Antibodies against Transforming Growth Factor-β1 Suppress Intimal Hyperplasia in a Rat Model

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Abstract

Intimal hyperplasia is induced by therapeutic vascular interventions and often results in clinically important narrowing of the vascular lumen. Examination of the role of TGF-β1 in a rat carotid artery injury model confirmed the presence of a previously reported increase in TGF-β1 mRNA in the media of injured arteries. Administration of neutralizing anti-TGF-β1 antibodies significantly ($P < 0.05$) reduced the size of the intimal lesions that developed after carotid balloon injury. A control antibody had no effect. The intimal/medial area ratio was also reduced in the anti-TGF-β1 group relative to controls ($P < 0.01$). Immunohistochemical staining showed that two TGF-β1-induced extracellular matrix components, EDA+ fibronectin and versican, were greatly increased in the untreated neointimal lesions, but were almost completely absent from the lesions of the anti-TGF-β1-treated animals. We conclude that TGF-β1 is causally involved in the development of intimal hyperplasia, and that anti-TGF-β1 agents may be useful in achieving at least partial control of this condition. (*J. Clin. Invest.* 1994. 93:1172–1178.) Key words: intimal hyperplasia • arterial injury • transforming growth factor-β • anti-transforming growth factor-β • extracellular matrix

Introduction

Intimal hyperplasia often causes occlusion of arteries that have undergone therapeutic vascular interventions. The main elements of intimal hyperplasia are vascular smooth muscle cell migration and proliferation, and the deposition of extracellular matrix by these cells (1, 2). TGF-β1 has been identified as an underlying factor in reparative processes after injury in various organs (3), and overproduction of this growth factor has been implicated as one of the causative agents in tissue repair processes that are characterized by increased production of extracellular matrix and fibrosis (4). Indeed, TGF-β1 mRNA and protein have been shown to increase in the arterial wall after balloon injury in rats (5). Moreover, in situ hybridization has revealed elevated levels of TGF-β1 mRNA in human coronary restenotic lesions removed by atherectomy (6). These correlations suggest TGF-β1 involvement in the restenosis process. We provide here direct evidence for a causal role of TGF-β1 in intimal hyperplasia by showing that neutralization of TGF-β1 activity suppresses the development of neointimal lesions after balloon injury of the rat carotid artery.

Methods

Preparation of TGF-β1 antibody. A polyclonal antiserum was raised by immunizing a rabbit with recombinant TGF-β1 (kindly provided by Daniel Twardzik, Oncogen, Inc., Seattle, WA). The TGF-β1 sample was denatured in Bouin's solution (formaldehyde, acetic acid, and picric acid) and dialyzed against PBS before injection. An initial dose of 200 μg of TGF-β1 with methylated BSA was injected subcutaneously. Subsequent doses of 100 μg were given every 4 wk. The IgG fraction of the antiserum was isolated on protein A. Another rabbit antiserum prepared in the same manner against a peptide, HRALQHRSKVQGEQSSETSDSD, of a nuclear protein (6a) served as control. The ability of the anti-TGF-β1 antibody to neutralize TGF-β1 was assayed by [^{3}H]thymidine incorporation on mink lung epithelial (Mv1Lu) cells as previously described (7, 8). The anti-TGF-β1 antibodies bound TGF-β1 in ELISA at a titer of 1:3,000 and reacted with TGF-β1 in immunoblotting (not shown).

Arterial injury and antibody treatment. Male Sprague-Dawley rats (Harlan Sprague-Dawley, Inc., Indianapolis, IN) weighing 350–400 g were anesthetized with Innovar, Pentobarbital, and Atropin, and underwent balloon injury of the left common carotid artery with a 2 F Fogarty balloon catheter (Baxter Scientific Products, Irvine, CA) (1). After the dosing of a similar antibody in an earlier glomerulonephritis study (8), the rats were given intravenous injections of 7.5 mg of IgG from anti-TGF-β1 antiserum or from control antibody, or only normal saline (0.9% NaCl). The injections were given immediately before arterial injury and daily thereafter, under isoflurane anesthesia. The antibody was administered for ≤ 14 d.

