Smad7 Gene Transfer Attenuates Adventitial Cell Migration and Vascular Remodeling After Balloon Injury

Chandike M. Mallawaarachchi, Peter L. Weissberg, Richard C.M. Siow

Objective—Migration of adventitial fibroblasts contributes to arterial remodeling after angioplasty. This study used vascular gene transfer of smad7 to investigate whether antagonism of transforming growth factor-β1 signaling alters luminal loss and adventitial cell migration after balloon injury in rat carotid arteries.

Methods and Results—Adenoviruses coordinating expression of β-galactosidase (β-gal) and smad7 or β-gal and green fluorescent protein (GFP) were applied to the perivascular surface of common carotid arteries. Balloon injury was performed 4 days after gene transfer, and animals were killed at 3, 7, and 14 days after injury. Uninjured arteries only expressed adventitial β-gal positive cells; however, after balloon injury in β-gal- and GFP-transfected arteries, β-gal-positive cells were observed within the medial layer of vessels and contributed to the population of cells within the neointima at 7 to 14 days. Overexpression of smad7 and β-gal resulted in a significant reduction in the number of β-gal–labeled cells in the neointima, concomitant with reduced luminal loss and decreased adventitial collagen content.

Conclusions—We provide the first evidence that vascular smad7 overexpression attenuates remodeling and contribution of adventitial fibroblasts to neointima formation after balloon angioplasty. Smad7 may represent a novel therapeutic target to reduce the incidence of restenosis. (Arterioscler Thromb Vasc Biol. 2005;25:1383–1387.)

Key Words: adventitia ■ restenosis ■ balloon angioplasty ■ smad7 ■ TGF-β1

Renarrowing of the vessel lumen follows percutaneous transluminal coronary angioplasty in humans and experimental vascular injury in animal models. Migratory and proliferative responses of cells within the vessel wall, in addition to deposition of extracellular matrix, play key roles in restenosis and atherosclerosis.1,2 Transforming growth factor (TGF)-β1 has been shown to play a key role in the vascular response to balloon angioplasty by stimulating smooth muscle cell migration and proliferation, whereas antagonism of vascular TGF-β1 signaling can attenuate these events and thereby limit vascular remodeling after angioplasty.3,4 Increasing evidence suggests that adventitial fibroblasts contribute to arterial remodeling5 through phenotypic transition under the influence of TGF-β1.6

To date, strategies to antagonise the effects of TGF-β1 in the vasculature have focused on quiescenting TGF-β1 by use of neutralizing antibodies4 or overexpression of soluble TGF-β receptors.7,8 However, studies to limit experimental lung9 and renal10 fibrosis have modulated TGF-β–mediated signaling events through inhibition of the “mothers against decapentaplegic homologues” (smad) pathway through overexpression of smad7 which inhibits phosphorylation of smad2,11 an early event in the classical TGF-β signaling cascade.12,13 We have previously used a localized in vivo adenoviral gene transfer approach to the adventitial surface to show that adventitial cells migrate toward the lumen and contribute to neointima formation after vascular injury.14 In the present study, we used this technique to determine whether adenoviral overexpression of smad7 in the adventitia modulates luminal loss, contribution of adventitial cells to neointima formation, and collagen deposition after balloon injury in rat carotid arteries to provide mechanistic insights to demonstrating that the smad signaling pathway contributes to vascular remodeling in response to balloon angioplasty.

Methods

Adventitial Gene Transfer and Carotid Artery Balloon Injury

Replication defective adenoviral vectors coordinating expression of nuclear-targeted β-galactosidase (Adβ-gal), green fluorescent protein (AdGFP), or smad7 (Adsmad7) were propagated and purified as described previously.14 For adventitial gene transfer, Adβ-gal and AdGFP (inactive controls) or Adβ-gal and Adsmad7 adenoviruses (10^9 pfu/mL) were suspended together in pluronic F127 gel (BASF, 25% wt/vol) and maintained at 4°C. Animal studies were carried out under the approval of the Home Office, UK. Male Sprague–Dawley rats (3 months old, weight range 380 to 450 g; n = 12 per time point for each treatment group; Charles River; Margate, Kent, UK) were anesthetized with ketamine and xylazine, allowed to recover after surgery, and killed by exsanguination and fixation in situ by retrograde aortic perfusion with saline containing formalin (2%) and glutaraldehyde (0.2%) as described previously before careful excision of the common carotid arteries to avoid damage to the adventitial layer.15 The left common carotid artery was exposed...
under aseptic conditions, and 200 μL of pluronic gel containing the adenovirus pairs was applied to the adventitial surface of the artery. After solidification of the gel, the wound was closed and animals recovered from anesthesia. The same rats were anesthetized again 4 days later and some animals killed to histologically assess transgene expression in carotid arteries. Four days after adventitial gene transfer, the left carotid artery was injured using a 2F Fogarty balloon catheter (Baxter) to elicit endothelial denudation and arterial distension. Rats were killed at 3, 7, or 14 days after adenoviral gene transfer only or at 3, 7, and 14 days after balloon injury and common carotid arteries excised for histological analyses.

