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M W Majesky, … , S M Schwartz, M A Reidy

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Research Article

Repair of arterial injury produced by balloon angioplasty leads to the formation of a neointima and a narrowing of the vascular lumen. In this study, we examined the possibility that smooth muscle cells (SMC) in injured rat carotid arteries are stimulated to produce type-1 transforming growth factor-beta (TGF-beta 1) during neointima formation in vivo. Levels of TGF-beta 1 transcripts (2.4 kb) were significantly increased within 6 h after carotid injury and reached a maximum (five to sevenfold) by 24 h. Regenerating left carotids had sustained increases in TGF-beta 1 mRNA levels (about fivefold) over the next 2 wk, during which time a substantial neointimal thickening was formed. No changes in basal TGF-beta 1 mRNA levels were found in contralateral uninjured carotids at any of the times examined. Immunohistochemical studies showed that a large majority of neointimal SMC were stained for TGF-beta 1 protein in an intracellular pattern, consistent with active TGF-beta 1 synthesis in this tissue. Neointima formation and TGF-beta 1 immunoreactivity were correlated with increases in fibronectin, collagen alpha 2(I), and collagen alpha 1(III) gene expression. Infusion of purified, recombinant TGF-beta 1 into rats with a preexisting neointima produced a significant stimulation of carotid neointimal SMC DNA synthesis. These results suggest that TGF-beta 1 plays an important role as an endogenous growth regulatory factor produced by neointimal SMC themselves […]

Production of Transforming Growth Factor β_1 during Repair of Arterial Injury

Mark W. Majesky, Volkhard Lindner, Daniel R. Twardzik,* Stephen M. Schwartz, and Michael A. Reidy
*Department of Pathology, University of Washington, Seattle, Washington 98195; and *Oncogen, Inc., Seattle, Washington 98121*

Abstract

Repair of arterial injury produced by balloon angioplasty leads to the formation of a neointima and a narrowing of the vascular lumen. In this study, we examined the possibility that smooth muscle cells (SMC) in injured rat carotid arteries are stimulated to produce type-1 transforming growth factor-β (TGF-β_1) during neointima formation in vivo. Levels of TGF-β_1 transcripts (2.4 kb) were significantly increased within 6 h after carotid injury and reached a maximum (five to sevenfold) by 24 h. Regenerating left carotids had sustained increases in TGF-β_1 mRNA levels (about fivefold) over the next 2 wk, during which time a substantial neointimal thickening was formed. No changes in basal TGF-β_1 mRNA levels were found in contralateral uninjured carotids at any of the times examined. Immunohistochemical studies showed that a large majority of neointimal SMC were stained for TGF-β_1 protein in an intracellular pattern, consistent with active TGF-β_1 synthesis in this tissue. Neointima formation and TGF-β_1 immunoreactivity were correlated with increases in fibronectin, collagen α_2(I), and collagen α_1(III) gene expression. Infusion of purified, recombinant TGF-β_1 into rats with a preexisting neointima produced a significant stimulation of carotid neointimal SMC DNA synthesis. These results suggest that TGF-β_1 plays an important role as an endogenous growth regulatory factor produced by neointimal SMC themselves during progressive neointimal thickening after balloon angioplasty. (*J. Clin. Invest.* 1991. 88:904–910.)
Key words: smooth muscle cell • neointima • carotid artery • extracellular matrix • atherosclerosis

Introduction

Progressive neointimal thickening after balloon angioplasty results from proliferation of arterial smooth muscle cells (SMC)[1] within the intima and abundant synthesis of extracellular matrix (1–4). In the rat carotid artery, neointimal SMC proliferation occurs mostly during the first two weeks after balloon angioplasty (5). Thereafter, SMC replication returns to basal values and continued intimal thickening is due to an active phase of new connective tissue formation (6). At 12 weeks after surgery, the neointima reaches a stable size that is maintained for at least one year (6).