Polymerase chain reaction. The carotid arteries were harvested and homogenized 24–48 h after the injury. Total cellular RNA was extracted with RNAzol B (Tel-Test, Inc., Houston, TX) (9), and 500 ng were subjected to reverse transcription with Mo-MuLV reverse transcriptase (Gibco Laboratories, Grand Island, NY). PCR was performed on a thermocycler (38 cycles) (Idaho Technologies, Inc., Idaho Falls, ID) in a 30-μl reaction volume including 0.5 U Taq DNA polymerase, 666 nM of each primer, and 10 μl cDNA template. Primers for TGF-β1 were sense TATAGCAACAATTCCTGGCG (position 932–951) and antisense TGCTGTCACAGGAGCAGTG (position 1094–1076) defining a fragment of 162 bp. Amplification of glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA served as control (25 cycles), and the primers were sense CCCTTCATTGACCTCAACTACATGG (position 166–190) and antisense CATGGTGGTGAAGACGCCAG (position 356–375), defining a fragment of 310 nucleotides. The primers were used after labeling with ^{32}P. The PCR products were separated by electrophoresis on 2.5% agarose gel and visualized by ethidium bromide staining. The bands were cut and solubilized in scintillation fluid, and the radioactivity was counted. Quantitative PCR was designed after optimization experiments, such that a dose-dependent PCR response could be obtained. To ensure that the correct number of cycles was used, 500 ng of reverse-transcribed RNA from a pool of RNA from normal and injured arteries were subjected to G3PDH and

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TGF-β PCR with different numbers of cycles. The number of cycles used in the quantitative experiments was chosen as the number of cycles that gave a weak signal in the optimization experiments, thereby ensuring that the PCR was done in a quantitative range.

Histomorphometry. Rats were killed 19 d after the injury and underwent perfusion-fixation through the abdominal aorta with 4% paraformaldehyde at a pressure of 100 mmHg. Both carotid arteries were harvested, and three segments from the proximal, middle, and distal parts of each vessel were embedded in paraffin. Sections measuring 5 μm and stained with hematoxylin-eosin were photographed under the light microscope. The intimal and medial areas were measured using a digitizer tablet and the computer software package Vidas, Videoplan (Kontron Instruments, Inc., Everett, MA).

Bromodeoxyuridine labeling. Smooth muscle cell proliferation was measured 6 and 19 d after the injury as described (10). 18 h before harvesting of the arteries, the rats were given 100 mg/kg 5-bromo-2′-deoxyuridine (BrdU)[1] (Sigma Immunochemicals, St. Louis, MO) and 75 mg/kg 2′-deoxycytidine (Sigma Immunochemicals) into a subcutaneous neck depot. In addition, 30 mg/kg BrdU and 25 mg/kg 2′-deoxycytidine were given intramuscularly 18 and 12 h before removal of the tissues. After fixation, embedding, sectioning, and staining as described elsewhere, the BrdU-positive nuclei were counted in media and neointima and expressed as positive cells per cross section.

Immunohistochemistry. Sections of carotid arteries were deparaffinized in xylene and hydrated in graded ethanol solutions. Endogenous peroxidase was quenched with 0.3% peroxide in methanol. The sections were then incubated with 0.4 mg/ml pepsin (Sigma Immunochemicals) in 0.1 M HCl for 20 min at 37° and washed with 0.5% Tween 20 (Sigma Immunochemicals). For staining of BrdU, the sections were then incubated in 4 M HCl for 20 min at 25° and neutralized in 0.1 M borate buffer (pH 8.5). All sections were incubated with 10% horse serum for 10 min and then incubated with the primary antibody. The antibodies used were monoclonal mouse antibodies reactive with an alternatively spliced form of fibronectin, EDA+, at 1:4 (kindly provided by Dr. Luciano Zardi, Istituto Nazionale per la Ricerca sul Cancro, Genoa, Italy; reference 11), affinity-purified rabbit antiversican antibody (12) or monoclonal mouse anti-BrdU 1:20 (Boehringer Mannheim Corp., Indianapolis, IN), polyclonal rabbit anti-fibronectin antibodies (13), monoclonal mouse anti-collagen type VI antibodies (14), and anti-collagen type III antibodies (provided by Dr. Heinz Furthmayr, Yale University, New Haven, CT). After overnight incubation with the antibodies at 4°, biotin-labeled anti-mouse (Sigma Immunochemicals) or anti-rabbit IgG (Vector Laboratories, Inc., Burlingame, CA) antibodies were applied. The avidin, biotinylated horseradish peroxidase complex (Vectastain; Vector Laboratories) was used and followed by color development with histo orange (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD) and counterstained with hematoxylin. The sections were evaluated in a blinded fashion.

