MMP) expression and cellular migration have been reported after different mechanisms of injury to rat carotids.\textsuperscript{27} The difference observed between the I:M ratio in our control groups (≈1.8) and those in injured rat carotids (≈1.0\textsuperscript{28} and ≈0.9\textsuperscript{29}) supports the hypothesis that the response of pig coronary arteries to PTCA differs significantly from the response of rat carotids to Fogarty balloon injury.

The principal action of TGF-β1 in vascular remodeling appears to be autocrine induction of the phenotypic modulation of adventitial fibroblasts to myofibroblasts,\textsuperscript{12} which subsequently synthesize collagen within the adventitia. How antagonism of TGF-β1 might reduce collagen degradation is uncertain. The effects of TGF-β1 on MMP expression are typically downregulatory, TGF-β1, however, is able to upregulate expression of MMP-2 and MMP-9 (72-kDa and 92-kDa type IV collagenases),\textsuperscript{28} which include collagens I, III, IV, V, and XI as substrates. MMP-2 and MMP-9 enhance cell migration through ECM and are expressed in injured arteries\textsuperscript{29,30} and adventitial explants from porcine coronaries.\textsuperscript{31} Because type IV collagenase activity is essential for cell migration through basement membranes,\textsuperscript{32} it is interesting to speculate that abrogation of TGF-β1-mediated induction of MMP-2 and MMP-9 within the adventitia and media might suppress collagen degradation and subsequent cellular migration. Furthermore, MMP-9 can also activate latent TGF-β1.\textsuperscript{33} If activation of TGF-β1 is reduced, its inhibition may further suppress phenotypic modulation of fibroblasts to myofibroblasts within the adventitia.

In conclusion, this study shows that adenovirus-mediated delivery of a truncated TβRII to porcine coronary arteries at the time of angioplasty results in a 40% increase in MLA 28 days after PTCA. This is achieved via inhibition of constrictive remodeling secondary to an increase in adventitial collagen content, implying inhibition of some effect of TGF-β1 on collagen degradation after PTCA. Our observations suggest that constrictive remodeling is inhibited by the creation of an adventitial collagen scaffold at the site of injury, which maintains the vessel circumference, preventing loss of area within the IEL and EEL. This further implies that restenosis occurs when the external support provided by the adventitia is diminished by collagen degradation after injury. Our findings also suggest that there are important differences between the responses of carotid arteries to Fogarty balloon injury and those of coronary arteries to PTCA and raise a query over the appropriateness of noncoronary models as a means of assessing mechanisms of or potential therapies for coronary pathology.

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