Immunohistochemical Analyses
Localization of nuclear β-galactosidase transgene expression was performed on arterial segments by X-gal staining as described previously to minimize loss of blue chromogen labeling. Expression of smad7 and α-actin was determined in common carotid arterial sections using monoclonal mouse antibodies (Santa Cruz Biotechnology) and secondary biotinylated anti-mouse antibody (Dako). Mouse IgG was used as a negative control instead of primary antibody. Immunostaining was visualized using the Vectastain Elite ABC system (Vector Laboratories). Collagen content in sections was assessed by picrosirius red staining, while the lack of vascular injury. Blue stained β-galactosidase–positive nuclei were restricted solely to the adventitial (a) compartment of arteries at 7 (A and B) and 14 (C and D) days after application of vectors in pluronic gel (n=12) with no expression in medial (m) layers. Smad7 immunostaining in control transfected arteries at 7 days after gene transfer (E) and Adsmad7 transfected arteries at 7 (F) and 14 (G) days after transfection. Scale bar represents 100 μm.

Assessment of TGF-β1 Antagonism
Carotid arteries were excised from rats killed at 3, 7, or 14 days (n=3 for each treatment group per time point from separate experiments) after adenoviral gene transfer of adenoviral vector pairs alone, cut into 200-μg sections, and incubated in serum-free Dulbecco’s modified Eagles medium containing 0 to 10 ng/mL recombinant TGF-β1 for 1 hour before extraction of proteins by homogenization in ice cold lysis buffer containing phosphatase and protease inhibitors. Changes in the level of phosphorylated smad2 were determined as an index of inhibition of TGF-β1 signaling by Western blot analyses of protein extracts using a specific mouse monoclonal primary antibody against phosphorylated smad2.

Adventitial Cell Migration, Collagen Content, and Morphometric Analyses
After computer image capture of sections from 12 separate animals per time point, software analysis (Photoshop 5.02; Adobe) was used to quantify morphological changes in vessel lumen area, wall thickness, and proportion of blue stained β-galactosidase positive nuclei of adventitial cells contributing to the medial and neointima after balloon injury in six representative areas for each compartment of the vessel wall as described previously. Quantification of collagen content was determined by software analysis of the area of non-zero pixels within a 200-μm perimeter of the external elastic lamina in picrosirius red–stained sections. Results are reported as mean±SEM, and data were compared using the unpaired Student t test with P<0.05 considered statistically significant.

Results
Adventitial Gene Transfer
After gene transfer with either Adβ-gal and Adsmad7 or Adβ-gal and AdGFP, the adventitia of left carotid arteries exhibited an equal proportion of cells with blue stained β-galactosidase–positive nuclei, which was restricted solely to the adventitial compartment at 7 and 14 days after adenoviral gene transfer regimens alone (Figure 1A through 1D). We reported previously that adenoviral β-gal gene delivery to the rat carotid adventitial surface resulted in a transfection efficiency of 34±4% determined by X-gal and nuclear fast red staining, while the lack of β-galactosidase transgene expression observed in the medial layer of uninjured vessels in the present study suggests that only adventitial cells were transfected and observed migrating toward the

**Figure 1.** X-gal staining of left carotid artery sections after perivascular gene transfer with either Adβ-gal and Adsmad7 or Adβ-gal and AdGFP in the absence of vascular injury. Beta stained β-galactosidase–positive nuclei are restricted solely to the adventitial (a) compartment of arteries at 7 (A and B) and 14 (C and D) days after application of vectors in pluronic gel (n=12) with no expression in medial (m) layers. Smad7 immunostaining in control transfected arteries at 7 days after gene transfer (E) and AdsMad7 transfected arteries at 7 (F) and 14 (G) days after transfaction. Scale bar represents 100 μm.
lumen after balloon injury. In the present study, compared with the low background smad7 immunoreactivity after Adβ-gal and AdGFP transfection of arteries (Figure 1E), Adsmad7 and Adβ-gal gene transfer resulted in enhanced adventitial smad7 expression at 7 and 14 days after transfection indicative of perivascular smad7 overexpression (Figure 1F and 1G). Adventitial transfer of adenoviral vector pairs alone did not result in changes in morphology of the vessel wall up to 14 days after adenovirus delivery, and expression of β-galactosidase was not observed at any time in control right untransfected carotid arteries (data not shown). Phosphorylation of smad2 in response to TGF-β1 was monitored ex vivo in carotid artery sections as an index of inhibition of TGF-β1 signaling after smad7 gene transfer. In response to exogenous TGF-β1 (0.01 to 10 ng/mL; 1 hour), smad2 phosphorylation was significantly reduced in lysates from arteries excised at 3, 7, and 14 days after transfection with Adβ-gal and Adsmad7 compared with those transfected with Adβ-gal and AdGFP or from untransfected arteries, indicating that TGF-β1 signaling in arteries had been effectively antagonised after smad7 overexpression (Figure 1, available online at http://atvb.ahajournals.org).