Statistics. Comparison of group means was performed with the unpaired two-tailed Student's t test. $P < 0.05$ was considered significant.

Results

The ability of the anti-TGF-β1 antibodies used here to study the role of TGF-β1 in intimal hyperplasia to reverse the biological activity of TGF-β1 was demonstrated by the [^{3}H]-thymidine incorporation assay in mink lung epithelial cells. Increasing concentrations of the antibodies reversed the suppressive effect of TGF-β1 on DNA synthesis in a dose-dependent manner in this assay (Fig. 1).

Balloon injury caused an increase of TGF-β1 mRNA in the injured arteries as measured by quantitative PCR (Fig. 2).

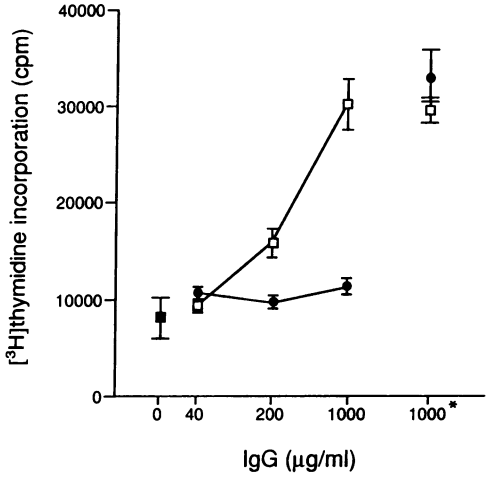
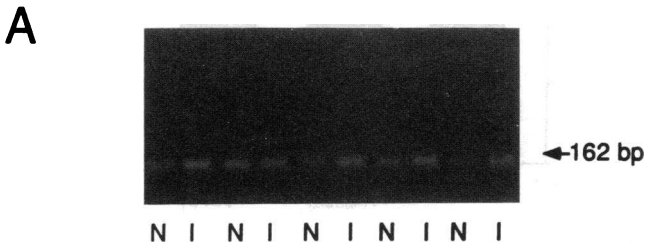


Figure 1. Neutralization of TGF-β1 activity by antibodies. [^{3}H]-thymidine incorporation into mink lung epithelial cells was measured. The cells were treated with 0.1 ng of TGF-β1 and various concentrations of anti-TGF-β1 IgG (□) or control IgG (●). The controls included no TGF-β1 addition in the presence of 1,000 μg/ml of the anti-TGF-β1 antibody (1,000*, □), or control IgG (1,000*, ●) and 0.1 ng TGF-β1 with no other additions (■).