The factors that control the early activation of SMC mitogenesis and sustained synthesis of extracellular matrix constituents in injured arteries are only beginning to be identified. One possibility is that the process of neointimal thickening is controlled, in large part, by regulatory molecules derived from sources within the damaged artery wall itself. Studies from our laboratory have shown that SMC growth and migration in the injured carotid media as well as chronic smooth muscle replication in the neointima can occur in the absence of platelets, a principle source of exogenous growth factors (7). Monocytes and lymphocytes are also present in developing neointima and are potential sources of locally acting mitogens (8), but their numbers are quite small (9). Alternatively, it is possible that arterial SMC themselves are an important source of mitogenic activity in vivo during the repair of vascular injuries. This idea is supported by the findings that cultured SMC can produce growth factors thought to be important for their own growth (10–14). Moreover, transcripts for platelet-derived growth factor (PDGF), basic fibroblast growth factor, type-1 transforming growth factor β (TGF-β_1) have been found in normal, hypertensive, and atherosclerotic artery wall in vivo (15–19).

In this study we have explored the possibility that repair of artery wall tissue injury caused by balloon angioplasty involves stimulation of TGF-β_1 production by the regenerating SMC population itself. An earlier report by Madri et al. (20) showed increased staining for TGF-β_1 protein in neointimal segments of balloon catheter–injured rat carotid arteries at 10 weeks after injury. However, it was not clear from those studies whether TGF-β_1 detected in the neointima was synthesized locally by neointimal SMC, deposited via platelet degranulation, or derived from the circulation. We report here that levels of TGF-β_1 transcripts in injured carotids were increased by six hours after wounding, before entry of SMC into S phase (21), and remained at elevated levels for at least two weeks after injury. Immunoreactive TGF-β_1 was found intracellularly in a large majority of neointimal SMC, consistent with the active synthesis of TGF-β_1 by these cells. Moreover, infusion of purified, recombinant TGF-β_1 into rats two weeks after carotid injury produced a marked stimulation of neointimal SMC proliferation. These findings suggest that TGF-β_1 plays an important role as an endogenous growth regulatory factor produced by neointimal SMC themselves during progressive intimal thickening after balloon angioplasty.

Methods

Arterial injury model. Male Sprague-Dawley rats (500 g, 5 mo old) (Tyler Laboratories, Bellevue, WA) were anesthetized and acute injury to the left common carotid artery was made with an inflated balloon catheter as previously described (5). At the indicated times after injury, animals were killed and both injured (left) and uninjured (right) common carotids were retrieved and stripped of periadventitial fatty and connective tissues in PBS at 4°C. Endothelium of the right carotid was removed by gently scraping the lumenal surface with the edge of a Teflon card. Efficacy of this procedure was verified by loss of the hybrid-

Address correspondence to Dr. Mark W. Majesky, Department of Pathology, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.

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1. *Abbreviations used in this paper:* PDGF, platelet-derived growth factor; SMC, smooth muscle cells; TGF-β, transforming growth factor-β.

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ization signal for von Willebrand's factor mRNA. Arteries were then snap frozen in liquid nitrogen for subsequent RNA isolation. In some cases, thickened neointima from regenerating carotid arteries was separated from underlying media by careful dissection under magnification (12) and RNA from the two tissue preparations was analyzed separately.

RNA isolation and blot hybridization. Frozen arterial tissue was ground to a fine powder under liquid nitrogen and total cellular RNA was prepared by acid guanidinium isothiocyanate–extraction as previously described (22). Agarose gel electrophoresis and RNA transfer to nylon membranes (Zeta Probe; Bio-Rad Laboratories, Richmond, CA) were carried out as previously described (16, 22). After transfer, RNA blots were exposed to short-wave UV light to cross-link RNA to the membrane and to visualize the major RNA bands. At this point assurance was made that equal amounts of total cellular RNA had transferred to the membrane. Blots were hybridized as previously described (16) using cDNA probes labeled with [^{32}P]dCTP by random primer extension (Amersham Corp., Arlington Heights, IL) and then washed at 65°C in two changes of 0.045 M NaCl/0.0045 M sodium citrate, pH 7.0/0.1% SDS for 10 min each and exposed to Kodak XAR-5 film at −70°C.

[^{32}P]-autoradiography. For quantitation of [^{32}P]-autoradiographic signals, blots were exposed to preflashed film and exposures were collected at increasing intervals (twofold) of time. Autoradiograms were scanned at 600 nm with a laser densitometer (Beckman Instruments, Inc., Fullerton, CA). The difference in exposure times required to produce signals of equal film density was determined for each comparison made. This difference was taken as the relative change in levels of a particular mRNA species. Transcript signals were normalized to total cellular RNA.

