Soluble Transforming Growth Factor-β Type II Receptor Inhibits Negative Remodeling, Fibroblast Transdifferentiation, and Intimal Lesion Formation But Not Endothelial Growth

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Abstract—Using the rat balloon catheter denudation model, we examined the role of transforming growth factor-β (TGF-β) isoforms in vascular repair processes. By en face in situ hybridization, proliferating and quiescent smooth muscle cells in denuded vessels expressed high levels of mRNA for TGF-β1, TGF-β2, TGF-β3, and lower levels of TGF-β receptor II (TGF-βRII) mRNA. Compared with normal endothelium, TGF-β1 and TGF-β2 mRNA were upregulated in endothelium at the wound edge. Injected recombinant soluble TGF-βRII (TGF-βRII:Fc) localized preferentially to the adventitia and developing neointima in the injured carotid artery, causing a reduction in intimal lesion formation (up to 65%) and an increase in lumen area (up to 88%). The gain in lumen area was largely due to inhibition of negative remodeling, which coincided with reduced adventitial fibrosis and collagen deposition. Four days after injury, TGF-βRII:Fc treatment almost completely inhibited the induction of smooth muscle α-actin expression in adventitial cells. In the vessel wall, TGF-βRII:Fc caused a marked reduction in mRNA levels for collagens type I and III. TGF-βRII:Fc had no effect on endothelial proliferation as determined by reendothelialization of the denuded rat aorta. Together, these findings identify the TGF-β isoforms as major factors mediating adventitial fibrosis and negative remodeling after vascular injury, a major cause of restenosis after angioplasty. (Circ Res. 1999;84:1212-1222.)

Key Words: intimal hyperplasia ■ collagen ■ fibrosis ■ smooth muscle α-actin ■ myofibroblast

Vascular remodeling is a response of blood vessels to both physiological and pathological stimuli, leading to either vessel enlargement (positive remodeling) or shrinking (negative remodeling). Several studies found that neointimal proliferation or intimal mass after angioplasty showed little correlation with restenosis because of permanent changes in vascular geometry. In humans, intravascular ultrasound has failed to show a correlation between lumen area and neointimal area, and vascular remodeling, as measured by a decrease in vessel area, was shown to account for most of the restenosis process. These observations clearly demonstrate that a successful therapeutic approach to restenosis would have to target negative vascular remodeling.

The transforming growth factor-β (TGF-β) family of cytokines can have a variety of effects on vascular cells, but very little is known about its role in vascular remodeling. TGF-β affects many functions, including proliferation of smooth muscle cells (SMCs). Grainger et al. have reported inhibition of SMC proliferation by TGF-β1 via extension of the G1 phase of the cell cycle, whereas others have shown that this inhibition is due to arrest in the late G1 phase. SMCs derived from atherosclerotic lesions responded to TGF-β1 with an increase in proliferation, and lower levels of TGF-β receptor II (TGF-βRII) have been implicated in the lack of inhibition by TGF-β1 in these cells. Several other groups have reported stimulation of SMC proliferation by TGF-β1 in vitro. Low doses of TGF-β1 were reported to stimulate SMC proliferation via platelet-derived growth factor (PDGF)-AA–dependent and PDGF-AA–independent mechanisms, whereas higher doses were inhibitory. Bifunctional effects of TGF-β1 were also seen in migration assays with SMCs. In one of the first studies on the role of TGF-β1 in intimal lesion formation, we reported a 5- to 7-fold induction of TGF-β1 mRNA in the balloonated rat carotid artery with elevated levels of TGF-β1 mRNA persisting for 2 weeks. During this period, elevated TGF-β1 mRNA levels correlated with increases in mRNA expression of fibronectin and the collagens α1 (I) and α1 (III). We also found that infusion of recombinant TGF-β1 caused an increase in intimal SMC proliferation in vivo.

Of clinical relevance is the study by Nikol et al., who reported increased TGF-β1 mRNA expression in restenotic lesions compared with primary atherosclerotic lesions. Additional functional data were provided by studies that used...
neutralizing antibodies to TGF-β, to reduce expression levels of TGF-β in the vessel wall. In the rat balloon injury model, treatment with TGF-β antibodies caused a small but significant reduction in neointima formation.16 Overexpression of TGF-β in the rat carotid artery by adenoviral gene transfer led to transient neointima formation with cartilaginous metaplasia that almost completely resolved within 8 weeks.17 TGF-β may also have effects on vascular tone, because the factor was found to suppress nitric oxide synthase expression18 while inducing antiapoptotic effects for SMCs have been demonstrated for the vasoconstrictor endothelin in SMCs in vitro.19 In addition, antiapoptotic effects for SMCs have been demonstrated for TGF-β.20

