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# Vascular Smooth Muscle Cells from Injured Rat Aortas Display Elevated Matrix Production Associated with Transforming Growth Factor-β Activity

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***The arterial response to injury is characterized by a short period of increased proliferation and migration of vascular smooth muscle cells, followed by an extended period of extracellular matrix accumulation in the intima. Transforming growth factor-β (TGF-β) has been implicated as a causative factor in the formation of extracellular matrix in this process, which leads to progressive thickening of the intima, known as intimal hyperplasia.* In vitro *analysis of vascular smooth muscle cells harvested from normal rat aortas and from aortas injured 14 days earlier showed that both types of cells attached equally well to culture dishes but that the initial spreading of the cells was increased in cells derived from injured vessels. Cells from the injured arteries produced more fibronectin and proteoglycans into the culture medium than the cells from normal arteries and contained more TGF-β1 mRNA. TGF-β1 increased proteoglycan synthesis by normal smooth muscle cells, and the presence of a neutralizing anti-TGF-β1 antibody reduced proteoglycan synthesis by the cells from injured arteries in culture. Fibronectin synthesis was not altered by these treatments. These results indicate that the accumulation of extracellular matrix components in neointimal lesions is at least partially caused by autocrine TGF-β activity in vascular smooth muscle cells. (Am J Pathol 1995, 147:1041–1048)***

Accumulation of extracellular matrix components is a feature of intimal hyperplasia both in human and experimental situations. This accumulation is believed to play a role in the progression of intimal lesions after an initial period dominated by cell proliferation.[1–3] Transforming growth factor-β (TGF-β) has been shown to be a causative factor in pathological accumulation of extracellular matrix components that characterizes tissue fibrosis.[4] Such pathologies include intimal hyperplasia, for which several lines of evidence suggest TGF-β involvement. First, TGF-β1 mRNA levels are increased in both experimental intimal hyperplastic lesions as well as in human restenotic lesions.[5,6] Secondly, neutralizing antibodies against TGF-β can reduce size and extracellular matrix accumulation in experimentally induced intimal hyperplasia.[7] Moreover, transfection of TGF-β1 cDNA into pig arteries elicited intimal lesions, dominated by a fibrotic element.[8] Thus, TGF-β, although it may inhibit smooth muscle cell activation in the early stages of arterial repair processes,[9] may be responsible for excessive matrix formation later on.

To gain information on the cellular sources of TGF-β and extracellular matrix in intimal hyperplasia, we have studied the role of TGF-β in extracellular matrix synthesis by smooth muscle cells taken into culture from injured arteries.

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## Materials and Methods

### Balloon Catheter Injury to Rat Aortas

Male Sprague-Dawley rats (Harlan Laboratories) weighing 350 to 400 grams were anesthetized with Innovar, pentobarbital and atropine. A 3 F Fogarty balloon catheter (America Edwards Laboratories) was inserted through the left common iliac artery and balloon injury was inflicted to the thoracic and abdominal aorta.