DNA probes. DNA probes used for RNA blot hybridization were as follows: TGF-β_1, a 2.1-kb EcoRI human cDNA fragment (23); TGF-β_2, a 442-bp EcoRI-XhoI murine cDNA fragment from pmTGF-β_2-9A (24); TGF-β_3, a 609-bp murine cDNA fragment from pmβ3-11B (25); fibronectin, a 1.2-kb EcoRI rat cDNA fragment released from pRLF-1 (26); collagen α_2(I), a 900-bp BamHI mouse cDNA fragment from pmCOL-1 (27); collagen α_1(III), a 1.1-kb EcoRI mouse cDNA fragment from pmCOL-3 (27); histone, a 1.7-kb AvaI-SalI mouse genomic fragment from pH312 (28); and glyceraldehyde-3-phosphate dehydrogenase, a 1.2-kb PstI human cDNA fragment released from pHcGAP (29).

Immunohistochemistry. Segments from normal and balloon catheter–injured rat common carotid arteries were embedded in O.C.T. compound (Tissue Tek, Miles, Inc., Elkhart, IN) and frozen sections were thaw-mounted onto glass slides. Sections were incubated in 0.3% hydrogen peroxide in cold methanol for 30 min to block endogenous peroxidase activity and to permeabilize the cells. Nonspecific binding of rabbit IgG was blocked by preincubation with normal goat IgG (1:50 in PBS/0.1% BSA). The sections were sequentially incubated at room temperature with rabbit anti–human TGF-β_1 (1:350) (30) for 1 h, biotinylated goat anti–rabbit IgG (1:500; Vector Laboratories, Inc., Burlingame, CA) for 30 min, and avidin-peroxidase (ABC Kit, Vector Laboratories) for an additional 30 min. Each incubation was followed by a wash in PBS and a 15-min incubation in PBS/0.1% BSA. Staining was visualized with 0.05% 3,3'-diaminobenzidine/0.03% hydrogen peroxide in 0.05M Tris, pH 7.6. The anti-TGF-β_1 antibody used in these studies recognizes an intracellular, but not secreted, form of TGF-β_1 (30, 31). Thus the antibody stains SMC profiles in tissue sections whereas no staining of extracellular matrix structures was observed. Substitution of anti-TGF-β_1 with an equivalent concentration of normal rabbit IgG (1:350) (Fig. 4), PBS, or an irrelevant antibody (anti–transforming growth factor-α) produced no staining of carotid artery sections.

Infusion of TGF-β_1 after carotid injury. 2 wk after left carotid denudation by a minimal trauma filament loop technique (32, 33), rats were given a single bolus injection intravenously of 100 μg of purified, recombinant TGF-β_1 (34) followed by an 8-h infusion of TGF-β_1 (1.2 μg/min). 8 h after this infusion rats were given single injections of TGF-β_1 (100 μg) at 8-h intervals for the next 24 h. Control rats were given saline vehicle for the same dosage schedule. Both groups of rats were injected with [^{3}H]-thymidine at 30, 39, and 47 h before killing at 48 h after the start of treatment with TGF-β_1. The frequency of SMC nuclei labeled with [^{3}H]-thymidine was determined as previously described (6). The preparation of purified recombinant TGF-β_1 was tested and found to be free of endotoxin contamination.

Results

Arterial injury stimulates TGF-β_1 gene expression. Transcripts for TGF-β_1 (2.4 kb) were present at low but readily detectable levels in normal carotid artery in adult rats (Fig. 1). A significant increase in levels of TGF-β_1 mRNA was first observed at 6 h after carotid injury and by 24 h, the levels of TGF-β_1 transcripts were five to sevenfold above those in uninjured contralateral right carotids. Over the next week, TGF-β_1 mRNA levels in the regenerating left carotid remained elevated approximately fivefold above uninjured artery.