Studies examining the expression of TGF-β ligand and receptor mRNAs by reverse transcriptase–polymerase chain reaction (PCR) revealed that TGF-β1, TGF-β2, and TGF-βRII mRNA levels were increased in the media of the injured rat carotid artery,21 and expression of TGF-β2 and TGF-β1 was also reported in SMCs of the lung vasculature.22,23 Reduced levels of TGF-βRII, however, were reported in human atherosclerotic lesions by McCaffrey et al.8 With regard to SMCs, the 3 TGF-β ligands have overlapping functions, and all of them induce expression of the α1 (I), α5 (I), and α1 (III) chains of collagen.24

Endothelial cells are growth inhibited by TGF-β in vitro,25 but little is known about the function and expression of TGF-β ligands in endothelial cells in vivo.

Because expression of all 3 TGF-β ligands has been reported in the vessel wall, the present study used a dimeric recombinant soluble TGF-βRII (TGF-βRII:Fc) to determine the importance of TGF-β ligands in the rat balloon injury model. We studied the effects of this reagent on SMC proliferation, neointima formation, remodeling, endothelial regeneration, and expression of matrix proteins. In addition, using an en face approach, we determined the time course of mRNA expression for the TGF-β1, TGF-β2, TGF-β3, and TGF-βRII in endothelium and SMCs after wounding by in situ hybridization.

Materials and Methods

Arterial Injury Model

All animal studies were approved by the Institutional Animal Care and Use Committee. Sprague Dawley rats (400 g, 3 to 4 months old) were bred from animals purchased from Taconic (Germantown, NY). All surgical procedures were carried out with the animals under general anesthesia by intraperitoneal injection of xylazine (2.2 mg/kg, AnaSed, Lloyd Laboratories) and ketamine (50 mg/kg body weight, Ketalar, Parke-Davis). The left carotid artery and the aorta were denuded with a 2F balloon catheter, as recently described.26 Starting at the day of surgery, TGF-βRII:Fc (see below) was injected into groups of rats (n=5) via the tail vein every other day at a concentration of 0.5, 2.0, and 5 mg/kg body weight in 0.2 mL of PBS. The control group (n=5) received 5 mg/kg of recombinant human IgG1 in an equal volume of PBS. Two hours before the rats were killed, 6 mg of 5-bromo-2’-deoxyuridine (BrDU; Boehringer Mannheim) was administered subcutaneously. Deendothelialized segments of arteries were identified by intravenous injection of Evans blue (0.5 mL of 5% solution in saline) 5 minutes before harvesting of the vessels. The animals were killed 14 days after balloon injury (7 injections of TGF-βRII:Fc), and perfusion fixation was carried out under physiological pressure with phosphate-buffered (0.1 mol/L, pH 7.4) 4% paraformaldehyde. Reproducibility of the data was further verified in a separate experiment by injecting groups of rats (n=5) with TGF-βRII:Fc (5 mg/kg) and vehicle following the same protocol as described above.

The effects of TGF-βRII:Fc on proliferation of medial SMCs and mRNA expression of matrix molecules were assessed in an additional experiment in which groups of rats (n=5) had balloon catheter denudation performed on both common carotid arteries. The treatment group received TGF-βRII:Fc (2 mg/kg) intravenously immediately after balloon injury, with an additional injection given 2 days later, whereas the control group received recombinant human IgG1. Two hours before killing at 4 days after denudation, 6 mg of BrDU was injected subcutaneously. The right carotid artery was excised and snap frozen in liquid nitrogen for RNA extraction. After perfusion fixation with 4% paraformaldehyde, the left carotid artery was then harvested for histology and determination of medial SMC proliferation.

Production of Soluble TGF-βRII (TGF-βRII:Fc)

The recombinant rabbit TGF-βRII:Fc fusion gene, comprising the extracellular domain of the rabbit type II TGF-β receptor fused to the Fc region of human IgG1 (Figure 1A and 1B), was constructed as follows. The extracellular domain of the rabbit type II TGF-β receptor was amplified from clone MIS-3f1127 by conventional PCR. Amplified sequences were flanked by a 5’ NotI and a 3’ SalI restriction site and subsequently digested with these restriction enzymes. The human IgG1 H chain C region containing the hinge region and the CH2 and the CH3 sequences was isolated with H restriction enzyme recognition site and were subsequently digested with these restriction enzymes. The human IgG1 H chain C region containing the hinge region and the CH2 and the CH3 sequences was isolated with H chain-specific primers by PCR amplification, as described.28 Amplified sequences were flanked by a 5’ SalI and a 3’ NotI restriction enzyme recognition site and were subsequently digested with these restriction enzymes. The receptor and IgG DNA fragments were cloned into the NotI restriction enzyme site in plasmid SAB1329 and transformed into competent bacteria. Plasmids recovered from the transformants were analyzed for the correct orientation of assembled fragments, and the entire coding sequence was confirmed by DNA sequencing.