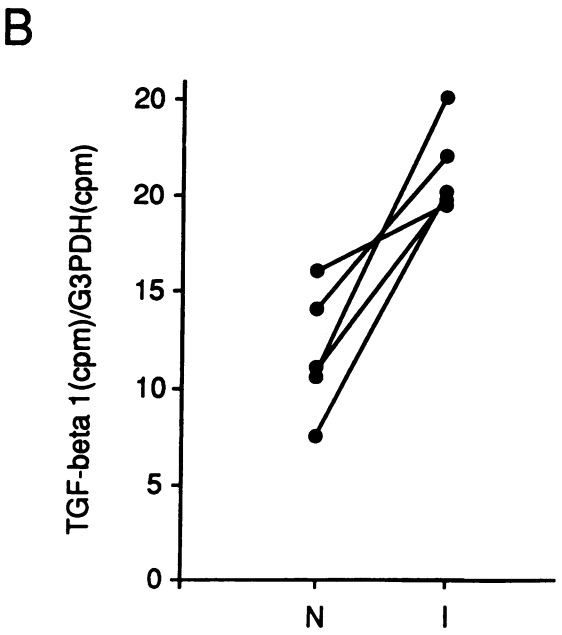


Figure 2. Quantitative PCR analysis of TGF-β1 mRNA in balloon-injured and uninjured carotid arteries. (*A*) Ethidium bromide–stained amplified products from TGF-β1 PCR resolved in 2.5% agarose gel. (*B*) The ratio between radioactivity incorporated in the TGF-β1 and G3PDH PCR products. The data are paired from each animal (*I*, injured carotid artery; *N*, noninjured artery). The results indicate that TGF-β1 mRNA is increased in injured arteries 24–48 h after the injury.

1. *Abbreviations used in this paper:* BrdU, 5-bromo-2′-deoxyuridine; G3PDH, glyceraldehyde-3-phospate dehydrogenase.

Treatment of the rats with anti-TGF-β1 antibodies did not affect the elevation of TGF-β1 mRNA levels after the injury. The TGF-β1 antibodies, when given intravenously for 14 d, significantly reduced the size of the arterial lesions after the injury. As summarized in Fig. 3, the neointimal lesions in the injured carotid arteries in the anti-TGF-β1-treated group measured 0.08±0.04 mm^2 and were decreased in size compared to the lesions in the control antibody group, which measured 0.12±0.05 mm^2, and the saline control group, which measured 0.12±0.06 mm^2 ($P < 0.05$). The intimal/medial ratio was 0.46±0.17 in the anti-TGF-β1 group, and it was significantly smaller than the intimal/medial ratios of 0.71±0.19 in the control antibody group and 0.68±0.22 in the saline group ($P < 0.01$) (Fig. 3). The uninjured right carotid arteries remained unchanged in both groups with an intima consisting of a flat monocellular layer. No untoward effects related to the administration of the antibodies were noted.

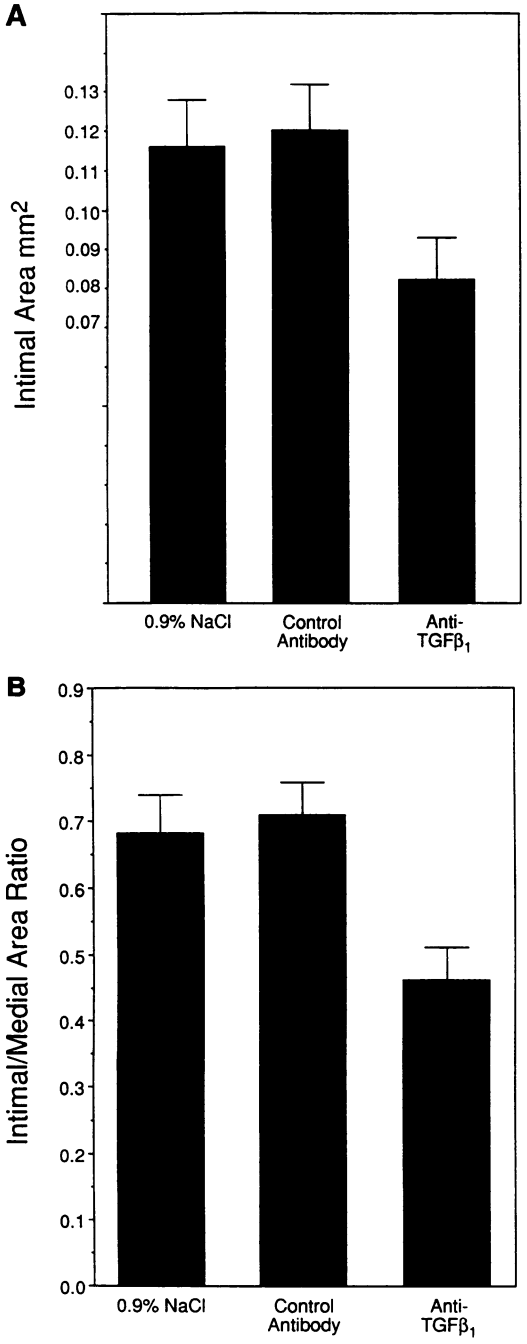


Figure 3. Size of intimal lesions after arterial balloon catheter injury. (*A*) Intimal area±SEM is given for arteries injured by balloon catheter and treated with anti-TGF-β1 ($n = 12$), control antibodies ($n = 14$), or normal saline ($n = 10$). The lesions were smaller in the TGF-β1 treatment group than in the control groups ($P < 0.05$). (*B*) The area ratio of the intima to the media was also significantly lower in the anti-TGF-β1 treatment group than in the control groups ($P < 0.01$).

