

# Adenovirus-Mediated Gene Transfer of Transforming Growth Factor- $\beta_3$ , but Not Transforming Growth Factor- $\beta_1$ , Inhibits Constrictive Remodeling and Reduces Luminal Loss After Coronary Angioplasty

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**Background**—Extracellular matrix (ECM) remodeling is central to the development of restenosis after PTCA. Substantial evidence implicates transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), a regulator of ECM deposition by vascular cells, in its pathogenesis. TGF- $\beta_3$  reduces TGF- $\beta_1$ —induced ECM deposition in cutaneous wounds. We therefore investigated the effects of intracoronary expression of TGF- $\beta_3$  and TGF- $\beta_1$  on luminal loss after angioplasty.

**Methods and Results**—Porcine coronary arteries received an adenovirus expressing TGF- $\beta_3$ , TGF- $\beta_1$ , or *lacZ* ( $\beta$ -galactosidase), or PBS only, at the site of angioplasty. Morphometric analysis 28 days after angioplasty confirmed reduced luminal loss in TGF- $\beta_3$  vessels ( $-0.65 \pm 0.10$  mm<sup>2</sup>) compared with *lacZ* ( $-1.18 \pm 0.19$  mm<sup>2</sup>) or PBS only ( $-1.19 \pm 0.17$  mm<sup>2</sup>;  $P=0.003$ ). Luminal loss was not reduced in TGF- $\beta_1$  vessels ( $-1.02 \pm 0.19$  mm<sup>2</sup>;  $P=0.48$ ). An increase in the external elastic lamina area in TGF- $\beta_3$ —treated vessels ( $+0.73 \pm 0.32$  mm<sup>2</sup>) contrasted with decreases in control vessels (mean,  $-0.53 \pm 0.17$  mm<sup>2</sup>;  $P=0.001$ ) and TGF- $\beta_1$  vessels ( $-0.87 \pm 0.34$  mm<sup>2</sup>;  $P=0.003$ ). Collagen content increased at the site of injury in TGF- $\beta_3$ —treated vessels ( $26.1 \pm 14.2\%$ ) but decreased in the *lacZ* ( $-22.8 \pm 6.6\%$ ) and PBS-only ( $-23.4 \pm 7.0\%$ ;  $P=0.002$ ) groups and was not significantly changed in TGF- $\beta_1$ —treated vessels.

**Conclusions**—Expression of TGF- $\beta_3$  inhibits constrictive remodeling after PTCA and reduces luminal loss. This is accompanied by increased adventitial collagen, which may act as an external “scaffold” preventing vessel constriction. These findings confirm the potential of gene therapies that modify ECM remodeling for prophylaxis of restenosis. (*Circulation*. 2003;108:2819-2825.)

**Key Words:** angioplasty ■ gene therapy ■ restenosis ■ collagen

Restenosis remains the principal constraint on the long-term success of coronary angioplasty (PTCA), significant luminal loss occurring in 30% to 50% of arteries within 6 months.<sup>1</sup> Stents may reduce restenosis, but rates of 20% to 30% can still occur after coronary stenting.<sup>1</sup> Extracellular matrix (ECM) deposition is central to the pathogenesis of restenosis: 7 days after injury, collagen accounts for  $\approx 25\%$  of vessel wall protein. At 30 days after injury, this proportion is  $>50\%$ .<sup>2</sup> ECM composes  $\approx 90\%$  of neointimal bulk in injured vessels.<sup>3</sup> Adventitial matrix accumulation is observed early after coronary injury and appears to contribute to constrictive remodeling.<sup>4</sup>

Transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) increases synthesis, by smooth muscle cells (SMCs) and fibroblasts in vitro, of numerous ECM components that modulate SMC migration/proliferation and are present in increased quantities in

vessels after injury (fibronectin,<sup>5</sup> thrombospondin,<sup>6</sup> osteopontin,<sup>7</sup> fibrillar collagens,<sup>8</sup> elastin,<sup>9</sup> and proteoglycans<sup>10</sup>). Accordingly, TGF- $\beta_1$  is implicated in the pathogenesis of restenosis: increased TGF- $\beta_1$  levels occur in restenotic vessels,<sup>11</sup> arteries exposed to TGF- $\beta_1$  after injury display increased neointimal hyperplasia (NH),<sup>12</sup> and TGF- $\beta_1$  appears to induce phenotypic modulation of adventitial fibroblasts to myofibroblasts.<sup>13</sup>

TGF- $\beta_3$  downregulates TGF- $\beta_1$ —induced ECM expression in fibroblasts,<sup>14</sup> suppresses phenotypic modulation of fibroblasts to myofibroblasts,<sup>15</sup> and reduces TGF- $\beta_1$ —induced ECM deposition in healing cutaneous wounds.<sup>16</sup> We have demonstrated that antagonism of TGF- $\beta_1$  after PTCA by adenovirus-mediated expression of a secreted type II TGF- $\beta$  receptor reduces vessel stenosis.<sup>17</sup> To test the hypothesis that localized expression of TGF- $\beta_3$  will inhibit luminal loss after

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angioplasty by antagonism of TGF- $\beta_1$ , we have undertaken a study using adenoviruses expressing TGF- $\beta_3$  (Ad5-TGF- $\beta_3$ ) and TGF- $\beta_1$  (RAD-TGF- $\beta_1$ ). We report a reduction in luminal loss and inhibition of constrictive remodeling in Ad5-TGF- $\beta_3$ -treated vessels, which is associated with increased adventitial collagen at the site of injury but is not observed in TGF- $\beta_1$ -treated vessels.