The recombinant rabbit TGF-βRII:Fc fusion gene was transfected into Chinese hamster ovary cells. After the initial transfection, cells were selected in 250 nmol/L methotrexate. The resulting colonies were expanded and analyzed for TGF-βRII:Fc fusion gene expression. Clones expressing the highest levels of TGF-βRII:Fc were selected for production of the fusion protein. TGF-βRII:Fc, which is dimeric, was
purified from culture supernatant by protein A-Sepharose affinity chromatography under sterile and endotoxin-free conditions.

The activity of this reagent was tested in mink lung epithelial cells (Mv1Lu, American Type Culture Collection). These cells were seeded in 96-well plates at a density of 1.8×10^4 cells per well in DMEM containing 10% FBS (Atlanta Biologicals). One nanogram of TGF-β1 (R&D Systems) was added in the absence or presence of various concentrations of soluble TGF-βRII and incubated for 72 hours at 37°C with 5% CO2. DNA synthesis was measured by adding 1 μCi per well of [3H]thymidine (Amersham) during the last 6 hours of the incubation. Subsequently, cells were harvested with a mechanical cell harvester, and [3H]thymidine incorporation was determined with a scintillation counter. The mean value±SEM from groups of 6 replicates is shown.

**SMC Proliferation, Morphometry, and Immunostaining**

The denuded and therefore Evans blue–stained part of the left carotid artery was divided into 3 segments that were embedded together in paraffin in 1 block. Sections (5 μm) were cut, and 3 or more sections spanning the length of the vessel were analyzed. Immunostaining with an antibody against BrdU (Cappel) was performed on the 4-day and the 2-week balloon-injured vessels as described previously.20 The number of total cells and the number of BrdU-positive cells were separately determined for the tunica media, neointima, and adventitia. The proliferation index was calculated as the percentage of BrdU-positive cells.

Masson trichrome staining was used to assess the composition and amount of extracellular matrix production. To localize the injected TGF-βRII-Fc and the control recombinant human IgG within the vessel wall, sections were stained with a biotin-labeled antibody against human IgG (DAKO) used at a dilution of 1:200. Subsequent steps followed previously published protocols.29 Expression of smooth muscle α-actin was examined by immunostaining on cross-sectioned carotid arteries 4 days after balloon injury and treatment with TGF-βRII-Fc (2 mg/kg) or vehicle (5 rats per group). Two injections were given, 1 immediately after surgery and the second 2 days later. A mouse monoclonal antibody against smooth muscle α-actin (Sigma, clone 1A4) was used at a 1:2500 dilution as described,30 and 3 or more sections from each animal were analyzed.

Morphometric analyses were carried out on the 2-week balloon-injured common carotid arteries in a blind manner. For each animal, 3 sections originating from the proximal, middle, and distal segments of the denuded vessel were analyzed for the morphometric studies. Digitized images of these vessels were analyzed using image analysis software for Apple Macintosh computers (NIH Image, version 1.60). The circumference (length) of the lumen and the lengths of the internal elastic lamina (IEL) and the external elastic lamina (EEL) were determined by tracing along the luminal surface, the perimeter of the neointima (IEL), and the perimeter of the tunica media (EEL). Very small folds were not included in the IEL and EEL data, and therefore the measurements more accurately reflect the perimeter of the neointima and media. Assuming a circular structure, these measurements were used to calculate the lumen area. The medial area was calculated by subtracting the area defined by the IEL from the area defined by the EEL, and intimal area was defined as the area defined by the luminal surface and IEL.

**Endothelial Regrowth**

After balloon denudation of the rat thoracic aorta, reendothelialization occurs by endothelial outgrowth from intercostal arteries. Reendothelialization was determined by injection of Evans blue as described above. The percentage of luminal surface covered by endothelium was determined 2 weeks after balloon denudation and IV injection of TGF-βRII-Fc every other day (5 mg/kg, n=5 animals) or control IgG (n=4 animals). Digitized images of the vessel surface were analyzed with NIH Image software.