### Vascular Smooth Muscle Cell Culture

After sacrifice of the rats, the thoracic aorta was removed and placed in Dulbecco's minimal essential medium (DMEM) containing 50 U/ml penicillin, 100 μg/ml streptomycin, and 300 mg/ml glutamine, preheated to 37°C. The adventitia was carefully removed under a dissection microscope and endothelial cells were removed during a 20-minute incubation of the vessels in a solution of 2 mg/ml collagenase (Worthington CLS1, 217 U/mg) in DMEM/HAM F-12 (1:1) with 10 mmol/L Hepes, 50 U/ml penicillin, 100 μg/ml streptomycin, and 300 mg/ml glutamine. Smooth muscle cells were then dispersed from the tissue in 2 mg/ml collagenase, 1 mg/ml elastase (Sigma E-7885, 80 U/mg; Sigma Chemical Co., St. Louis, MO), and 0.5 mg/ml soybean trypsin inhibitor (Sigma T-6522, 10,000 BAEU/mg) in DMEM/HAM during a 2.5-hour incubation at 37°C under constant orbital shaking. The liberated cells were washed twice in DMEM containing 10% fetal calf serum (FCS), dissolved in 0.5 ml of 10% FCS in DMEM, and counted in a hemocytometer. More than 90% of the cells were viable as judged by exclusion of trypan blue. The average yield of cells after collagenase and elastase dispersing of the cells from noninjured vessels was approximately 400,000 and from injured vessels approximately 750,000. Freshly enzyme-dispersed smooth muscle cells were plated in either 12-well tissue culture dishes (Corning Glass Works, Corning, New York) at a density of 50,000 cells/ml (1000 μl/well) for morphology studies or at a density of 150,000 cells/ml (100 μl/well) in 96-well plates (Corning) for metabolic labeling studies. The cells were plated in 10% FCS in DMEM and, after 20 hours, washed twice with serum-free medium and for the rest of the experiments maintained in serum-free medium containing 0.1% bovine serum albumin. In some studies neutralizing chicken polyclonal antibodies against TGF-β1 or control chicken immunoglobulins were added to the incubation media at concentrations of 25 μg/ml (AB-101-NA and AB-101-C, R&D Systems, Minneapolis, MN). The chicken anti-human TGF-β1 recognizes TGF-β1 but not TGF-β2 and -3 according to the supplier. This antibody has previously been used to neutralize TGF-β of murine source.[10] The antibodies were present in the initial plating solution and were added when the cells were changed to serum-free conditions after 20 hours and again after a 24-hour incubation. For studies of the effect of TGF-β1 on [$^{35}$S]sulfate and [$^{35}$S]methionine incorporation in vascular smooth muscle cells, cells from normal arteries were grown to confluency in 96-well plates and metabolic labeling experiments were performed with these cells after 20 hours in serum-free conditions.

### Metabolic Labeling with Sulfate and Methionine

After a 12-hour incubation of the primary harvested cells in serum-free conditions, the cultures were pulsed with either 200 μCi/ml [$^{35}$S]sulfate or 100 μCi/ml [$^{35}$S]methionine for an additional 48 hours. The culture media were then removed; phenylmethylsulfonyl fluoride (Sigma Chemical Co.), pepstatin, and aprotinin (Calbiochem, La Jolla, CA) were added as protease inhibitors; and samples were either electrophoresed immediately or stored at −20°C. Incubations with [$^{35}$S]sulfate were performed in antibiotic-free media.

### Immunoprecipitation

Samples for immunoprecipitation were first precleared by incubating for 30 minutes with nonimmune rabbit IgG and protein-A-Sepharose beads. Subsequently, 100-μl aliquots of the supernatants were mixed with rabbit antiserum against fibronectin and incubated overnight at 4°C. Protein-A-Sepharose beads (Sigma Chemical Co.) were added to precipitate the antigen-antibody complex and washed eight times in RIPA buffer. The pellets were dissolved in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS and 5% mercaptoethanol and heated at 100°C for 10 minutes.

### Chondroitinase ABC Treatment

For molecular characterization of labeled proteoglycans, the glycosaminoglycan side chains were removed by treating conditioned media with 5 U/ml chondroitinase ABC (Seikagaku 100330, 110 U/mg,

protease-free) in 0.1 mol/L Tris-HCl, pH 8.0, containing 0.03 mmol/L sodium acetate for 5 hours at 37°C.

### *Separation of Metabolically Labeled Macromolecules by SDS-PAGE*

Samples for SDS-PAGE were mixed with sample buffer to a final concentration of 2% SDS/5% mercaptoethanol and heated for 2 minutes at 100°C. Aliquots were applied to 4 to 12% gradient gels (Novex, Encinitas, CA). Prestained molecular size markers were from GIBCO BRL (Bethesda, MD). Fluorography was performed by incubating gels in Enlightning (New England Nuclear, Boston, MA), drying, and exposing the gels to x-ray films. To quantitate individual separated molecules, the dried gels were scanned with a gas detector on an AMBIS scanner.