## Methods

### Generation of Recombinant Adenoviruses

Viruses were generated as described previously.<sup>17</sup> Ad5-TGF- $\beta_3$  and RAD-TGF- $\beta_1$  contain cDNAs for human TGF- $\beta_3$  and TGF- $\beta_1$ , respectively under transcriptional regulation of the major immediate-early human cytomegalovirus enhancer/promoter. Stocks were purified by CsCl gradient centrifugation and titered by serial-dilution end-point assay.

### Cell Culture and Infection

Porcine SMCs were cultured as described previously.<sup>17</sup> For preparation of conditioned media, SMCs were infected with Ad5-TGF- $\beta_3$ , RAD-TGF- $\beta_1$ , or Ad5-*lacZ* (expressing  $\beta$ -galactosidase) at multiplicity of infection of 100. At 48 hours after infection, media were assayed for TGF- $\beta$  activity.

### In Vitro Assay of TGF- $\beta$ Activity

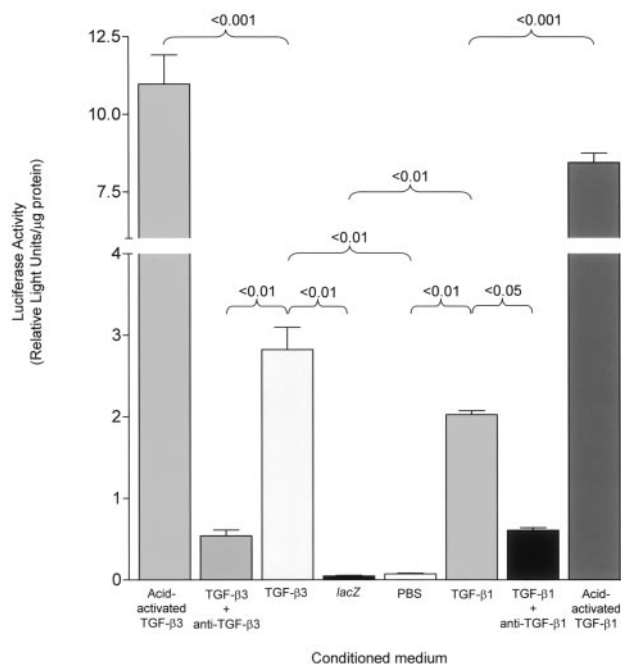
Mink lung epithelial cells for plasminogen activator inhibitor/luciferase assay [MLEC(PAI/L)] were cultured as described previously.<sup>17</sup> Conditioned media from infected SMCs were added to 24-well plates seeded with MLEC(PAI/L). After overnight incubation, luciferase activity and protein content in each cell lysate were assayed.

### PTCA and Adenovirus Injection

Thirty-seven Large White pigs (20 to 28 kg) underwent PTCA. Procedures conformed to the Animals (Scientific Procedures) Act of 1986 and were authorized by the Home Office. Animals received 150 mg of aspirin 24 hours before and every 48 hours after PTCA. Vessels were randomized to receive  $2 \times 10^9$  IU of Ad5-TGF- $\beta_3$ , RAD-TGF- $\beta_1$ , or Ad5-*lacZ*, or PBS only. Anesthesia was induced by isoflurane inhalation. An endotracheal tube was inserted, and anesthesia was maintained with 2% isoflurane. The left carotid artery was exposed, an 8F guide catheter inserted, and 2500 IU of heparin injected. Then 200  $\mu$ g of glyceryl trinitrate was injected into each coronary artery, and angiography was performed. Segments of the left anterior descending and/or right coronary artery were selected by an operator blinded to the allocated treatment group. Suitable segments were 2.0 to 2.5 mm in diameter, were  $\geq 15$  mm long, and had no significant side branches. A 3.0-mm Infiltrator catheter was used for injury (8 atm,  $2 \times 30$  seconds), followed by virus/PBS injection. A postinjury angiogram was performed after injection of a further 200  $\mu$ g of glyceryl trinitrate. The carotid artery was ligated, the neck incision was closed, and the animals were allowed to recover. To assess transgene expression *in vivo*, 3 animals received nonrandomized injection of virus. These were euthanized 3 days after PTCA. Blocks of infected vessel were embedded in OCT and snap-frozen in liquid nitrogen for immunohistochemistry.

### Detection of Transgene Expression by Immunohistochemistry

Immunohistochemistry was performed on 7- $\mu$ m-thick cryostat sections. Primary anti-TGF- $\beta_3$  antibody (Santa Cruz) or primary anti-TGF- $\beta_1$  antibody (Serotec) was diluted to 1:1000. Secondary anti-rabbit-horseradish peroxidase conjugate (Dako) was diluted to 1:2000. Sections were counterstained with neutral red or hematoxylin.