Migration of Adventitial Cells After Balloon Injury
Cells exhibiting blue nuclear β-galactosidase staining were observed in the medial layer of the vessel wall at 3 (6.3±1.9% of medial cells), 7 (8.5±2.2%), and 14 (3.8±1.3%) days after balloon injury of smad7-transfected left carotid arteries. At 7 days after vascular injury, perivascular smad7 overexpression significantly attenuated the number of adventitial cells that had migrated as shown by the reduction in blue stained nuclei in the neointima by 33.4±5.4% (P=0.035; n=12) compared with the number in the control Adβ-gal– and AdGFP-transfected arteries, whereas at 14 days after injury there was a 36.8±4.6% (P=0.016; n=12) reduction compared with control arteries (Figures 2A through 2D and 3A).

Luminal Area and Arterial Wall Thickness
Smad7 overexpression significantly attenuated the loss in luminal area 14 days after balloon injury from 0.28±0.06 mm² in control injured arteries transfected with Adβ-gal and AdGFP to 0.36±0.05 mm² in β-gal- and smad7-transfected arteries (P=0.01; n=12; Figure 3B). The increase in lumen area was associated with a significant decrease in thickness of medial and neointimal compartments after balloon injury in Adsmad7- and Adβ-gal–transfected arteries; however, the overall medial and neointimal areas were not significantly altered due to the increase in overall circumference of these arteries compared with arteries transfected with AdGFP and Adβ-gal (Figure 2E and 2F). Neointimal compartment thickness at 14 days after injury was 29.3±6.9 μm in arteries overexpressing smad7 compared with 37.5±5.2 μm in control transfected vessels (P=0.03; n=12), whereas medial compartment thickness at 14 days after injury in arteries treated with Adsmad7 was 42.6±10.8 μm compared with 57.3±11.5 μm in the control transfected arteries (P=0.043; n=12).

Smooth Muscle α-Actin Expression and Collagen Synthesis
Overexpression of smad7 and β-gal in the adventitial compartment of carotid arteries significantly attenuated adventitial, medial, and neointimal smooth muscle α-actin expression at 14 days after balloon injury (Figure 4A) compared with levels in arteries transfected with Adβ-gal and AdGFP (Figure 4B). Picrosirius red staining revealed a 26.6±4.3% decrease in adventitial collagen staining in sections from Adsmad7 and Adβ-gal–transfected arteries 14 days after balloon injury in comparison with arteries transfected with inactive controls Adβ-gal and AdGFP (P=0.01; n=12; Figures 3C, 4C, and 4D).

Discussion
Our previous study together with other reports have provided evidence that adventitial cells contribute to constrictive remodeling after vascular injury by migrating to the neointima and synthesizing extracellular matrix. TGF-β1 plays a key role in the remodeling processes after vascular balloon angioplasty through its contribution to phenotypic modulation and migration of adventitial cells. Our present study is the first to demonstrate that in vivo perivascular smad7 gene transfer antagonizes TGF-β1 signaling in the carotid.
arteries. Our findings demonstrate that adventitial smad7 overexpression results in antagonism of TGF-β1 signaling for up to 14 days as shown by diminished smad2 phosphorylation in arterial sections in response to exogenous TGF-β1, leading to attenuation of adventitial cell migration to the neointima, loss of luminal area and perivascular collagen content, and decreased smooth muscle α-actin expression in the adventitia after vascular balloon injury.