### *Isolation of Total RNA, Reverse Transcription, and Polymerase Chain Reaction (PCR)*

Total RNA from cultured cells was isolated by lysing cells in guanidinium isothiocyanate in the presence of sodium sarkosyl and mercaptoethanol, with the RNAzol reagent (Teltest Inc., Friendswood, TX) as described by Chomczynsky and Sacchi.[11] RNA was precipitated, quantitated by absorbance at 260 nm, and stored at −70°C. RNA (500 ng) was reverse transcribed with random hexamer primers in 30 μl of PCR buffer (50 mmol/L KCl, 10 mmol/L Tris-HCl, pH 8.0, 1.5 mmol/L $MgCl_2$, 0.001% gelatin, 0.1 mg/ml bovine serum albumin) containing 1 mmol/L each dNTP (Boehringer Mannheim, Indianapolis, IN) 80 U of RNAse inhibitor (Boehringer Mannheim), 1 μmol/L random hexamers (Pharmacia, Uppsala, Sweden), and 1000 U of murine Moloney leukemia virus reverse transcriptase (GIBCO BRL) at 37°C for 1.5 hours. Aliquots of cDNA were then subjected to PCR in 30 μl of the same buffer as mentioned above for the reverse transcription reaction with 0.3 mmol/L each dNTP, 0.5 U of Taq polymerase (Boehringer Mannheim), and 0.6 μmol/L specific 5′ and 3′ primers for TGF-β1. 5′-TACAGCAACAATTCCTGGCG-3′ (h-TGF-β1–1363(+)) and 5′-GCAGGAAAGGCCGGTTCATG-3′ (h-TGF-β1–1618(−)) were used as 5′ and 3′ primers, respectively. The primers were from two different exons.[12] PCR was performed in glass capillary tubes in a thermocycler from Idaho Technology with denaturing at 95°C for 4 seconds, annealing at 45°C for 1 second, and extension at 74°C for 40 seconds. The number of cycles used was determined after initial optimizing experiments to make sure that a dose-response relation could be obtained for TGF-β1 mRNA molecules. These experiments included non-reverse-transcribed RNA and water as negative controls.

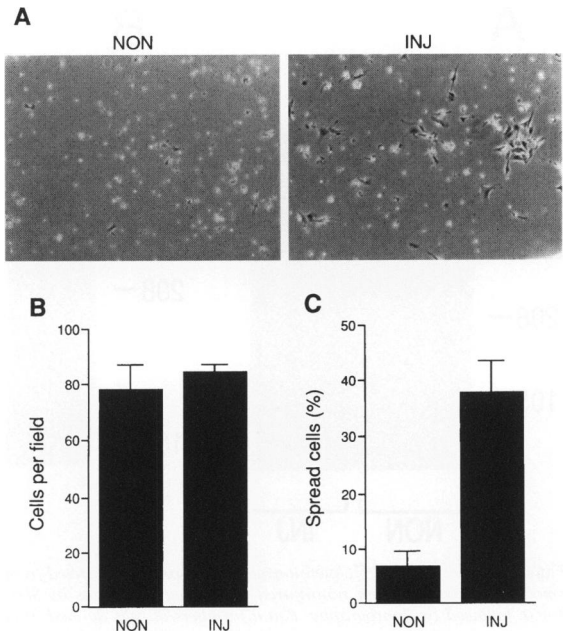


**Figure 1.** *Attachment and spreading of smooth muscle cells cultured from injured and noninjured rat aortas. The cells were enzyme dispersed from the tissue, incubated in 10% FCS for 20 hours, washed in serum-free medium twice, and incubated for an additional 16 hours.* **A:** *Comparison of the morphology of smooth muscle cells from noninjured (NON) and injured (INJ) rat aortas.* **B:** *Attachment of cells from noninjured and injured aortas. Attached cells were counted in fields defined by a grid. Results are presented as mean ± SEM (n = 5 aortas in both groups).* **C:** *Spreading of attached cells from noninjured and injured aortas. The number of spread cells were counted and the results are presented as percentage of cells spread in relation to total number of attached cells (mean ± SEM; n = 5 aortas in both groups, 2P < 0.05).*

### *Statistical Evaluation*

Student's *t*-test (two-tailed) for nonpaired observations were used to evaluate differences between injured and noninjured cells. To evaluate differences between anti-TGF-β1-treated and control cells, Student's *t*-test for paired observations was used.