**Figure 1.** TGF- $\beta_3$  and TGF- $\beta_1$  activity in conditioned medium from Ad5-TGF- $\beta_3$ - and RAD-TGF- $\beta_1$ -infected SMCs. Induction of luciferase expression is seen in MLEC(PAI/L) exposed to conditioned medium from Ad5-TGF- $\beta_3$ - and RAD-TGF- $\beta_1$ -infected SMCs. Expression is almost undetectable in MLEC(PAI/L) exposed to control conditioned medium. Preincubation of conditioned media with specific neutralizing antibodies results in  $\approx 4$ - to 5-fold reduction in luciferase expression. Acid activation of conditioned medium before incubation with MLEC(PAI/L) results in an  $\approx 4$ -fold increase in luciferase expression.

### Morphometric Analysis and Quantification of Collagen Content

Processing and morphometric analysis of sections from the site of minimum luminal area (MLA) and most distal uninjured vessel segment proximal to the angioplasty site (proximal reference segment, PRS) were performed as described previously<sup>17</sup> by an operator blinded to the identity of each vessel. Explanted hearts were compared with post-PTCA angiograms to ensure that correct vessel segments were harvested. Vessels without internal elastic lamina (IEL) fracture in  $\geq 2$  adjacent blocks were excluded on the basis of inadequate injury. Collagen content was evaluated as the total area of nonzero pixels in picrosirius red-stained sections viewed by use of circularly polarized light.<sup>17</sup>

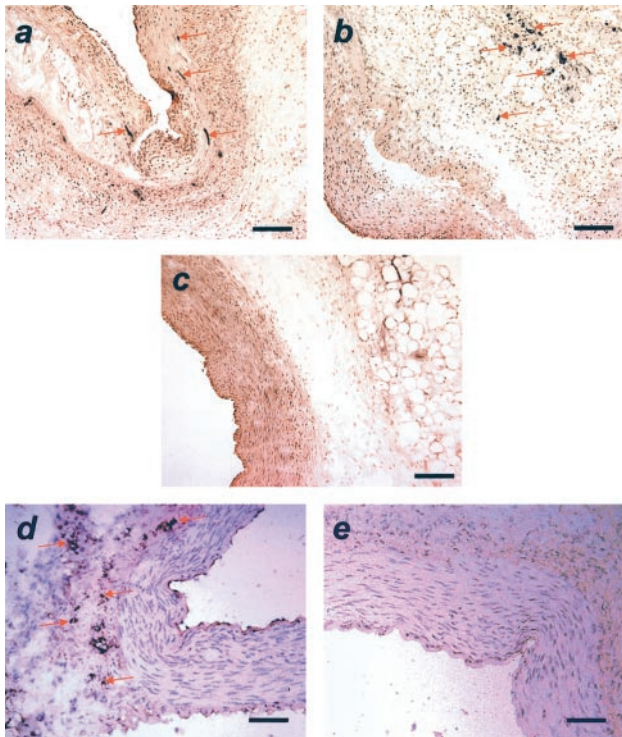
### Statistical Analysis

Multiple groups were analyzed by 1-way ANOVA and Newman-Keuls post hoc test. Paired morphometric parameters and collagen areas from PRS and MLA sites were compared by Wilcoxon matched-pairs test.

## Results

### Biological Activity of TGF- $\beta_3$ and TGF- $\beta_1$ In Vitro

Conditioned medium from Ad5-TGF- $\beta_3$ - and RAD-TGF- $\beta_1$ -infected SMCs induced  $\approx 40$ - to 50-fold greater luciferase activity in MLEC(PAI/L) than control conditioned medium (Figure 1; all  $P < 0.01$ ). Preincubation of Ad5-TGF- $\beta_3$ -conditioned medium with anti-TGF- $\beta_3$  antibodies and RAD-TGF- $\beta_1$ -conditioned medium with anti-TGF- $\beta_1$  reduced luciferase induction by both to levels not significantly different from control. Acid-activation of Ad5-TGF- $\beta_3$ - and RAD-



**Figure 2.** Transgene expression in Ad5-TGF- $\beta_3$ - and RAD-TGF- $\beta_1$ -infected coronary arteries. Immunohistochemistry reveals TGF- $\beta_3$ -immunopositive cells in media (a) and adventitia (b) of Ad5-TGF- $\beta_3$ -infected vessels probed with anti-TGF- $\beta_3$  (arrows). TGF- $\beta_1$  immunopositivity is visible in adventitia (d) of RAD-TGF- $\beta_1$ -infected vessels probed with anti-TGF- $\beta_1$  (arrows). No immunopositivity is visible in Ad5-*lacZ*-infected artery probed with anti-TGF- $\beta_3$  (c) or anti-TGF- $\beta_1$  (e). Bars=50  $\mu$ m.

TGF- $\beta_1$ -conditioned medium before incubation increased luciferase expression  $\approx$ 4-fold in both conditioned media. Conditioned media from SMCs infected with Ad5-TGF- $\beta_3$  and RAD-TGF- $\beta_1$  thus contain wild-type human TGF- $\beta_3$  and wild-type TGF- $\beta_1$ , respectively: activity is inhibited by specific neutralizing antibodies, and most is secreted in latent form.

### Transgene Expression in Ad5-TGF- $\beta_3$ - and RAD-TGF- $\beta_1$ -Infected Coronary Arteries

Immunohistochemistry revealed TGF- $\beta_3$ -immunopositive cells within the media and adventitia of Ad5-TGF- $\beta_3$ -infected arteries (Figure 2, a and b). TGF- $\beta_3$  immunopositivity was not observed in Ad5-*lacZ*-infected arteries (Figure 2c). Immunostaining for TGF- $\beta_1$  was observed in the adventitia of the RAD-TGF- $\beta_1$ -infected vessel (Figure 2d), but not in Ad5-*lacZ*-infected vessels (Figure 2e).

