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Transforming growth factor type β specifically stimulates synthesis of proteoglycan in human adult arterial smooth muscle cells

(atherosclerosis)

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ABSTRACT **Myo-intimal proteoglycan metabolism is thought to be important in blood vessel homeostasis, blood clotting, atherogenesis, and atherosclerosis. Human platelet-derived transforming growth factor type β (TGF-β) specifically stimulated synthesis of at least two types of chondroitin sulfate proteoglycans in nonproliferating human adult arterial smooth muscle cells in culture. Stimulation of smooth muscle cell proteoglycan synthesis by smooth muscle cell growth promoters (epidermal growth factor, platelet-derived growth factor, and heparin-binding growth factors) was <20% of that elicited by TGF-β. TGF-β neither significantly stimulated proliferation of quiescent smooth muscle cells nor inhibited proliferating cells. The extent of TGF-β stimulation of smooth muscle cell proteoglycan synthesis was similar in both nonproliferating and growth-stimulated cells. TGF-β, which is a reversible inhibitor of endothelial cell proliferation, had no comparable effect on endothelial cell proteoglycan synthesis. These results are consistent with the hypothesis that TGF-β is a cell-type-specific regulator of proteoglycan synthesis in human blood vessels and may contribute to the myo-intimal accumulation of proteoglycan in atherosclerotic lesions.**

Proteoglycans are components of the extracellular matrix that influence cell adhesion, migration, and proliferation during morphogenesis (1) and have been implicated in atherogenesis and atherosclerosis (2). The metabolism of proteoglycans in the myo-intimal space between smooth muscle cells (SMC) and endothelial cells (EC) may have profound effects on homeostasis and function of blood vessel walls. Glycosaminoglycans have been implicated in diverse molecular processes during interactions between blood components and the blood vessel wall, including platelet attachment and aggregation, blood clotting, and lipid metabolism (2). Atherosclerotic lesions are characterized by myo-intimal thickening presumably due to SMC hyperplasia. Accumulation of proteoglycan in the myo-intima accompanies the SMC hyperplasia (3). The early and abnormal presence of blood platelets and monocytes/macrophages at atherosclerotic sites suggests that factors derived from one or both cell types might be involved in the SMC hyperplasia and proteoglycan accumulation (4, 5). Both platelets and macrophages release the well-characterized SMC mitogen platelet-derived growth factor (PDGF); therefore, PDGF has been proposed as the most likely molecule to cause abnormal SMC proliferation in the atherosclerotic lesion (6, 7). Platelets and monocytes/macrophages have been shown to be the source of additional hormone-like polypeptide factors (8–10). Outstanding among these factors is transforming growth factor type β (TGF-β), which has been purified from platelets with yields comparable to that of PDGF (11). These observations and the dramatic effects of TGF-β on the proliferation and differentiation of a number of isolated cells (12–15) warrant an evaluation of the effect of purified TGF-β on proliferation and proteoglycan metabolism of human blood vessel cells. In this report, we show that human platelet-derived TGF-β exerts a cell type-specific and factor-specific stimulation of biosynthesis of secreted and cell-associated proteoglycan molecules by human adult arterial SMC.

MATERIALS AND METHODS

Materials. Medium MCDB 107 was prepared from high quality chemicals as described (16). Collagenase (type 1, 140 units/mg) was obtained from Cooper Biomedical (Malvern, PA). Purified human platelet-derived TGF-β was a generous gift from M. Sporn (National Cancer Institute, Bethesda, MD). EGF and PDGF were purchased from Collaborative Research (Waltham, MA), and collagen (Vitrogen 100) was from Flow Laboratories. [^{3}H]Glucosamine (29.0 Ci/mmol; 1 Ci = 37 GBq) and sodium [^{35}S]sulfate (37.4 mCi/mmol) were from New England Nuclear and Amersham, respectively. Glycosaminoglycan standards were generously provided by C. P. Dietrich (Departamento Bioquimica, Escola Paulista de Medicina, Sao Paulo, Brazil). All other chemicals were from Sigma. Human adult arterial tissue was obtained from National Diabetes Research Interchange (Philadelphia, PA) and Comprehensive Cancer Center (Birmingham, AL).

Cell Culture Methods. The SMC and EC were isolated from arterial tissues (aortas and large arteries) of nonatherogenic human autopsy specimens as described (17, 18). Cells were seeded in collagen-coated T-25 tissue culture flasks (19) in 5 ml of medium MCDB 107 supplemented with EGF (10 ng/ml), partially purified, bovine brain-derived growth factors (20), and 2% (vol/vol) fetal bovine serum (FBS). For the growth of EC, the medium was further supplemented with heparin (50 μg/ml). Cultures were incubated in a humidified atmosphere of 5% CO_2/95% air. After 24 hr, the medium was replaced. At confluence, cells were harvested by trypsin treatment (0.025% trypsin) and subcultured at 1:3 cell split.