## *Results*

Smooth muscle cells isolated from injured (iSMC) and non-injured (nSMC) vessels attached equally well to tissue culture plastic in the presence of 10% serum, but the injured tissue cells were better spread during the initial several days of culture (Figure 1). The difference in spreading suggested that the two cell populations could differ with regard to the synthesis of adhesive extracellular matrix proteins. Met-

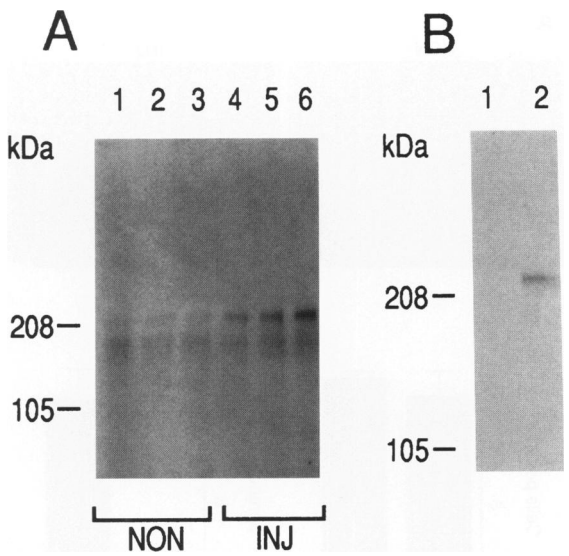


Figure 2. *Separation of [$^{35}$S]methionine-labeled proteins secreted from smooth muscle cells from noninjured or injured rat aortas by SDS-PAGE followed by fluorography. Equal numbers of cells derived from noninjured and injured aortas were plated in 10% FCS, changed to serum-free conditions after 20 hours and maintained in culture for an additional 3 days. During the last 48 hours the cells were labeled with [$^{35}$S]methionine. Aliquots of labeled medium were subjected to SDS-PAGE and fluorography.* **A:** **Lanes 1** *to* **3** *show labeled medium of cells from three noninjured (NON) arteries and* **lanes 4** *to* **5** *show medium of cells from three injured (INJ) aortas.* **B:** *Immunoprecipitation with anti-fibronectin* **(lane 2)** *or a control nonimmune serum* **(lane 1)** *from a pool of [$^{35}$S]methionine-labeled conditioned media from injured cells. Molecular weight markers are shown to the left.*

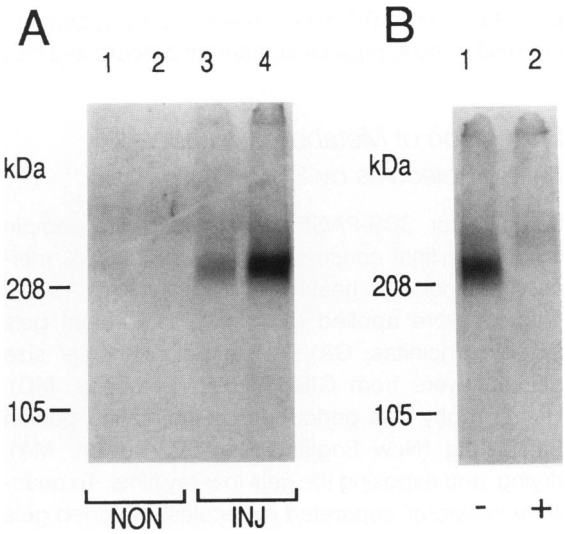


Figure 3. *Separation of [$^{35}$S]sulfate-labeled proteoglycans in conditioned medium from cells derived from noninjured and injured aortas by SDS-PAGE followed by fluorography. Experimental design was as described in the legend of Figure 2 except that [$^{35}$S]sulfate was used for metabolic labeling.* **A:** *Proteoglycans synthesized from cells from two normal (NON) aortas* **(lanes 1** *and* **2)** *and two injured (INJ) aortas* **(lanes 3** *and* **4)** *are shown.* **B:** *Chondroitinase ABC treatment of a pool of [$^{35}$S]sulfate-labeled conditioned media from injured cells.* **Lane 1** *is control-treated, conditioned medium;* **lane 2** *is conditioned medium treated with chondroitinase ABC.*