### Effect of Transgene Expression on Luminal Loss and Constrictive Remodeling

Twelve arteries were allocated to each group. Eleven Ad5-*lacZ*-infected arteries, 10 RAD-TGF- $\beta_1$ -infected vessels, and 9 each from the Ad5-TGF- $\beta_3$ -infected and PBS groups were suitable for analysis. No differences in MLA, areas within the internal and external elastic laminae (IEL and EEL areas) at the site of MLA, or severity of injury were observed (Table). Comparison of PRS morphometric parameters, however, revealed significant differences between groups (Table), suggesting that comparison of absolute morphometric values at the site of MLA could miss significant effects on outcome after PTCA and gene transfer. Changes in morphometric parameters at the MLA site relative to the PRS were therefore compared.

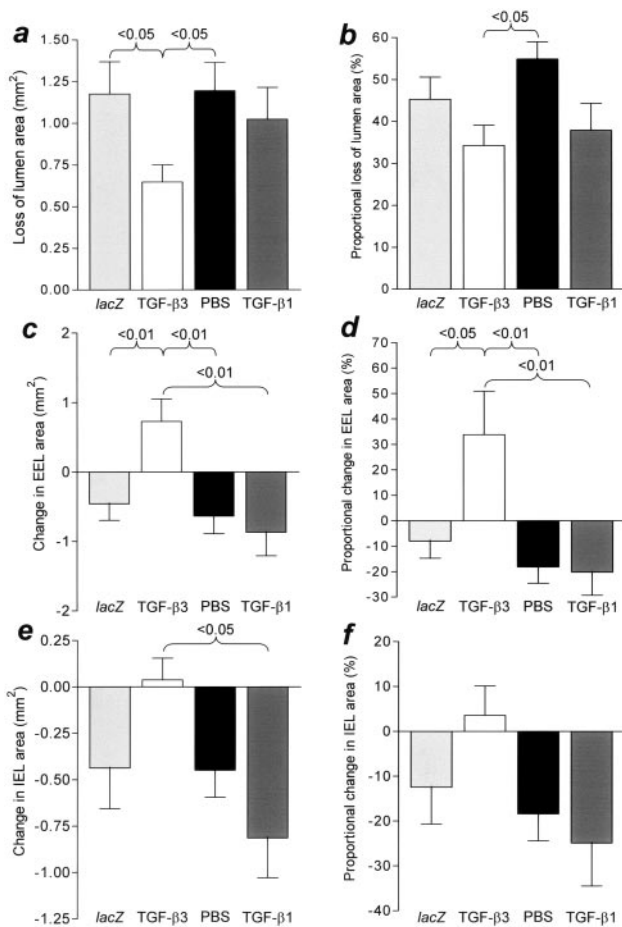
Significant differences between actual (Figure 3a) and proportional (Figure 3b) luminal loss and actual (Figure 3c) and proportional (Figure 3d) loss of EEL area at the site of MLA were observed. Proportional loss of lumen area in the TGF- $\beta_3$  group ( $34.2 \pm 4.9\%$ ) was 31% less than mean loss in the *lacZ* and PBS groups ( $49.3 \pm 3.6\%$ ;  $P=0.02$ ). Actual luminal loss was reduced by 45% in the TGF- $\beta_3$  group (TGF- $\beta_3$ ,  $0.65 \pm 0.10$  mm<sup>2</sup>; *lacZ*+PBS,  $1.18 \pm 0.13$  mm<sup>2</sup>;  $P=0.01$ ). Proportional and actual luminal loss in the TGF- $\beta_1$  group ( $37.9 \pm 6.4\%$  and  $1.02 \pm 0.19$  mm<sup>2</sup>, respectively) were 23% less and 14% less than the mean losses in the *lacZ* and PBS groups. Neither reduction was significant.

Increased EEL area was observed in the TGF- $\beta_3$  group (actual,  $+0.73 \pm 0.32$  mm<sup>2</sup>; proportional,  $+32.6 \pm 15.1\%$ ; Figure 3, c and d) in contrast to reduced mean EEL areas in *lacZ*