Cell proliferation measured by staining for BrdU was slightly reduced in the anti-TGF-β1 treatment group, but the difference compared to the control group was not significant (Table I). 6 d after the injury, there was no discernible intimal lesion and all BrdU-positive cells (presumed to be smooth muscle cells; references 1, 2) were in the media. After 19 d, most of the positive cells were in the neointima and the media contained only occasional dividing cells (Table I and Fig. 4).

To examine the effect of anti-TGF-β1 treatment on extracellular matrix deposition and composition, we stained sections with antibodies against two substances known to be selectively induced by TGF-β1, the EDA+ form of fibronectin and versican (15–18). Staining for both substances localized almost exclusively in the neointima of the control group (Fig. 5, *A* and *C*) and was essentially undetectable in the anti-TGF-β1 group (Fig. 5, *B* and *D*). In contrast, staining for fibronectin (Fig. 5, *E* and *F*) and collagen types III and VI was seen throughout the vessel wall and was not modified by the anti-TGF-β1 treatment (not shown).

Discussion

Our results show that elevated levels of TGF-β1 and TGF-β1-inducible extracellular matrix components are present in intimal hyperplastic lesions, and that the size of these lesions can be reduced by in vivo neutralization of TGF-β1. TGF-β1 has been previously implicated in intimal hyperplasia in correlative studies that have demonstrated increased levels of TGF-β1 mRNA and protein in injured arteries (5, 6). Our study confirms the association of elevated TGF-β1 mRNA with arterial injury, and more importantly, provides proof for a causal role for TGF-β1 in the pathogenesis of the lesion. While the antibodies used were raised against the TGF-β1 isoform,

*Table I. Proliferating Cells in Carotid Arteries**

	6 d		19 d	
	Control antibody	Anti-TGF-β1	Control antibody	Anti-TGF-β1
Media	$n = 10$	$n = 10$	$n = 12$	$n = 11$
	13.3±5.4	11.3±4.1	1.8±1.1	1.4±1.2
Intima	—	—	$n = 12$	$n = 11$
			17.8±6.4	14.3±5.4

* Proliferating cells were quantitated by counting nuclei that had incorporated BrdU in histological sections of carotid arteries harvested at 6 or 19 d after balloon catheter injury. The results are expressed as positive nuclei per cross section (mean±SEM). The rats were treated with anti-TGF-β1 or control antibody during the development of the neointimal lesions.