abolic labeling of secreted macromolecules with [$^{35}$S]methionine showed that, whereas some proteins were secreted in equal amounts from iSMCs and nSMCs, the injured cells produced more of a polypeptide migrating with a molecular mass of 220 kd (Figure 2A). The 220-kd protein was identified as fibronectin by immunoprecipitating with fibronectin antibodies (Figure 2B). The variation between samples was small in these experiments, as a background band at approximately 190 kd did not vary significantly between samples (Figure 2A).

Labeling with [$^{35}$S]sulfate showed increased levels of radioactivity in several secreted components from iSMCs. Both a band at 200 to 250 kd and a band that barely penetrated the SDS-polyacrylamide gel showed increased sulfate incorporation (Figure 3A). Chondroitinase ABC treatment of [$^{35}$S]sulfate-labeled conditioned medium identified the 200 to 250-kd band and part of the high molecular weight band as chondroitin sulfate/dermatan sulfate proteoglycans (Figure 3B).

Reverse transcription PCR quantitation showed an increase of TGF-$\beta$1 mRNA in cells from injured arteries (Figure 4). Attempts to measure TGF-$\beta$ activity in conditioned media from both nSMCs and iSMCs were done with mink lung epithelial growth inhibition assays. However, the content of TGF-$\beta$ activity was below the sensitivity limit in this assay (data not shown).

Treatment of cells from normal arteries with TGF-$\beta$1 increased proteoglycan synthesis, without having a major effect on total protein or fibronectin in the medium (Figure 5). To test whether TGF-$\beta$1 action is involved in the differences we observed between injured and noninjured cells, we performed

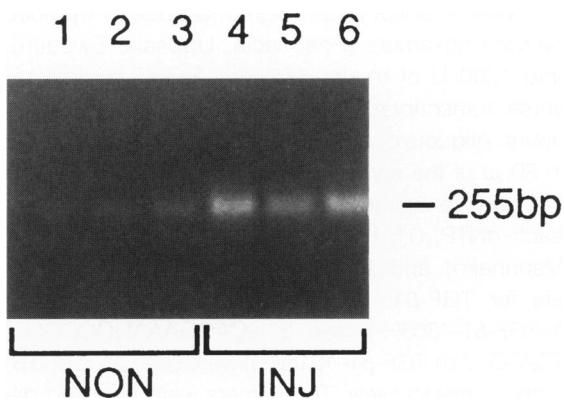


Figure 4. *TGF-$\beta$1 mRNA in smooth muscle cells from noninjured and injured aortas. Total RNA (500 ng) from cells cultured for 2 days after plating were subjected to reverse transcription PCR (32 cycles) to amplify TGF-$\beta$1. PCR products were separated in a 2% agarose gel. The size of the specific band is indicated on the right.* **Lanes 1** *to* **3** *show results from cells obtained from three noninjured aortas (NON) and* **lanes 4** *to* **6** *show results obtained from three injured aortas (INJ).*