**Northern Blotting and cDNA Probes**

The right common carotid artery was harvested from rats 4 days after balloon injury and injection of TGF-βRII-Fc or control IgG. Vessels were stripped of periadventitial fat and then snap frozen in liquid nitrogen. Frozen arterial tissue was ground to a fine powder under liquid nitrogen, and total cellular RNA was prepared by acid guanidinium thiocyanate extraction.31 Agarose gel electrophoresis of RNA (15 μg total RNA per lane) and transfer to nylon membranes (Zeta Probe, BioRad Laboratories) were carried out as previously described.32 After transfer, RNA blots were exposed to short-wave UV light both to cross-link RNA to the membrane and to visualize the major ribosomal RNA bands stained with ethidium bromide. The blot was hybridized using cDNA probes labeled with [32P]dCTP by random primer extension (Amersham), washed at 65°C in 2 changes of 0.015 mol/L NaCl-0.0015 mol/L sodium citrate (pH 7.0)-0.1% SDS for 30 minutes each, and then exposed to Kodak X-AR5 film at −70°C.

All probes used for Northern blotting were rat-derived cDNAs and included α1 type I collagen, α2 type III collagen, collagen XV, tropoelastin, osteopontin, and fibronectin. The identity of these cDNAs was verified by DNA sequence analysis.

**In Situ Hybridization**

In situ hybridization was carried out on en face preparations of vessel segments as recently described.29 Segments from normal rat carotid arteries and aortae were used to determine gene expression in normal, quiescent endothelium. Gene expression in migrating and proliferating endothelium (at the wound edge) was examined in the thoracic aorta 8 days after denudation during active reendothelialization from intercostal arteries. mRNA expression in proliferating and migrating SMCs was studied in 8-day-denuded carotid arteries and aortae. In the rat balloon injury model, SMCs return to a quiescent state within several weeks after denudation, and we examined mRNA expression in quiescent luminal SMCs of carotid arteries and aortae at 4 weeks after injury. A minimum of 4 specimens from 3 different animals was hybridized with antisense probes, and 2 specimens were hybridized with sense probes, to assess background hybridization. A cDNA for rat TGF-βRII was kindly provided by Dr W.W. Vale (Salk Institute, La Jolla, CA),33 and a 700-bp Prot1 fragment was subcloned into Bluescript II (Stratagene). cDNAs for rat TGF-β1, rat TGF-β2, and mouse TGF-β1 were kindly provided by Dr Anita Roberts (NIH, Bethesda, MD).34 5S-labeled UTP-labeled sense and antisense riboprobes were synthesized with T3 and T7 RNA polymerases from linearized plasmids. Following hybridization in situ hybridization procedure for en face preparations,29 the slides were coated with autoradiographic emulsion (NTB2, Kodak), exposed for 3 weeks, and developed (D-19, Kodak).
Preparations were observed under the light microscope using dark-field and bright-field illumination.

**Statistical Analysis**

ANOVA was used to determine whether significant differences between the means of treatment groups were present ($P \leq 0.05$). Multiple comparisons between groups were then performed using the Scheffé test. The Student $t$ test was used to compare the means between 2 groups, and differences were considered significant if $P \leq 0.05$. All data are shown as mean ± SEM.

**Results**

Similarly to native TGF-βRII, TGF-βR:Fc binds active TGF-β1 and TGF-β3, but not TGF-β2, which in addition requires betaglycan (data not shown).

The ability of the recombinant dimeric soluble TGF-βR:Fc to inhibit TGF-β-mediated responses was tested in the mink lung epithelial cell line. $[^3]H$Thymidine incorporation in these cells is inhibited by TGF-β1, and as shown in Figure 2, the TGF-βR:Fc neutralized this inhibitory effect in a dose-dependent manner, with 50 ng/mL of TGF-βR:Fc completely neutralizing the effects of 1 ng/mL of TGF-β1. TGF-βR:Fc had no inhibitory effect on proliferation mediated by PDGF-BB (10 ng/mL) or fibroblast growth factor-2 (10 ng/mL) in 3T3 cells (data not shown).

**Expression of TGF-β Ligands and TGF-βRII in the Vessel Wall**

We have used in situ hybridization to determine the expression of mRNA for TGF-β1, TGF-β3, and TGF-βRII on en face preparations. We have previously demonstrated that this is a very sensitive and semiquantitative method for the detection of mRNA expression in cells on the luminal surface of rat carotid arteries and aortae. Normal, quiescent endothelium revealed low levels of expression for TGF-β1, TGF-β2, and TGF-βRII. Wound-edge endothelium revealed a dramatic upregulation of TGF-β1, TGF-β3, and TGF-βRII mRNA, with TGF-β2 being restricted to only a few cells deep into the monolayer. SMCs expressed all 3 TGF-β isoforms at high levels at 8 days and 4 weeks after injury, and TGF-β1 mRNA was the most abundant form. SMCs also expressed TGF-βRII, but levels were lower than in wound-edge endothelium. Hybridization with the sense probe is shown for background evaluation. All specimens are seen under dark-field illumination at ×200 original magnification after staining with hematoxylin.