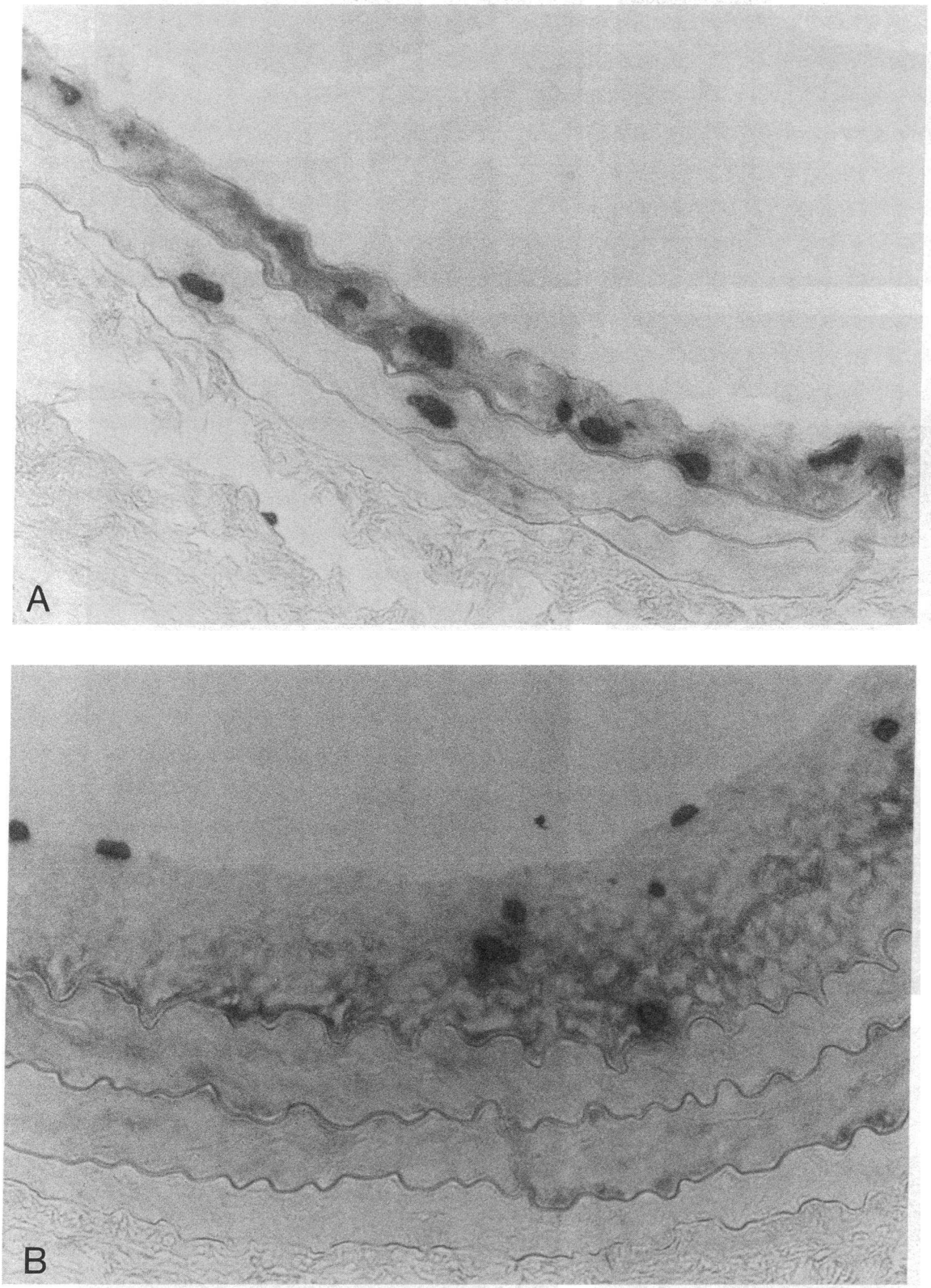


Figure 4. Quantitation of proliferating cells after balloon catheter injury. Cell proliferation was assayed by labeling with and staining for bromodeoxyuridine. (*A*) Arterial section 6 d after the injury. Active proliferation takes place within the media. (*B*) 19 d after the injury, the majority of the proliferating cells are in the neointima. Differences between the anti-TGF-β1 treated group and the control group were not statistically significant.