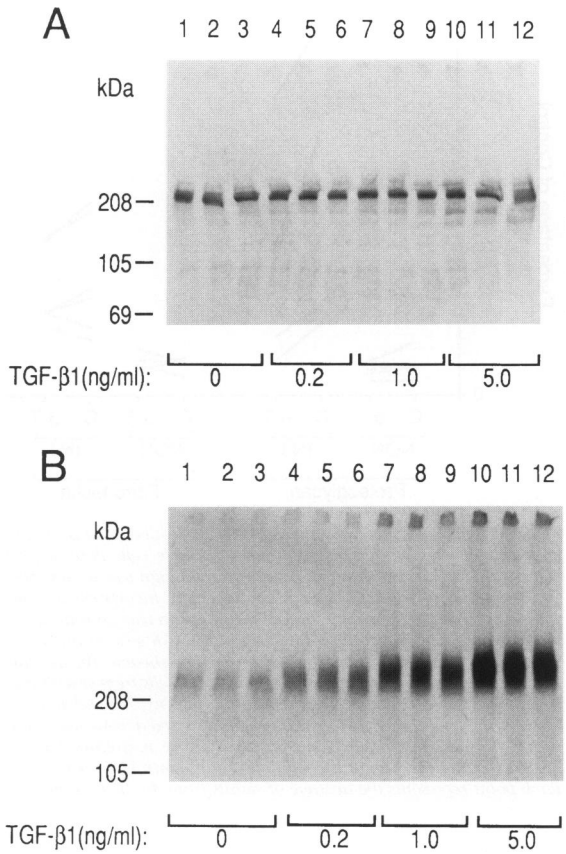


Figure 5. *Effect of TGF-β1 on [$^{35}$S]methionine and [$^{35}$S]sulfate incorporation into macromolecules secreted by vascular smooth muscle cells from noninjured aortas. Smooth muscle cells were serum starved for 20 hours before being subjected to TGF-β1 treatment and either [$^{35}$S]methionine* (**A**) *or [$^{35}$S]sulfate* (**B**) *labeling for 48 hours. Labeled macromolecules were separated by SDS-PAGE and visualized by fluorography. Results from control-treated cells* (**lanes 1** *to* **3**) *and cells treated with 0.2 ng/ml* (**lanes 4** *to* **6**), *1 ng/ml* (**lanes 7** *to* **9**), *or 5 ng/ml* (**lanes 10** *to* **12**) *TGF-β1 are presented. Molecular weight markers are shown at the left.*

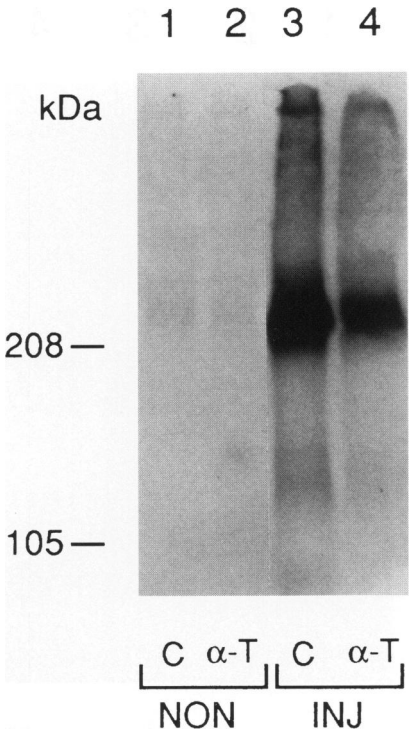


Figure 6. *Effect of a neutralizing TGF-β antibody on proteoglycan synthesis by smooth muscle cells derived from injured and noninjured aortas. Conditioned medium from cells derived from noninjured (NON) or injured (INJ) aortas was analyzed by SDS-PAGE followed by fluorography. Experimental design was as described in the legend of Figure 3. The cells were incubated in the presence of either a control chicken IgG (C) or a neutralizing antibody against TGF-β1 (α-T).*

metabolic labeling experiments with a neutralizing antibody against TGF-β1 present in the growth medium. This treatment reduced the [$^{35}$S]sulfate labeling of proteoglycans in the medium of the cells from injured arteries. Figure 6 shows a representative example of such results. Quantitation showed on average a sevenfold increase in the synthesis of the 200- to 250-kd proteoglycan in the cells from injured arteries relative to the noninjured cells. This increase was reduced by an average of 33% in the presence of the neutralizing TGF-β1 antibody ($2P < 0.01$; paired Student's *t*-test; see Figure 8). A fourfold increase was seen in fibronectin levels in the injured cell populations and, although some experiments showed a small decrease in the synthesis of fibronectin in the presence of the neutralizing TGF-β antibody, no consistent reduction was seen (Figures 7 and 8).