Figure 3. In situ hybridization with $^{35}S$-labeled UTP-labeled riboprobes for TGF-β isoforms and TGF-βRII were carried out on en face preparations. Representative photomicrographs are shown. Expression of mRNA in endothelium was examined in the uninjured aorta (normal EC) and in proliferating endothelium 8 days after denudation (8d EC edge). Expression of mRNA in SMCs on the luminal surface was determined in carotid arteries at times of rapid proliferation 8 days after balloon injury (8d SMC) and at 4 weeks after injury (4 wk SMC) when these cells had returned to quiescence. Normal endothelium showed low levels of expression for TGF-β1, TGF-β3, and TGF-βRII. Wound-edge endothelium revealed a dramatic upregulation of TGF-β1, TGF-β3, and TGF-βRII mRNA, with TGF-β2 being restricted to only a few cells deep into the monolayer. SMCs expressed all 3 TGF-β isoforms at high levels at 8 days and 4 weeks after injury, and TGF-β1 mRNA was the most abundant form. SMCs also expressed TGF-βRII, but levels were lower than in wound-edge endothelium. Hybridization with the sense probe is shown for background evaluation. All specimens are seen under dark-field illumination at ×200 original magnification after staining with hematoxylin.

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![Figure 3](http://circres.ahajournals.org/)

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migrating endothelium at the wound edge in the thoracic aorta 8 days after denudation showed upregulated expression of TGF-β1, TGF-β2, and TGF-βRII, but expression of TGF-β3 was similar to that in normal endothelium (Figure 3). Unlike TGF-β1 and TGF-βRII, the increased TGF-β2 mRNA expression at the wound edge was restricted to only a few cells into the endothelial monolayer. Proliferating SMCs on the luminal surface of carotid arteries and aortae at 8 days after injury showed high levels of expression for all 3 TGF-β isoforms, with TGF-β1 being the most abundant mRNA (Figure 3). These proliferating SMCs expressed TGF-βRII mRNA, but the levels were lower than in wound-edge endothelium. At 4 weeks after injury, SMCs on the surface of these deendothelialized vessels have returned to quiescence, and at this time point, mRNA expression levels for TGF-β1, TGF-β2, TGF-β3, and TGF-βRII were similar to those at 8 days (Figure 3).

Soluble TGF-βRII and the Balloon-Injured Carotid Artery

The left common carotid artery was denuded with a balloon catheter, and groups of animals received TGF-βR:Fc at concentrations of 0.5, 2, and 5 mg/kg by intravenous injection every other day over a period of 2 weeks. Representative sections of the vessels from the treatment group and control group injected with control IgG harvested after 2 weeks are shown in Figure 4. Control vessels developed an extensive neointima and adventitial fibrosis with abundant collagen production seen in blue on the Masson trichrome–stained sections, and some contraction of the elastic laminae was evident (Figure 4A and 4C). Vessels from soluble TGF-βRII–treated rats revealed little intimal lesion formation and a dramatic reduction in adventitial collagen (B and D). Staining with an anti-human IgG antibody was used to localize the injected reagent within the vessel wall (E and F). In the control group, only diffuse immunoreactivity throughout the arterial wall was seen (E), whereas soluble TGF-βRII was localized predominantly in the adventitia and neointima. Strong immunoreactivity for smooth muscle α-actin was present in the outer adventitia (arrow) of 4-day balloon-injured control vessels (G). Smooth muscle α-actin immunostaining revealed reduced expression or absence of staining in the adventitia (arrow) of vessels from soluble TGF-βRII–treated rats at 4 days (H). Original magnification: ×100 (A and B), ×400 (C through H).
recognizing the human IgG domain of the recombinant fusion protein. Immunoreactivity was concentrated in the neointima and adventitia (Figure 4F), suggesting the presence of active TGF-β ligands at these sites. Control vessels showed the presence of the control IgG in a very diffuse distribution throughout the vessel wall (Figure 4E).