The neointima formed 2 wk after injury is sufficiently large to allow its dissection from the underlying media. Levels of the 2.4-kb TGF-β_1 mRNA in these neointimal strippings were about threefold higher than in the underlying medial tissue (Fig. 2). In addition, a second mRNA species of ~ 4 kb was detected in the neointima, but not the media, of the injured left carotid artery. This mRNA species was sufficiently related to the 2.4-kb TGF-β_1 mRNA to hybridize under the conditions used here, but the cDNA probe appeared to wash off the 4-kb band more readily with increasing stringency than it did for the 2.4-kb mRNA (data not shown). Whether the 4-kb mRNA species is a product of alternative splicing from the TGF-β_1 gene (35) or is the product of a different gene with sufficient sequence similarity to portions of our cDNA probe to permit hybridization is not yet clear.

To examine the possibility that the 4-kb mRNA species represents one of the other members of the TGF-β family of growth factors (24, 36), the RNA samples shown in Fig. 2 were reprobed for the presence of TGF-β_2 and TGF-β_3 transcripts. We were unable to detect expression of the TGF-β_2 gene in these carotid RNAs. A single TGF-β_3 transcript species of the expected size (3.8 kb) (25) was found in carotid neointima, but at low levels that were not different from underlying media or uninjured artery (data not shown). Therefore it is unlikely that the 4-kb mRNA shown in Fig. 2 can be explained by expression patterns of the related genes for TGF-β_2 or TGF-β_3 in the developing rat carotid neointima.

Arterial injury stimulates extracellular matrix protein gene expression. One consistent effect of addition of TGF-β_1 to a variety of cell types in vitro, including arterial SMC, is a stimu-

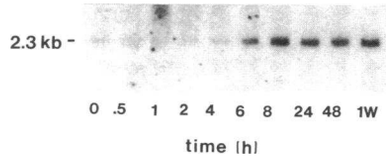

Figure 1. TGF-β_1 gene expression after carotid injury. Total cellular RNA was isolated from left carotid arteries at the times indicated after balloon catheter injury (10 vessels pooled per time point). 12 μg was electrophoresed, transferred to a nylon membrane, and hybridized with a [^{32}P]-labeled human TGF-β_1 cDNA probe (kindly provided by Dr. G. I. Bell, University of Chicago). The blot was exposed to film for 18 h. W, week.

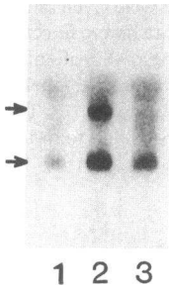

Figure 2. TGF-β_1 gene expression in left carotid neointima. At 2 wk after left carotid injury, the neointima was dissected from the underlying media and total cellular RNA was isolated from the two artery wall compartments separately. 12 μg per lane of total cellular RNA was electrophoresed, blotted, and hybridized as described in the legend to Fig. 1. The lanes shown are as follows: (*1*) normal left carotid artery before injury; (*2*) left carotid neointima 2 wk after wounding; and (*3*) underlying media of left carotid artery 2 wk after injury. The arrows mark the position of the 2.4-kb TGF-β_1 mRNA (*lower*) and the 4-kb TGF-β_1-related mRNA (*upper*) species.

lation of extracellular matrix protein synthesis (37–39). To examine the relation between the timing of stimulated TGF-β_1 gene expression in injured arteries and the production of a new extracellular matrix in the neointima (40), RNA blots were probed for fibronectin, collagen α_2(I), and collagen α_1(III) mRNAs. As shown in Fig. 3, all three gene products are increased at late times ($\geq$ 1 wk) after injury. In particular, the timing of the increase in mRNA levels for fibronectin, collagen α_2(I), and, to a lesser extent, collagen α_1(III) correlates best with the onset of neointima formation rather than an increase in TGF-β_1 mRNA levels per se in injured arteries.