## Discussion

We show here that cultured cells from 14-day-old arterial lesions display greatly elevated production of extracellular matrix components, such as fibronectin and proteoglycans, we also provide direct evidence that autocrine TGF-β activity within the smooth muscle cells in the injured arteries is at least partially responsible for the matrix producing phenotype.

Previous studies have shown increased migratory ability, an epithelial-like morphology, and increased platelet-derived growth factor expression in smooth muscle cells obtained from injured arteries.[13–16] These studies, however, have not provided information on the extracellular matrix accumulation that has been suggested as the major mechanism whereby intimal lesions grow in the late stages after arterial injury.[1–3] Our results add extracellular matrix production and TGF-β expression to the characteristics that distinguish iSMCs from nSMCs. Our finding that iSMCs decreased proteoglycan synthesis when cultured in the presence of a neutralizing TGF-β antibody indicates that autocrine TGF-β activity is responsible for at least some of the elevated proteoglycan production in these cells. The cells

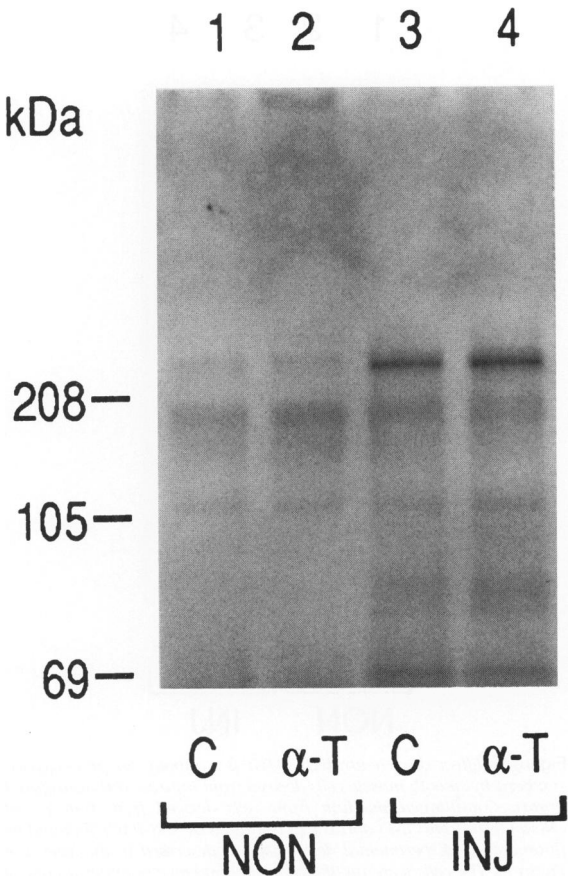


Figure 7. *Effect of a neutralizing TGF-β antibody on protein synthesis by smooth muscle cells derived from injured and noninjured aortas. Conditioned medium from cells derived from noninjured (NON) or injured (INJ) aortas was analyzed by SDS-PAGE followed by fluorography. Experimental design was as described in the legend of Figure 2. The cells were incubated in the presence of either a control chicken IgG (C) or a neutralizing antibody against TGF-β1 (α-T).*