TGF-β has been implicated in myofibroblastic transdifferentiation,³⁷⁻³⁹ causing fibroblasts to transiently express smooth muscle α-actin.⁴⁰ We therefore examined the expression of smooth muscle α-actin in the carotid artery by immunostaining at 4 days after balloon denudation, when proliferation of adventitial fibroblasts is rapid. Immunoreactive smooth muscle α-actin was either completely absent or markedly reduced in the outer adventitia of vessels from rats treated with TGF-βR:Fc (2 mg/kg, Figure 4G) compared with controls (Figure 4H).

Morphometric analysis of the carotid arteries showed significant increases in lumen area in all rats treated with TGF-βR:Fc (Figure 5A), with an ≈88% increase in the 2 mg/kg group. Intimal lesion formation and ratios of intimal/medial areas were significantly inhibited in the 2 mg/kg (=65%) and 5 mg/kg groups when compared with control animals (Figure 5B and 5C).

Loss of lumen area as a result of negative remodeling in the balloon-injured carotid arteries was assessed by measuring the length of the IEL and EEL. All treatment groups revealed significantly less shrinking of IEL and EEL compared with control animals, with an 18.3% increase in the IEL length in the 2 mg/kg group (Figure 6). Overall, the 2 mg/kg dose had the most pronounced effects on all of the parameters measured in this study.

Subsequent experiments sought to address the mechanism by which intimal lesion formation was inhibited in response to TGF-βR:Fc administration. To determine whether the reduced intimal lesion was the result of decreased intimal SMC proliferation, we performed BrdU labeling to identify the percentage of replicating cells. The proliferation index of intimal SMCs at 2 weeks after injury showed no significant differences between groups (Figure 7A). As expected from the intimal area data, however, the number of intimal cells was significantly lower in the 2 and 5 mg/kg treatment groups (Figure 7B). We then examined whether reductions in the intimal area were also affected by differences in extracellular matrix accumulation. This was done indirectly by determining the intimal cell density as nuclear profiles per mm². The differences between groups were not statistically significant, even though cell density was increased up to 30% in the 2 mg/kg treatment group (Figure 7C). In an additional experiment, we assessed whether medial SMC proliferation was inhibited by TGF-βR:Fc within the first 4 days after balloon injury, but no significant difference was found between groups (Figure 8A). In the same experiment, however, significantly fewer cells (44% reduction) had accumulated within the adventitia (Figure 8B).

### Expression of Matrix Molecules

Masson trichrome staining of sections indicated that adventitial collagen deposition was reduced in response to TGF-βR:Fc treatment. Northern blot analysis of total RNA isolated

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**Figure 5.** Morphometric analysis of rat carotid arteries 2 weeks after balloon injury. Groups of animals (n=5 per group) received injections of soluble TGF-βRII (0.5, 2, or 5 mg/kg) or control IgG every other day. Lumen area (A), intimal area (B), and intimal area/medial area (C) ratios are shown. *Significant difference from the control group (P<0.05). Data are mean±SEM.
from balloon-injured carotid arteries 4 days after treatment with TGF-βR:Fc was performed to determine expression of matrix molecules that are thought to be under transcriptional control of TGF-β. Marked reductions in mRNA levels of fibrillar collagens, ie, α1 chain of type I and α1 chain of type III collagen, were seen with TGF-βR:Fc treatment, whereas levels of collagen XV were not affected (Figure 9). No obvious differences in mRNA levels were seen for osteopontin, tropoelastin, and fibronectin (Figure 9), genes of which the expression is induced during vascular injury. The Northern blot shown in Figure 9 is representative of 2 independent experiments.

Endothelial Growth in the Aorta

The in situ hybridization data demonstrate that TGF-β1 and TGF-β2, as well as the TGF-βRII, show increased expression in proliferating and migrating endothelium compared with normal endothelium. The effects of TGF-β signaling on reendothelialization of the denuded rat aorta were therefore examined. The surface area covered with endothelium 2 weeks after denudation and treatment with TGF-βR:Fc or control IgG was measured, and in both groups 53.5% of the surface area was reendothelialized (Figure 10).