Immunolocalization of TGF-β_1 in carotid neointima. To examine the distribution of TGF-β_1 protein synthesis within the developing carotid neointima, an antibody recognizing an intracellular form of TGF-β_1 (30, 31) was used for immunohistochemical analysis. Neointimal SMC were strongly positive for TGF-β_1 (Fig. 4). A vast majority of SMC throughout the thickness of the neointima were positive for TGF-β_1 staining at 2 wk after wounding (see Fig. 4, *C* and *D*). Certain SMC present in the underlying media were also positive for TGF-β_1 staining, but at a lower frequency ($\sim$ 50%). Since the intensity of staining for TGF-β_1 on a per cell basis was similar in neointimal and medial SMC of regenerating left carotid arteries, the more significant difference is that the frequency of TGF-β_1–positive SMC is much higher in the neointima than underlying media. Although the vast majority of immunopositive cells in the neointima must be SMC, a small percentage of neointimal cells present at this time are monocyte/macrophages (9). Since macrophages in vivo are capable of producing TGF-β_1 (41), the possibility that at least some of the immunopositive cells in the carotid neointima are macrophages should be considered. Substitution of anti-TGF-β_1 with an equivalent concentration of normal rabbit IgG (Fig. 4 *A*), PBS, or an irrelevant antibody (anti–transforming growth factor-α) produced no staining of carotid artery sections. Moreover, the staining pattern shown in Fig. 4 is not likely to represent cross-reactivity to related members of the TGF-β growth factor family because: (*a*) we could detect no expression of the TGF-β_2 gene within developing carotid neointima; and (*b*) levels of TGF-β_3 mRNA in the neointima were not different than in underlying media or uninjured carotid (data not shown) despite clear differences in TGF-β immunostaining in these arterial segments.

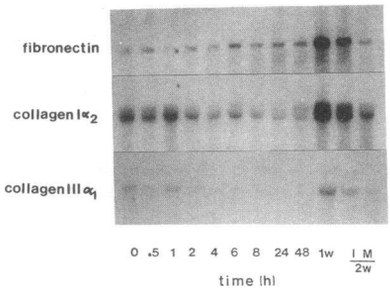

Figure 3. Expression of extracellular matrix genes in wounded carotid arteries. Total cellular RNA was isolated from left carotid arteries at the times indicated after balloon catheter injury. 12 μg was electrophoresed, blotted, and probed sequentially with [^{32}P]-labeled cDNA probes for transcripts encoding the extracellular matrix proteins fibronectin, collagen α_2(I), and collagen α_1(III). Blots were exposed to film for 14, 30, and 68 h, respectively. W, weeks; I, intima; M, media.

Effects of infusion of purified TGF-β_1 on DNA synthesis by neointimal SMC. TGF-β_1 can stimulate (42–44) or inhibit (45, 46) aortic SMC proliferation in vitro depending upon cell density (43, 45). However, when SMC are suspended in soft agar (a state that may more closely resemble SMC in the artery wall than monolayer cultures), TGF-β_1 stimulates cell replication and promotes the formation of large focal colonies in vitro (42).

To examine the possibility that TGF-β_1 produced locally by neointimal SMC might be an important source of SMC mitogenic activity in regenerating artery wall, we infused purified, recombinant TGF-β_1 beginning 2 wk after carotid injury produced by a gentle nylon filament loop technique (32, 33). The cumulative dose given over a 48-h period was 1 mg per rat. At these levels, exogenous TGF-β_1 produced a significant increase in SMC DNA synthesis in the carotid neointima but not in the underlying media (Table I). The frequency of [^{3}H]-thymidine–labeled SMC nuclei in rats treated with TGF-β_1 was highest in those neointimal SMC that line the luminal surface, although a significant increase in SMC thymidine index was found throughout the entire intima after TGF-β_1 infusion. No changes in SMC replication were detected in the underlying media following infusion of TGF-β_1.

Discussion

We report here that TGF-β_1 gene expression in rat carotid artery is stimulated during neointima formation after a balloon catheter injury. Moreover, a majority of neointimal SMC are positive for immunoreactive TGF-β_1 in an intracellular pattern, consistent with active TGF-β_1 synthesis in this tissue. In addition, infusion of purified, recombinant TGF-β_1 protein into rats with a preexisting neointima stimulated DNA synthesis in neointimal, but not medial, SMC. Levels of stimulated SMC replication after TGF-β_1 infusion were particularly high in neointimal SMC that line the luminal surface. These results suggest that TGF-β_1 is an important endogenous growth regulatory factor for neointimal SMC during the progressive neointimal thickening that occurs following balloon angioplasty in vivo.