### Pre-PTCA Angiographic Vessel Areas and Morphometric Parameters at the PRS and Site of MLA

	<i>lacZ</i>	TGF- $\beta_3$	PBS	TGF- $\beta_1$	<i>P</i>
Angiographic pre-PTCA area	$4.37 \pm 0.18$	$4.01 \pm 0.18$	$4.41 \pm 0.19$	$4.19 \pm 0.19$	0.43
PRS					
Lumen area	$2.50 \pm 0.20$	$1.81 \pm 0.14$	$2.15 \pm 0.22$	$2.57 \pm 0.30$	0.064
IEL area	$2.54 \pm 0.20$	$1.85 \pm 0.14$	$2.17 \pm 0.22$	$2.70 \pm 0.30$	0.063
EEL area	$3.32 \pm 0.24$	$2.52 \pm 0.15$	$2.99 \pm 0.28$	$3.75 \pm 0.44$	0.033
MLA					
Lumen area	$1.33 \pm 0.13$	$1.20 \pm 0.10$	$1.08 \pm 0.17$	$1.54 \pm 0.22$	0.26
IEL area	$2.07 \pm 0.13$	$1.92 \pm 0.10$	$1.83 \pm 0.19$	$1.89 \pm 0.23$	0.77
EEL area	$2.82 \pm 0.19$	$3.29 \pm 0.27$	$2.50 \pm 0.21$	$2.87 \pm 0.42$	0.32
Injury score	$2.77 \pm 0.12$	$2.50 \pm 0.17$	$2.67 \pm 0.17$	$2.70 \pm 0.15$	0.63
Fracture length, mm	$1.51 \pm 0.20$	$1.23 \pm 0.15$	$1.54 \pm 0.19$	$1.66 \pm 0.24$	0.53

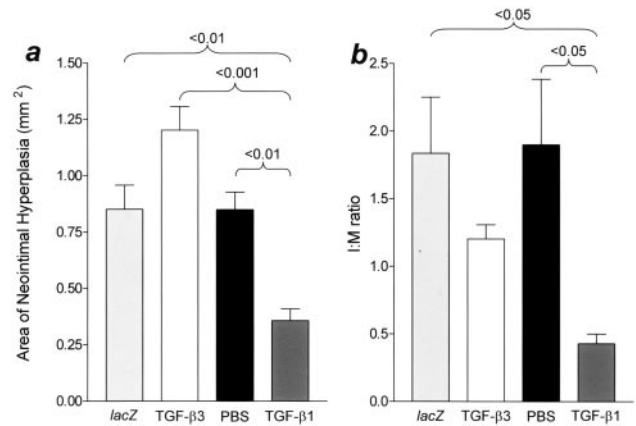
Values are mean  $\pm$  SEM. All areas are measured in mm<sup>2</sup> unless otherwise indicated.



**Figure 3.** Luminal loss and constrictive remodeling at site of MLA 28 days after PTCA. Actual and proportional changes of lumen area (a and b), EEL area (c and d), and IEL area (e and f) at site of MLA compared with PRS are all reduced in Ad5-TGF-β<sub>1</sub> group. No significant difference was detected between RAD-TGF-β<sub>1</sub>, Ad5-lacZ, and PBS groups for any parameter. Proportional loss =  $100 \times [1 - (\text{parameter}_{\text{MLA}} / \text{parameter}_{\text{PRS}})]$ .

and PBS groups (actual,  $-0.53 \pm 0.17$  mm<sup>2</sup>; proportional,  $-14.0 \pm 4.7\%$ ; both  $P < 0.001$ ). In contrast, the mean EEL area at the site of MLA was reduced in the TGF-β<sub>1</sub> group (actual,  $-0.87 \pm 0.34$  mm<sup>2</sup>; proportional,  $-20.2 \pm 9.1\%$ ). This reduction was greater than that in either the lacZ or PBS group, although not significantly. Loss of EEL area in the TGF-β<sub>1</sub> group differed significantly from the increase in the TGF-β<sub>3</sub> group (Figure 3, c and d).

Reduced IEL area in the Ad5-lacZ and PBS groups (actual,  $-0.44 \pm 0.14$  mm<sup>2</sup>; proportional,  $-15.1 \pm 5.3\%$ ) contrasted with increased IEL area in the Ad5-TGF-β<sub>3</sub> group (actual,  $+0.04 \pm 0.12$  mm<sup>2</sup>; proportional,  $+4.7 \pm 5.9\%$ ; Figure 3, e and f). Comparison of mean change in IEL area in the control groups with the TGF-β<sub>3</sub> group revealed significant beneficial effects on actual ( $P = 0.04$ ) and proportional ( $P = 0.03$ ) change in TGF-β<sub>3</sub>-treated vessels. Loss of IEL area in the TGF-β<sub>1</sub> group (actual,  $-0.81 \pm 0.21$  mm<sup>2</sup>; proportional,  $-21.9 \pm 12.0\%$ ) was greater than that in the lacZ and PBS groups, although neither proportional nor actual loss was increased significantly. Actual loss of IEL area in TGF-β<sub>1</sub> vessels was significantly greater than that in the TGF-β<sub>3</sub> group (Figure 3e).



**Figure 4.** Neointima formation at site of MLA 28 days after PTCA. Neointimal area and intima-to-media ratio are significantly reduced in RAD-TGF-β<sub>1</sub>-treated vessels. Ad5-TGF-β<sub>3</sub>, Ad5-lacZ, and PBS groups do not differ significantly.