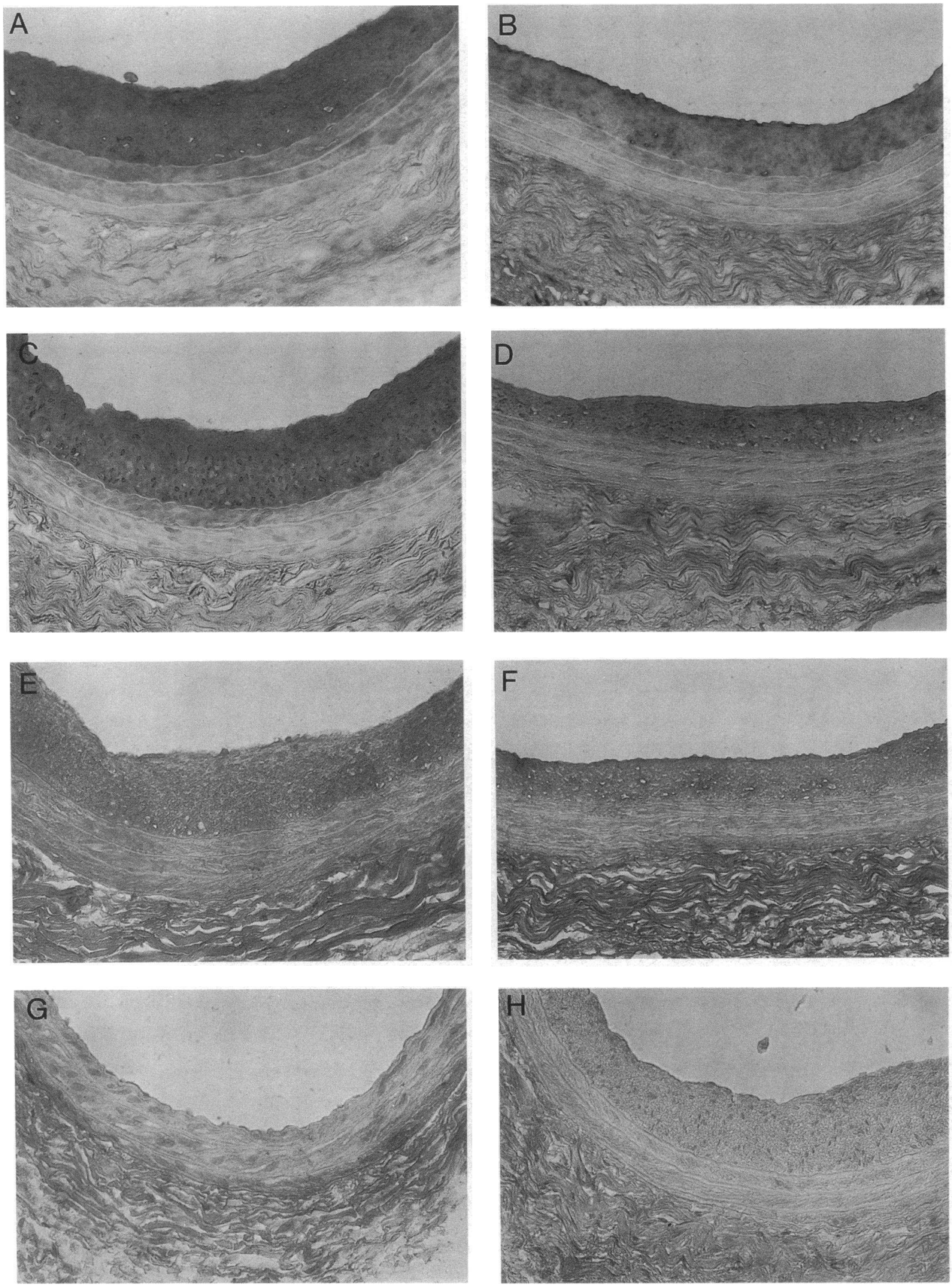


Figure 5. Immunohistochemical staining of sections of carotid arteries. Effect of anti–TGF-β1 treatment on the expression of EDA+ form of fibronectin, versican, and other matrix components in injured arteries. (*A*) EDA+ fibronectin in an artery from the control group and (*B*) from an anti–TGF-β1–treated animal. (*C*) Staining for versican in an injured artery from the control group and (*D*) from the treatment group. (*E*) Staining for fibronectin in injured arteries from the control group and (*F*) anti–TGF-β1 treatment group. (*G*) Staining for the EDA form of fibronectin in an uninjured artery does not reveal an appreciable focal staining. (*H*) Injured artery stained with control antibody.

their effect could have been mediated through inhibition of other TGF-β isoforms. Antibodies prepared by the method we used can neutralize TGF-β2 and TGF-β3 activity, albeit less potently than TGF-β1 (Yamaguchi, Y., and E. Ruoslahti, unpublished results). However, it is unlikely that TGF-β2 or TGF-β3 would be involved, because the mRNAs for these isoforms are not elevated in intimal hyperplasia (reference 5 and our unpublished results).