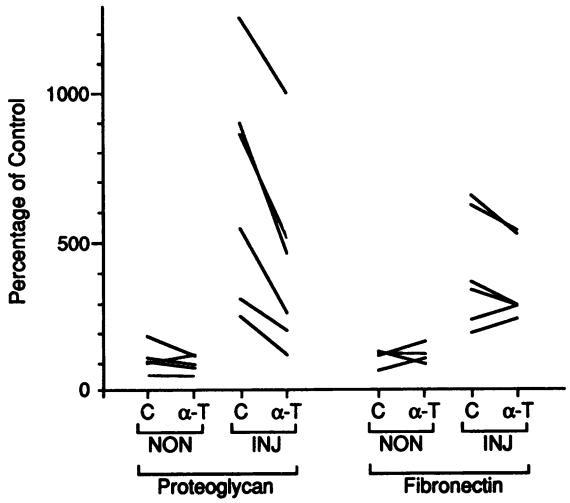


Figure 8. *Effect of a neutralizing anti-TGF-β1 antibody on proteoglycan and fibronectin synthesis by smooth muscle cells derived from injured and noninjured aortas. Experimental design was as described in the legends of Figures 6 and 7. Radioactivity incorporated (cpm) into the 200- to 250-kd proteoglycan or fibronectin was quantitated by scanning the bands directly from dried SDS-PAGE gels on an AMBIS scanner. Results from several experiments are shown. The average radioactivity obtained from noninjured (NON) cells treated with control immunoglobulin was set to 100% in each experiment and the results from the injured (INJ) groups were expressed accordingly. Lines indicate results from cells from the same artery maintained in the presence of neutralizing anti-TGF-β (α-T) or control (C) antibodies. Each point represents the average of results from duplicate samples.*

were plated in the presence of 10% FCS and we cannot completely exclude the possibility that TGF-β present in the serum could have contributed to the TGF-β activity in our experimental system. However, because we also found augmented TGF-β1 mRNA in the injured cells and because the proteoglycan production was evaluated after the cells had been maintained for 24 hours in serum-free conditions, it seems quite unlikely that serum TGF-β would have been a factor in the proteoglycan synthesis. Instead, it appears that TGF-β activity is produced by the iSMCs in an autocrine fashion.

The autocrine production of active TGF-β requires not only synthesis of the growth factor but also conversion from the latent form into the active form.[17] Our *in vitro* results suggest that both increased production and conversion of TGF-β to its active form is carried out by the iSMCs *in vitro*. Normal rat vascular smooth muscle cells produce inactive TGF-β in culture but generate no detectable TGF-β activity into the growth medium.[18–20] To exert its activity in an autocrine fashion, TGF-β would have to be activated by some mechanism in the population of cells from the injured aortas. Activation of smooth muscle cell-derived TGF-β by endothelial cells has been described in a coculture system.[18–20] It is not likely that a substantial number of endothelial cells would be present in our cell preparation, because regrowth of endothelium has taken place only near the ends of the injured area after 14 days[21] and because most luminal cells were removed by a collagenase treatment of the tissue before harvesting the smooth muscle cells. Therefore, it seems likely that the TGF-β precursor is activated by the smooth muscle cells themselves. The mechanisms in the endothelial/smooth muscle cell coculture system that activates TGF-β is believed to involve activation of plasmin, which in turn cleaves the TGF-β precursor, thereby activating the latent complex.[19] Although increased local production of plasminogen activators has been found in tissue after arterial injuries,[22] the mechanism of TGF-β activation is probably not via plasmin in our study, because our experiments were performed in the absence of serum. It may be that the injury activates an alternative mechanism of TGF-β processing.

TGF-β is a potent stimulator of smooth muscle cell proteoglycan synthesis, as shown previously[23] and in the present study. We could also show that anti-TGF-β treatment of iSMCs suppressed much of the proteoglycan synthesis in these cells. These results suggest that the accumulation of proteoglycans in the intimal hyperplasia matrix at least partly can be ascribed to TGF-β effects. The situation appears to be more complex with fibronectin. Whereas TGF-β enhances the synthesis of the alternative spliced $EDA^+$ form of fibronectin,[24] previous studies[25] and our present data show that TGF-β does not increase the total synthesis of fibronectin by nSMCs in culture. The increased fibronectin synthesis in injured cells could also not be decreased by neutralizing TGF-β. Therefore, the augmented fibronectin production must be dependent on some other factor. Considering the known ability of TGF-β to induce fibronectin synthesis by other types of cultured cells,[26] it seems possible that the elevated fibronectin synthesis was originally induced by TGF-β in the tissue environment but has become refractory to TGF-β or TGF-β neutralization under the culture conditions.

In summary, these data provide strong evidence for an autocrine TGF-β effect in the accumulation of extracellular matrix in neointimal lesions that follow arterial injury.

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