### Effect of RAD-TGF-β<sub>1</sub> on Neointima Formation

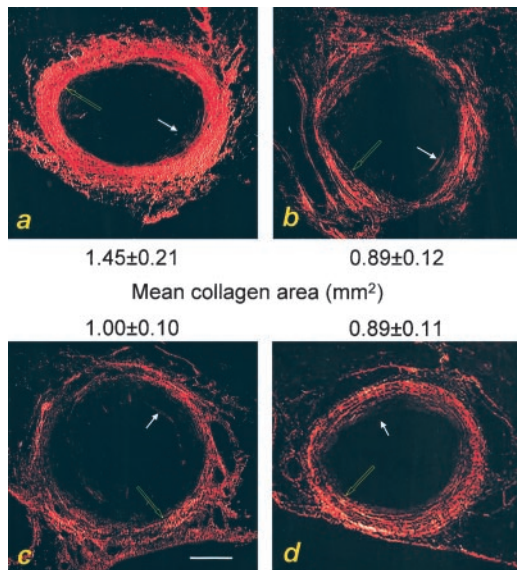
Neointimal area did not differ between the lacZ, PBS, and TGF-β<sub>3</sub> groups (Figure 4a) but was significantly reduced in TGF-β<sub>1</sub> vessels. Neointimal area in the TGF-β<sub>1</sub> group ( $0.36 \pm 0.05$  mm<sup>2</sup>) was 58% less than the mean neointimal area of the lacZ and PBS groups ( $0.85 \pm 0.07$  mm<sup>2</sup>;  $P < 0.0001$ ) and 66% less than the neointimal area in the TGF-β<sub>3</sub> group ( $1.05 \pm 0.18$  mm<sup>2</sup>;  $P < 0.001$ ). Intima-to-media (I:M) ratio was reduced significantly in the TGF-β<sub>1</sub> group compared with the lacZ and PBS groups (TGF-β<sub>1</sub>,  $0.43 \pm 0.07$ ; lacZ+PBS,  $1.86 \pm 0.31$ ;  $P = 0.003$ ), although it did not differ significantly from the TGF-β<sub>3</sub> group ( $1.40 \pm 0.29$ ; Figure 4b).

### Effect of Ad5-TGF-β<sub>3</sub> and RAD-TGF-β<sub>1</sub> on Collagen Content

Total collagen content ( $P = 0.02$ ), adventitial collagen ( $P = 0.02$ ), and medial plus neointimal collagen ( $P < 0.0001$ ) at the site of MLA were all greater in the Ad5-TGF-β<sub>3</sub> group than in the RAD-TGF-β<sub>1</sub>, Ad5-lacZ, or PBS groups (Figures 5, a to d, and 6a). Comparison of the site of MLA with the PRS (Figure 6b) revealed decreased collagen at the site of angioplasty in the Ad5-lacZ and PBS groups (both  $P = 0.01$ ) and increased collagen in the Ad5-TGF-β<sub>3</sub> group ( $P = 0.05$ ). Increased collagen in TGF-β<sub>3</sub>-treated vessels was attributable principally to increased adventitial collagen, although medial plus neointimal collagen was also increased (Figure 6b; both  $P = 0.004$ ). In contrast, collagen was decreased in the adventitia (both  $P < 0.02$ ) and media plus neointima (both  $P < 0.004$ ) of the Ad5-lacZ and PBS groups (Figure 6b). Collagen content did not change significantly in any layer of the vessel wall in the TGF-β<sub>1</sub> group. Within the media plus neointima, this represented a response significantly different from that in the lacZ and PBS groups (Figure 6b). For all groups, collagen content<sub>MLA</sub> and proportional loss of collagen correlated with luminal loss. Correlation coefficients were  $-0.40$  for collagen content ( $P = 0.0035$ ) and  $0.45$  for collagen loss ( $P = 0.0012$ ).

### Discussion

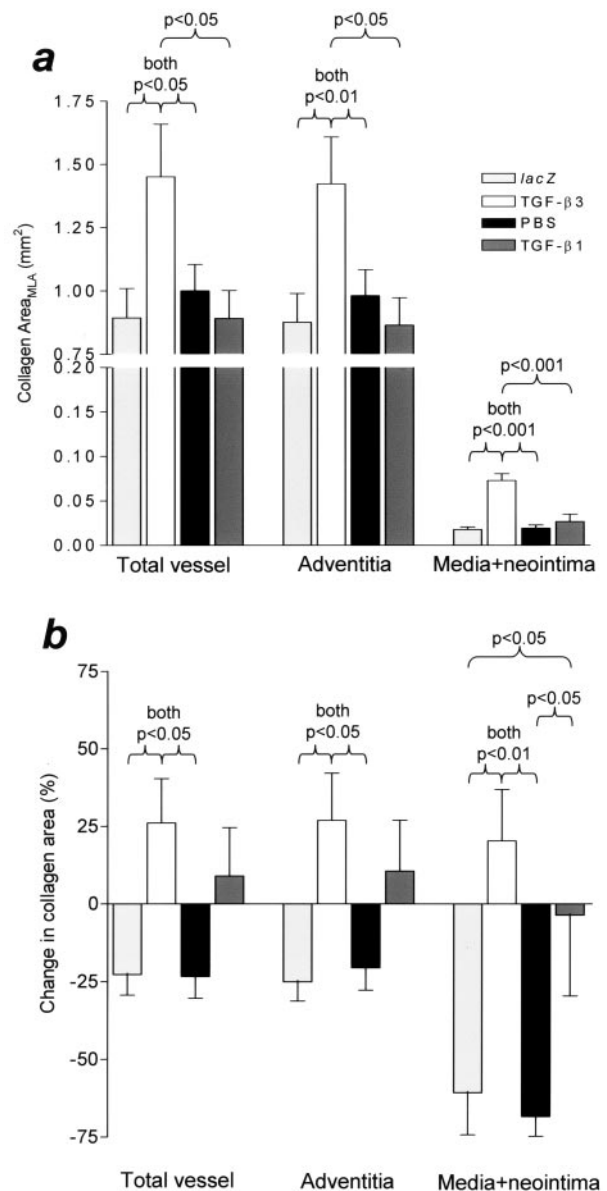
We have investigated the potential for localized expression of TGF-β<sub>3</sub> to inhibit (re)stenosis after PTCA. Comparison of