Formation of a neointima in injured arteries requires that normally quiescent, contractile SMC acquire new functions. The most important of these new functions for intimal thickening are cell proliferation, a transition to a motile phenotype, and production of a new extracellular matrix (5, 47, 48). Since the addition of exogenous TGF-β_1 to cultures of arterial SMC in vitro is reported to stimulate SMC mitogenesis (43–45), chemotaxis (49), and extracellular matrix production (37, 50), it is conceivable that TGF-β_1 produced by SMC within injured arteries in vivo plays an important role in fibrocellular intimal

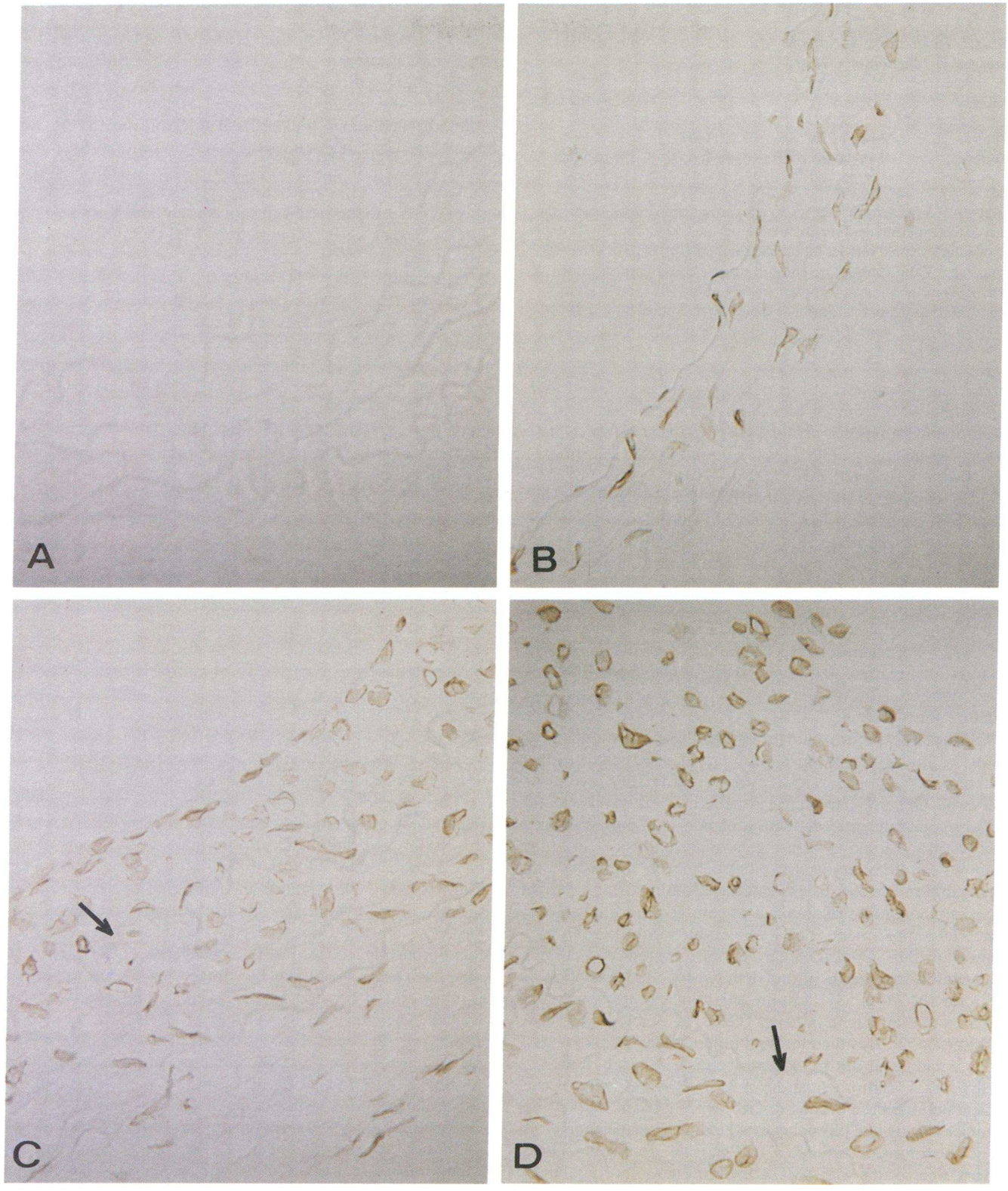

Figure 4. Immunohistochemical localization with anti-TGF-β_1 antibody in rat carotid neointima. Frozen sections of regenerating left carotid artery 2 wk after wounding were stained with a polyclonal anti–human TGF-β_1 IgG (30, 31) (1:350). (*A*) Normal uninjured left carotid stained with a control rabbit IgG (1:350). (*B*) Normal uninjured left carotid stained with anti-TGF-β_1. The lumen is at top left. The staining pattern is intracellular, in agreement with previous studies with this antibody (30, 31). Approximately 50% of medial SMC profiles stain positively for intracellular TGF-β_1. (*C*) Left carotid 1 wk after injury. Arrow marks the position of the internal elastic lamina. The lumen is at top left. Note that a majority of SMC in the developing neointima, and some cells in the adventitia, are positively stained for TGF-β_1. (*D*) Left carotid 2 wk after injury. The lumen is at upper right. The arrow marks the internal elastic lamina. Note that a majority of neointimal SMC are positive for TGF-β_1 immunoreactivity. All photomicrographs are ×100. Substitution of anti-TGF-β_1 antibody with an equivalent concentration of normal rabbit IgG (1:350), PBS, or an irrelevant antibody (anti–transforming growth factor-α) produced no staining of normal or injured carotid artery sections.

Table I. Replication of Smooth Muscle Cells in Rat Carotid Artery after Infusion of TGF-β_1

Treatment*	Intima	Media	Luminal SMC
Vehicle	2.38±1.22‡	0.12±0.01	7.6±1.6
TGF-β_1	8.55±3.56§	0.08±0.02	14.1±4.4‖