Previous reports have shown that sequestration of TGF-β1, through overexpression or administration of a soluble TGF-β type II receptor,7,8 decrease luminal loss after angioplasty. However, our present study provides novel mechanistic evidence that perivascular smad7 gene transfer to antagonize TGF-β1–mediated smad signaling events attenuates phenotypic modulation of quiescent adventitial cells, as shown by decreased adventitial smooth muscle α-actin expression, reduced adventitial cell migration, and perivascular collagen content elicited by vascular injury. Modulation of changes in the adventitial compartment by smad7 overexpression contributes to the attenuation of luminal area loss and reduced thickness of medial and neointimal compartments after vascular injury. It is possible that restriction of smad7 gene transfer to the adventitia in this study did not effect the medial smooth muscle cell contribution to neointimal hyperplasia; however, it is of note that endogenous smad7 expression observed within medial cells was significantly lower than in the adventitial compartment after smad7 gene transfer. A deficiency in smad7 expression has been associated with enhanced skin fibrosis in scleroderma,16 and overexpression of smad7 in cultured rat aortic smooth muscle cells has been reported to inhibit the proliferative effects of TGF-β1.17 Nevertheless, our present findings provide further evidence that adventitial cells play a significant role in the remodeling process after angioplasty under the influence of TGF-β1. Overexpression of smad7 in rodent models of lung9 and renal renal fibrosis has been shown to inhibit morphological changes, myofibroblast differentiation, and collagen accumulation; however, apart from the present report, there remains a paucity of data regarding the effects of in vivo smad7 gene transfer in the vasculature. It is conceivable that antagonism of the smad signaling pathway by smad7 overexpression attenuates the phenotypic modulation of adventitial cells to myofibroblasts which express smooth muscle α-actin and that subsequently migrate and synthesize extracellular matrix after balloon injury.

An imbalance between matrix metalloproteinases (MMPs) and their inhibitors (TIMPs) contributes to vessel remodeling in restenosis.18 This may be mediated in part by facilitation of adventitial cell migration through degradation extracellular matrix proteins by enhanced matrix metalloproteinase (MMP) activity and decreased TIMP expression.5,15 Antagonism of TGF-β signaling by perivascular smad7 overexpression also leads to diminished MMP-2 and enhanced TIMP-1 expression in the adventitial and medial compartments of balloon injured carotid arteries (data not shown), which may partially account for the attenuation of adventitial myofibroblast migration and invasion toward the lumen after injury because TGF-β has been shown to modulate MMP and TIMP expression in fibroblasts.19

Our present findings provide mechanistic insights to demonstrate that the TGF-β1–mediated smad signaling cascade

**Figure 3.** A, Quantification of β-gal–positive cells in the neointima after balloon injury in Adβ-gal alone, AdGFP and Adβ-gal, or Adsmad7– and Adβ-gal–transfected carotid arteries. B, Morphometric assessment of lumen loss in transfected vessels after balloon injury. C, Perivascular collagen content quantified by pixel densitometry of the adventitia within a 200 μm border of the external elastic laminae. Values denote Mean±SEM. (P<0.01 relative to values in arteries transfected with Adβ-gal and AdGFP; n=12).

**Figure 4.** Vascular smooth muscle α-actin immunostaining (A and B) and collagen content (C and D) after balloon injury in Adsmad7– and AdGFP-transfected arteries (A and C) and control Adβ-gal– and AdGFP–transfected vessels (B and D) at 14 days after balloon injury. a indicates adventitia; m, media; n, neointima. Scale bar represents 100 μm.
plays a key role in adventitial cell migration and remodeling after vascular injury. It is conceivable that smad7 overexpression can modulate smad-mediated signaling elicited by other members of the TGF-β super-family, such as the bone morphogenetic proteins to attenuate adventitial cell migration. However, TGF-β1 can also activate smad-independent mitogen-activated protein kinase (MAPK) pathways, including c-Jun-NH2-terminal kinase and p38 MAPK, which contribute to phenotypic modulation of myofibroblasts and vascular remodeling; however, these interactions between TGF-β and MAPK signaling remain to be elucidated in vivo in the vasculature. It is also exciting to speculate that TGF-β-mediated signaling events may contribute to the involvement of progenitor cells in vascular remodeling. It is possible that smooth muscle progenitor cells present in the adventitia may be stimulated to differentiate under the influence of TGF-β1. In this context, it has recently been shown that smad7 can modulate the fate of multipotent cells, and thus it is conceivable that perivascular smad7 expression of smad7 may represent a novel therapeutic target for gene therapy to reduce the incidence of restenosis after angioplasty.

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References

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Online Figure I.
Representative western blots showing vascular smad7 overexpression reduces smad2 phosphorylation in response to TGF-β1. Arteries transfected with Adsmad7 and Adβ-gal or the control adenoviral pairs, AdGFP and Adβ-gal, were excised at 3, 7 and 14 days following gene transfer and treated ex vivo with recombinant TGF-β1 (10 ng/ml) for 1 h (A) or arteries excised at 7 days exposed to TGF-β1 (0-1.0 ng/ml) for 1 h (B). Control untransfected arteries were also treated with 0-1.0 ng/ml TGF-β1 for 1 h. Tissue protein lysates were probed using an antibody against phosphorylated smad2 (n=3).