**Figure 5.** Effects of Ad5-TGF- $\beta_3$  on collagen content 28 days after PTCA. Representative picosirius red-stained sections of coronary artery at site of MLA 28 days after angioplasty (a, Ad5-TGF- $\beta_3$ ; b, Ad5-*lacZ*; c, PBS; d, RAD-TGF- $\beta_1$ ). Collagen content is increased in Ad5-TGF- $\beta_3$  group ( $P=0.023$ ) compared with Ad5-*lacZ*, PBS, and RAD-TGF- $\beta_1$  groups (white arrows indicate luminal surface; yellow arrows, external elastic lamina). Bar=500  $\mu\text{m}$ .

PRS parameters revealed vessels randomized to receive Ad5-TGF- $\beta_3$  to be smaller than those in the control groups. Consequently, we observed no difference in MLA between groups. Loss of lumen area ( $LA_{\text{PRS}} - \text{MLA}$ ), however, was 45% less in the TGF- $\beta_3$  group than in the *lacZ* and PBS groups, and a 31% reduction in proportional luminal loss (intrinsically correcting for differences in vessel size) was observed in Ad5-TGF- $\beta_3$ -treated vessels. Reduced constrictive remodeling accompanied luminal preservation, manifesting in increased EEL and IEL areas in TGF- $\beta_3$ -treated vessels in contrast to reduced areas in control vessels. The difference between the mean  $LA_{\text{PRS}}$  of the groups is similar to the differences between calculated pre-PTCA angiographic luminal areas (Table). Because pre-PTCA angiographic dimensions were measured before gene transfer, they could not be influenced by transgene expression. It therefore seems unlikely that the smaller PRS parameters in the Ad5-TGF- $\beta_3$  group are a consequence of TGF- $\beta_3$  expression.

In healing cutaneous wounds, TGF- $\beta_3$  antagonizes the effects of TGF- $\beta_1$  on ECM deposition.<sup>16</sup> We set out to test the hypothesis that TGF- $\beta_3$  would have similar effects after vascular injury. Because our hypothesis was based on the premise that TGF- $\beta_3$  antagonizes TGF- $\beta_1$ , we also investigated the effects of TGF- $\beta_1$  gene transfer. In keeping with TGF- $\beta_3$  acting as an antagonist of TGF- $\beta_1$ , contrasting effects were observed at the site of PTCA. TGF- $\beta_1$  expression was not associated with reduced luminal loss, and indices of constrictive remodeling were increased. Furthermore, whereas TGF- $\beta_3$  had no effects on NH, TGF- $\beta_1$  significantly reduced neointima formation. Expression of Ad5-TGF- $\beta_3$  was associated with increased collagen content in all layers of the vessel wall, whereas TGF- $\beta_1$  gave rise to no significant changes in content. This effect of TGF- $\beta_1$  was qualitatively



**Figure 6.** Vessel collagen content at site of MLA 28 days after angioplasty. Collagen content in all layers is greater in Ad5-TGF- $\beta_3$  group (a). Comparison of collagen content at site of MLA with PRS shows increased total collagen in Ad5-TGF- $\beta_3$  group and decreased collagen in Ad5-*lacZ* and PBS groups (b). Collagen content is not changed significantly in RAD-TGF- $\beta_1$  group. Increased collagen in Ad5-TGF- $\beta_3$  group is attributable to increased collagen content in all layers of vessel wall. No significant difference was detected between Ad5-*lacZ* and PBS groups for any parameter.

distinct from the *lacZ* and PBS groups, but a significant difference was present only within the neointima plus media.

Comparisons of the effects of TGF- $\beta_1$  and TGF- $\beta_3$  on vascular cells in vitro have been infrequent. Their effects on SMC proliferation and migration are similar in all models that have been studied.<sup>18</sup> In the sole comparison of effects on expression of ECM components, both isoforms stimulated thrombospondin-5 expression by SMCs.<sup>19</sup> More comparisons have been made in dermal fibroblasts, in which both isoforms have similar effects in some models in vitro. In others,

however, the effects of TGF- $\beta_1$  and TGF- $\beta_3$  are distinct. TGF- $\beta_3$  increased expression of hyaluronic acid by confluent cells in a 3D collagen matrix and thereby stimulated migration, whereas TGF- $\beta_1$  stimulated neither. Coincubation with TGF- $\beta_1$  abolished the promigratory effects of TGF- $\beta_3$ .<sup>20</sup> Both isoforms in isolation stimulated collagen expression by dermal fibroblasts cultured in monolayers on plastic.<sup>14</sup> Downregulation by TGF- $\beta_3$  of TGF- $\beta_1$ -induced collagen expression was observed only when fibroblasts were exposed to both isoforms simultaneously. The reported stimulation of thrombospondin-5 expression in SMCs by TGF- $\beta_3$ <sup>19</sup> does not, therefore, preclude the possibility that coincubation of TGF- $\beta_3$  with TGF- $\beta_1$  will suppress expression of ECM components by SMCs.