* Rats (n = 3 per group) were given left carotid injury 2 wk before the start of the experiment. Treatment with purified, recombinant TGF-β1 or saline vehicle was for 48 h. The total dose of TGF-β_1 given was 1 mg/rat. ‡ Values shown are smooth muscle cell thymidine index (%±SEM) in the indicated compartments of the left carotid artery wall. § TGF-β-treated value differs from saline control ($P < 0.05$). ‖ TGF-β-treated value differs from saline control ($P < 0.01$).

thickening. In support of this possibility, we have found that neointimal SMC contain transcripts for at least one form of TGF-β_1 and stain positively for TGF-β_1 using an antibody that recognizes an epitope (30, 31) present on intracellular (probably newly synthesized) TGF-β_1.

It is tempting to speculate that TGF-β_1 produced by neointimal SMC acts locally to stimulate SMC proliferation and extracellular matrix production in injured arteries in vivo. However, TGF-β_1 is usually synthesized and secreted in an inactive precursor form (51–53). Once secreted from cells in complex tissues in vivo, inactive TGF-β_1 can either: (*a*) form a complex with α_2 macroglobulin and be cleared from the site of secretion (52, 53); (*b*) be retained locally by binding to components in the extracellular matrix (30, 54); or (*c*) become activated by proteolysis, or by other means, to release the biologically active 24-kD mature form of TGF-β_1 (55–57). While we have shown that TGF-β_1 gene expression and protein synthesis are stimulated in SMC within injured arteries, we do not know the fate of this nascent TGF-β_1 once secreted into the regenerating carotid artery wall. Of particular interest is the recent finding that carotid injury stimulates the production of both tissue plasminogen activator and urokinase during carotid repair (48). Since the injured carotid artery wall is more permeable to plasma proteins due to the loss of an endothelial lining (4), one might expect circulating plasminogen to gain access to the smooth muscle compartment. The local production of plasminogen activators during repair of carotid injury suggests that plasmin generation may occur in the vicinity of TGF-β_1 secretion by carotid SMC (48, 58). Plasmin activity has been shown to be able to convert latent, inactive TGF-β_1 to a biologically active form (56, 57). It is therefore not unlikely that TGF-β_1 activation could occur during carotid neointima formation in vivo. However, plasmin activation of TGF-β_1 is only one mechanism that has been proposed; the ways that latent TGF-β_1 precursor is activated physiologically are not clearly defined.