Discussion

The present study sought to determine the role of TGF-β in arterial repair processes after balloon catheter injury. Our expression analysis by en face in situ hybridization revealed that all 3 TGF-β isoforms were abundantly expressed in intimal SMCs, and all of these isoforms were also present in endothelium with dramatically upregulated expression of TGF-β1 and TGF-β2 in migrating and proliferating endothelium. Among the TGF-β isoforms, mRNA for TGF-β1 showed the highest expression levels in intimal SMCs, suggesting that this isoform might be the most relevant. Since the TGF-β1, TGF-β2, and TGF-β3 isoforms have distinct and overlapping functions, our approach was to interfere with receptor-mediated signaling that is shared by the different isoforms, thereby inhibiting the biological responses of several TGF-β ligands. Recombinant human soluble TGF-βRII has been shown to inhibit responses mediated by TGF-β1, TGF-β2, and TGF-β3, but not TGF-β2. After verifying the efficacy of the TGF-βR:Fc to inhibit TGF-β1-mediated responses in the mink lung epithelial cell assay, we tested the role of TGF-β signaling in the rat carotid artery balloon injury model. Even the lowest dose used (0.5 mg/kg) caused nearly a 60% increase in lumen area despite the fact that intimal lesion formation was not affected by this dose. This indicates that loss of lumen area is in large part due to negative remodeling (vessel shrinking) and measurements of the perimeter of the neointima (IEL) and media (EEL) demonstrate that all doses of TGF-βR:Fc used in this study significantly inhibited the reduction in IEL and EEL. We envision the following mechanism for this phenomenon. Assuming that IEL and EEL are elastic structures that can be stretched and shortened in a reversible manner and, further, that in a normal vessel under physiological blood pressure the elastic laminae are stretched to a certain extent (not maximally shortened), one would measure a shorter IEL and EEL in a more relaxed state. We believe that the more relaxed state is forced on the elastic laminae by the constriction resulting from the fibrosis in the adventitia.

The effect of TGF-βR:Fc on remodeling is highly relevant to the clinical situation in which this is the major cause for restenosis after angioplasty. Immunostaining with an anti-human IgG antibody demonstrated that the TGF-βR:Fc primarily localized to the adventitia and neointima, suggesting that these are the predominant sites of TGF-β activity. One obvious effect of soluble TGF-βRII was on collagen synthesis, and this was particularly striking in the adventitia of Masson trichrome–stained sections. We further investigated the effects of TGF-βR:Fc on collagen expression by Northern blot analysis of RNA isolated from carotid arteries 4 days after injury. A marked reduction in mRNA levels was apparent for collagen types I and III, despite the differences in RNA loading. Interestingly, expression of other genes that are thought to be regulated by TGF-β, including tropoelastin and fibronectin, were not affected, and expression of the nonfibrillar collagen XV and osteopontin was also not changed. The increased cell density within the neointima of TGF-βR:Fc–treated rats suggests that extracellular matrix production may also be reduced in the intimal lesion. Therefore, TGF-βR:Fc is a very potent inhibitor of negative vascular remodeling by inhibiting collagen production, particularly in the adventitia.

Intimal lesion formation is the result of cell migration, cell proliferation, and matrix accumulation. The studies per-
formed here did not reveal a statistically significant effect of TGF-βR:Fc on proliferation of intimal SMCs, despite the fact that intimal cell numbers were significantly lower in the treated rats. In addition, the increase in intimal cell density as an indirect measure of extracellular matrix synthesis in the TGF-βR:Fc group also did not reach statistical significance. A potential explanation for the differences in intimal SMC number could be via an effect of TGF-βR:Fc on cell death, but staining for apoptotic cells with the terminal deoxynucleotidyl transferase–mediated nick end labeling (TUNEL) technique revealed no differences, with very few TUNEL-positive cells present in all groups (data not shown). Proliferation of SMCs after balloon injury of the rat carotid artery begins with medial SMC proliferation, and we therefore asked whether early proliferative events are inhibited by TGF-βR:Fc, by measuring medial SMC replication at 4 days after injury. The difference in the replication between the control and treatment groups was not significant, and in both groups only very few TUNEL-positive cells were detected at this time point (data not shown). Interestingly, the number of fibroblasts in the adventitia was significantly lower in the TGF-βR:Fc group, suggesting inhibition of fibroblast proliferation; however, the adventitial cell replication index at 4 days was not significantly different between groups (data not shown). An additional experiment measuring medial SMC proliferation at 4 days with a longer BrdU labeling period (3 injections over 24 hours) also showed no significant difference, although ≈30% more BrdU-positive cells were present in the media compared with the experiment with a single BrdU injection (data not shown). In both experiments, however, there was a trend with a decreased average replication index in the soluble TGF-βRII group. Therefore, it is possible that a larger number of animals per group may have revealed a significant difference. In the present study we did not examine time points earlier than 4 days after injury. Because the first wave of medial SMC and adventitial fibroblast replication occurs within 48 hours and maximal SMC apoptosis is seen within hours after balloon injury, it is still possible that TGF-βR:Fc treatment was affecting early cell proliferation, apoptosis, or both.