In our previous study, expression of a transgene proven to antagonize TGF- $\beta_1$  in vitro had effects on luminal loss and collagen content after PTCA similar to those observed after expression of TGF- $\beta_3$ .<sup>17</sup> We speculated that this was the consequence of downregulation of TGF- $\beta_1$ -induced expression of type IV collagenase, and a similar mechanism could account for the effects of TGF- $\beta_3$  observed here. Within the dermis, however, suppression of the phenotypic modulation of fibroblasts to myofibroblasts by TGF- $\beta_3$  was accompanied by an increase in collagen deposition.<sup>15</sup> The effects of TGF- $\beta_3$  expression within the coronary adventitia may simply represent a similar effect without the need to invoke an alteration in collagen degradation to account for it. It is more difficult to explain the unchanged vessel collagen content in the TGF- $\beta_1$  group, although an increase in type IV collagenase activity with a subsequent increase in new collagen synthesis could account for the overall lack of change by comparison with the *lacZ* and PBS groups.

The evidence suggesting a role for TGF- $\beta_1$  in the pathogenesis of restenosis is based on observations of increased TGF- $\beta_1$  expression within the neointima of injured vessels<sup>11,21</sup> and significantly increased NH after delivery of TGF- $\beta_1$  to the vasculature.<sup>12,22,23</sup> It is surprising, therefore, that in the work presented here, expression of TGF- $\beta_1$  was associated with reduced NH. In all previous studies of vascular TGF- $\beta_1$  delivery, however, vessel exposure was effectively luminal, and in neither study of TGF- $\beta_1$  gene transfer<sup>22,23</sup> was expression reported within the adventitia. This is in direct contrast to the adventitial expression of TGF- $\beta_1$  observed here. Furthermore, no previous study has used the coronary vasculature, and as we have previously discussed,<sup>17</sup> the behavior of cultured coronary cells (including responses to TGF- $\beta_1$ ) differs from that of noncoronary cells. Subsequent to the variations observed in the response of dermal fibroblasts cultured on different substrata to differing TGF- $\beta$  isoforms, it has been proposed that the bioactivity of cytokines and ECM macromolecules may be considered only in the context of a "tissue response unit" comprising the target cell population at a particular activation state, the macromolecular matrix in contact with the cells, and the full complement of cytokines within their microenvironment.<sup>20</sup> If this is the case, there is no pressing reason to believe that the effects of expression of a constitutively active mutant TGF- $\beta_1$  in the media and intima of porcine iliofemoral arteries<sup>22</sup> or the endothelium of rat carotids<sup>23</sup> should be similar to the

effects of overexpression of wild-type TGF- $\beta_1$  within the coronary adventitia.

It is more difficult to reconcile the observations made here with previous reports that endogenous TGF- $\beta_1$  expression within the adventitia of injured coronary arteries stimulates phenotypic modulation of adventitial fibroblasts to myofibroblasts with subsequent neointima formation.<sup>13</sup> However, comparison of Figure 2, d and e, shows that the level of adventitial TGF- $\beta_1$  expression achieved by transfer of RAd-TGF- $\beta_1$  is vastly supraphysiological at 72 hours after PTCA. TGF- $\beta_1$  induces apoptosis of SMC<sup>24</sup> and was recently shown to sensitize fibroblasts to apoptosis induced by oxidative stress.<sup>25</sup> Induction of apoptosis in adventitial fibroblasts sensitized by TGF- $\beta_1$  (but seemingly not by TGF- $\beta_3$ ) to the oxidative stress induced by balloon injury could account for the differences observed in NH and vessel collagen content between TGF- $\beta_1$  and TGF- $\beta_3$  groups. Alternatively, the absence of a reduction in neointima formation by TGF- $\beta_3$  might be accounted for by increased motogenesis<sup>20</sup> affecting those adventitial fibroblasts that survived this putative increase in apoptosis.

In conclusion, few studies have assessed the effects of modulation of ECM expression after coronary injury. This study shows that adenovirus-mediated delivery of TGF- $\beta_3$  to porcine coronary arteries at the time of angioplasty results in a 45% reduction in luminal loss 28 days after PTCA, which is achieved by inhibition of constrictive remodeling. In contrast, expression of TGF- $\beta_1$  was associated with increased constrictive remodeling, although luminal loss was not increased by TGF- $\beta_1$  expression because of a 58% reduction in NH in this group. These findings support our previous observation that constrictive remodeling appears to occur within the coronary vasculature when external support by the adventitia is diminished after injury and that inhibition of TGF- $\beta_1$  activity after angioplasty seems to inhibit constrictive remodeling by allowing creation of an adventitial collagen "scaffold" at the site of injury, which prevents loss of area within the IEL and EEL. In combination with our previous study,<sup>17</sup> we have provided evidence that localized modulation of ECM deposition related to physiological expression of TGF- $\beta_1$  after PTCA offers the potential to favorably alter the outcome after coronary angioplasty, although further work is clearly needed to define the mechanisms by which this is achieved. Because the majority of coronary interventions are accompanied by stent deployment, however, and in-stent restenosis is the consequence of NH alone,<sup>26</sup> the most clinically significant observation made here may be of the potential for overexpression of TGF- $\beta_1$  within the adventitia to inhibit neointima formation after PTCA.

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