TGF-β1 appears to be responsible for the increased extracellular matrix accumulation that characterizes intimal hyperplasia, especially in its advanced stages. TGF-β can induce in vitro matrix production in the predominant cell type of the hyperplasic lesion, the vascular smooth muscle cells. Proteoglycans, in particular, are produced in large amounts under the influence of TGF-β (18, 19). Moreover, vascular smooth muscle cells produce latent TGF-β1 in vitro (20) and can activate it in coculture with endothelial cells (21, 22). In agreement with the postulated role of TGF-β1 in the stenotic vascular processes, extracellular matrix is abundant (≤ 80% of the lesion; reference 2) in human restenotic lesions and rat lesions caused by balloon catheter injury.

Our ability to partially block the matrix accumulation in rat intimal hyperplasia with anti-TGF-β1 conclusively demonstrates a role for TGF-β1 in the development of these lesions and is in agreement with the involvement of this growth factor in a number of diseases characterized by pathological accumulation of matrix (4). These diseases include experimental glomerulonephritis, where similar to our present findings, anti-TGF-β1 antiserum has been used to suppress the increased matrix production, with resultant attenuation of the histological manifestations of the disease (8). A natural inhibitor of TGF-β, decorin, has also been used successfully to suppress glomerulonephritis (23). We did not see significant reduction of the arterial lesion size in a single experiment that used decorin as an inhibitor instead of the antibody (unpublished). It may be that glomerulonephritis is a more suitable target for decorin therapy than arterial injury because intravenously injected decorin accumulates in the kidney (23, 24).

The extracellular matrix in the neointimal lesions appears to be qualitatively different from the normal matrix in the arterial wall. We found that the EDA+ form of fibronectin was localized almost exclusively to the neointimal matrix, as has been reported by Glukhova (25). Moreover, such matrices have been shown to be particularly rich in proteoglycans (26), and we find that versican, a large proteoglycan produced by fibroblasts and smooth muscle cells (18, 27), is far more abundant in the lesions than in normal vessels. The synthesis of both EDA+ fibronectin and versican is known to be greatly enhanced by TGF-β (15–18). The absence of an increase in EDA+ fibronectin and versican after anti-TGF-β1 therapy indicates that TGF-β1 is responsible for these special features of the neointimal matrix in injured arteries. However, the fact that we were able to essentially completely suppress the appearance of these TGF-β1-inducible matrix components within the lesions, but failed to suppress the entire lesion, suggests that other factors in addition to TGF-β1 may contribute to the process.

The component of the hyperplastic lesions that is resistant to anti-TGF-β1 treatment may be accounted for by smooth muscle cell proliferation. TGF-β1 has a concentration-dependent bimodal effect on smooth muscle cell proliferation in vitro (28–30), and in vivo administration of recombinant TGF-β1 at high doses in the setting of a neointimal lesion increases DNA synthesis in vascular smooth muscle cells (5). However, anti-TGF-β antibodies did not have statistically significant suppressive effect on smooth muscle cell proliferation in our model. Indeed, growth factors that are capable of affecting smooth muscle cell proliferation more strongly than TGF-β, such as PDGF (31, 32) or FGF (33, 34), have also been implicated in the formation of the neointimal lesion. The effect of PDGF on matrix accumulation in intimal hyperplasia has not been studied in vivo, but it is known to promote matrix production in vitro, although not as strongly as TGF-β (35, 36). FGF does not appear to control extracellular matrix deposition in intimal hyperplasia because its administration does not result in increased matrix production (34), and because its neutralization with antibodies decreases smooth muscle cell proliferation without affecting the size of the lesion (37). Thus, these growth factors could be responsible for the proliferative aspects of intimal hyperplasia, while TGF-β1 may be the main cause of the matrix accumulation.

Reducing the overproduction of extracellular matrix is clearly important in intimal hyperplasia. Such matrix-directed treatment may be particularly beneficial because the matrix production coincides with the late and more persistent phase in the development of this lesion as opposed to the initial proliferative surge. Neutralization of TGF-β1 may hold promise in this regard and its effects may be synergistic with agents that decrease smooth muscle cell proliferation.

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Editor's note. One of the authors of this paper (E. Ruoslahti) has disclosed to the Editor a substantial interest in a company that is working on TGF-β1 inhibitors.

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