The timing of stimulated TGF-β_1 gene expression reported here is correlated with SMC growth initiation during the first 24 h after carotid injury (21). Moreover, TGF-β_1 transcripts remain elevated throughout the first two weeks after wounding, when SMC proliferation is highest in this model of arterial wound repair (5). These results argue against a dominant SMC growth inhibitory effect of TGF-β_1 in this particular setting in vivo. Rather, they suggest that TGF-β_1 (derived from platelets and/or carotid SMC) could act to promote SMC mitogenesis during repair of an arterial injury. A growth-promoting effect of TGF-β_1 in wounded arteries would be consistent with previous reports that TGF-β_1 stimulates SMC replication at confluent cell densities in vitro (43–45) and when SMC are suspended within a three-dimensional soft agar matrix (42). To directly test the possibility that local production of TGF-β_1 within the neointima could stimulate SMC replication, we treated rats that had a preexisting neointima with purified, recombinant TGF-β_1 (34) for 48 h and then examined SMC DNA synthesis in the regenerating left carotid artery. Exogenous TGF-β_1 produced a significant stimulation of neointimal, but not medial, SMC proliferation. [^{3}H]-thymidine–labeled SMC nuclei were particularly frequent in cells localized along the lumenal surface. We previously reported that platelet-derived growth factor A-chain (PDGF-A) synthesis was highly active in carotid lumenal SMC by in situ hybridization and immunohistochemistry (59). Since TGF-β_1 has been shown to promote rat aortic SMC mitogenesis in vitro indirectly via stimulation of PDGF-AA production (43), it is reasonable to consider that a similar mechanism may explain luminal SMC replication in vivo. It should also be noted that TGF-β_1 has antiinflammatory properties (60). Since production of γ-interferon by infiltrating T lymphocytes has been proposed to act as a local growth inhibitor for neointimal SMC (9), TGF-β_1 could contribute to SMC mitogenesis by reducing the source of this negative growth regulator. Moreover, since bimodal SMC responses in vitro have been observed with increasing amounts of TGF-β_1 (44), we caution against drawing firm conclusions about the effects of locally produced TGF-β_1 within the neointima from the results obtained here using pharmacological doses of TGF-β_1 in vivo. The lack of stimulation of DNA synthesis in underlying medial SMC of injured carotids is consistent with the suggestion that neointimal SMC have special properties and growth controls in vivo compared to normal medial SMC (4, 12, 61). However, the possibility that medial SMC were not exposed to sufficient concentrations of infused TGF-β_1 to stimulate replication cannot be ruled out.

Formation of a neointima in vivo requires the synthesis and assembly of a new extracellular matrix. In particular, fibronectin, elastin, and type I collagen are major constituents of the matrix produced by neointimal SMC (40). A likely function for SMC-derived TGF-β_1 during the formation of a neointima is stimulation of extracellular matrix synthesis by neointimal SMC (37, 50, 62). Consistent with this possibility, we found that fibronectin, collagen α_2(I), and collagen α_1(III) transcripts are selectively increased at the times when the neointima is forming and at the sites where TGF-β_1 immunostaining was located. Therefore, TGF-β_1 production by SMC in the carotid neointima could contribute to neointimal thickening in two ways: (*a*) stimulation of SMC proliferation, possibly via local induction of PDGF-AA synthesis; and (*b*) stimulation of SMC extracellular matrix synthesis and accumulation.

Arterial injury following balloon angioplasty leads to the formation of a new layer of artery wall, a neointima, that can compromise the flow of blood (63). Progressive neointimal thickening may be stimulated by growth regulatory factors produced by neointimal SMC themselves. Our recent studies have identified two such endogenously produced growth factors, namely TGF-β_1 (this report) and PDGF-AA (59). In addition, neointimal SMC at the luminal surface have a morphological and functional specialization that provides a "pseudoendothelium" in areas of incomplete endothelial regeneration (4, 12, 61). Our finding that purified, recombinant TGF-β_1 stimulated neointimal, but not medial, SMC proliferation in vivo might

suggest that functional differences between neointimal and medial SMC may extend to the level of growth control as well. If true, this may have important implications for mechanisms of progressive neointimal thickening after balloon angioplasty.

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