Migration of SMCs from the media into the intima is difficult to assess in this in vivo model, particularly because virtually all SMCs arriving in the intima are also replicating. TGF-β has been reported to prolong the duration of the cell cycle. Although this remains a possibility, it is difficult to reconcile this potential mechanism with the present findings of reduced neointimal formation in response to TGF-βR:Fc treatment. In a previous study, using a high dose of TGF-β, we observed a significant increase in intimal SMC proliferation.14 Together, these data suggest that the net effect of TGF-β signaling in SMCs and fibroblasts in vivo is stimulatory. Although in the experiments performed here we could not detect a significant effect of TGF-βR:Fc on SMC proliferation, it should be emphasized that loss of lumen area is predominantly a result of negative remodeling, which is dramatically inhibited by TGF-βR:Fc. This is particularly evident by the observation that even the lowest dose of TGF-βR:Fc (0.5 mg/kg), which had absolutely no effect on intimal lesion formation, caused nearly a 60% increase in

Figure 7. A, SMC proliferation in the neointima at 2 weeks was determined by BrdU labeling and showed no significant difference between groups. B, Intimal cell numbers were significantly lower in rats injected with 2 and 5 mg/kg of soluble TGF-βRII. C, Number of nuclear profiles per mm² within the intimal lesion. *Significant difference from the control group (P<0.05). Data are mean±SEM.
lumen area. Thus, regardless of its effects on SMC proliferation, TGF-βR:Fc is a potent inhibitor of negative vascular remodeling. Another potential mechanism that could explain the TGF-βR:Fc effects on remodeling is related to the ability of TGF-β to induce myofibroblastic transdifferentiation, caus- ing fibroblasts to express smooth muscle α-actin. The reduction in adventitial smooth muscle α-actin expression after treatment with TGF-βR:Fc was striking, indicating that the induction of smooth muscle α-actin expression by adventitial fibroblasts is mediated by TGF-β. Whether expression of smooth muscle α-actin by adventitial myofibroblasts is directly affecting negative remodeling by exerting an active constrictive effect remains to be determined.

Unlike SMCs and fibroblasts, growth of endothelial cells in the aorta was not affected at all by the TGF-βR:Fc treatment. This result is surprising, because numerous studies have demonstrated that at least in vitro, TGF-β inhibits endothelial cell proliferation. It was also interesting to note that, compared with normal endothelium, much higher levels of mRNA for TGF-β₁, TGF-β₂, and TGF-βRII were expressed by endothelial cells near the wound edge, where active migration and proliferation occur. To our knowledge, there is currently no information as to the effects of TGF-β on large vessel endothelium in vivo, and the expression patterns for TGF-β ligands and TGF-βRII observed here do not support the concept of TGF-β as an inhibitor of endothelial cell growth in vivo. One issue that the present study did not address relates to activation of TGF-β, which is synthesized by most cells in an inactive form that does not bind to cellular receptors. Both plasmin and thrombospondin have been identified as physiologically relevant activators of TGF-β₁. Plasmin generation occurs in the arterial wall, and thrombospondin is expressed by many cell types, including SMCs in the balloon-injured vessel wall. It is therefore likely that active TGF-β is present in injured arteries. The TGF-βR:Fc binds only active TGF-β, and given that TGF-βR:Fc localized preferentially to the neointima and adventitia, it seems reasonable to assume that active TGF-β is present in these locations. The differential effects of TGF-βR:Fc on adventitia and neointima versus endothelium are
Soluble TGF-βRII

Reendothelialization (%): 53.5 ± 1.2

Control

Reendothelialization (%): 53.5 ± 1.7

Figure 10. Reendothelialization of the rat aorta 2 weeks after denudation and treatment with soluble TGF-βRII or control IgG (control) was determined by Evans blue staining. Endothelial regeneration occurring from the intercostal arteries (white areas) was 53.5% of the total surface area in both groups.

interesting, because they indicate that the effects seen in SMCs and fibroblasts are specific or that localization of active TGF-β is not near endothelial cells. One difference between endothelium and SMCs was that wound-edge endothelium expressed much higher levels of TGF-βRII mRNA than SMCs, and thus there is the possibility that the doses of TGF-βR:Fc used in the experiments were sufficient to block TGF-β signaling in SMCs and fibroblast but not in endothelium.

In summary, the present study demonstrates that TGF-β signaling is responsible for negative remodeling with adventitial fibrosis and neointima formation in an arterial balloon injury model. These TGF-β-mediated effects can be inhibited with a soluble TGF-βRII without affecting reendothelialization of the denuded vessels. This reagent may be useful in a clinical setting for targeting restenosis after angioplasty.

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