Cell Growth Assays. Cell growth was measured by increase in cell number after 5 days of incubation. Cells were plated at 10^4 cells per well in a 16-mm collagen-coated multiwell tissue culture plate in 1 ml of medium MCDB 107 containing 2% (vol/vol) FBS and indicated growth factors. After incubation, cell number was determined from duplicate wells by harvesting cells by trypsin treatment and then counting cells in a Coulter counter (model Z_f).

Cell Labeling and Proteoglycan Isolation. Subconfluent nonproliferating and proliferating cells were labeled with 0.26 mM [^{35}S]sulfate and/or 1 μM [^{3}H]glucosamine for 48 hr in medium MCDB 107 containing 2% (vol/vol) FBS and indi-

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Abbreviations: TGF-β, transforming growth factor type β; SMC, smooth muscle cells; EC, endothelial cells; PDGF, platelet-derived growth factor; EGF, epidermal growth factor; HBGF-1, heparin-binding growth factor type 1; FBS, fetal bovine serum.

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cated growth factors. The radioactive medium was then harvested, and the cell layers were washed twice with cold phosphate-buffered saline. The medium and washes were combined and centrifuged to remove floating cells, and solid guanidine hydrochloride was added to a final concentration of 4 M. The cell layers were extracted twice with 1 ml of 50 mM sodium acetate, pH 6.0/4 M guanidine hydrochloride/0.2% Triton X-100/50 mM EDTA/1 mM phenylmethylsulfonyl fluoride at room temperature for 2 hr. The extracts were combined and centrifuged to remove cell debris. Radiolabeled proteoglycans were separated from unincorporated radionuclide using Sephadex G-25 columns made from 10-ml disposable serological pipettes. Radioactivity of the cold trichloroacetic acid-insoluble material from aliquots of the high molecular weight fractions was quantitated by liquid scintillation.

Pronase Digestion. [^{35}S]Sulfate-labeled macromolecules isolated from the SMC culture medium and cell layer were completely digested with Pronase, and the digested materials were analyzed by Sepharose CL-6B column chromatography. The digestion was done at 37°C for 24 hr in 100 mM Tris acetate, pH 7.4/1 mM $CaCl_2$/1 mg of Pronase per ml. The digests were centrifuged to remove insoluble material, pellets were washed once with the same buffer, and the wash was combined with the digest supernatant. Sepharose CL-6B columns (1 × 60 cm) were equilibrated with 50 mM sodium acetate, pH 6.0/50 mM NaCl/0.2% $NaDodSO_4$. After sample application, the column was eluted with the same buffer at a flow rate of 12 ml/hr, and 1-ml fractions were collected. Radioactivity in each fraction was determined in a scintillation counter.

Analysis of Proteoglycans by DEAE-Sephacel Chromatography. Proteoglycan samples were fractionated on a DEAE-Sephacel column (0.6 × 10 cm) equilibrated with 50 mM sodium acetate, pH 6.0/8 M urea/0.5% Triton X-100/0.1 M NaCl (21, 22). After sample application (0.5 ml), the column was eluted with 10 ml of the same solution and then with 36 ml of a linear 0.1–0.8 M NaCl gradient. Fractions (1 ml) were collected at a flow rate of 3.3 ml/hr.

Agarose Gel Electrophoresis. Aliquots of [^{35}S]sulfate-labeled proteoglycan samples were completely digested with Pronase in the presence of chondroitin sulfate (2 μg), dermatan sulfate (2 μg), and heparan sulfate (2 μg) standards. After digestion, glycosaminoglycans were ethanol precipitated, redissolved in 5 μl of water, and electrophoresed on a 0.5% agarose gel at pH 9.0 (23). The gel was fixed with 0.1% (wt/vol) Cetavlon and stained with 0.1% toluidine blue in acetic acid/ethanol/water, 0.1:5:5 (vol/vol). The radioactive bands corresponding to the [^{35}S]sulfated glycosaminoglycans were detected by autoradiography.

RESULTS

Specific Stimulation of SMC Proteoglycan Synthesis by TGF-β. Proteoglycan synthesis was investigated in nonproliferating subconfluent cultures of SMC and EC at a cell population doubling level of 8 to 17 in medium MCDB 107 containing only 2% (vol/vol) FBS. Over a 48-hr period, the addition of TGF-β (1 ng/ml) stimulated [^{35}S]sulfate incorporation by 4- to 5-fold into the secreted and 2- to 3-fold into the cell-associated fractions of SMC cultures (Table 1). The incorporation of [^{3}H]glucosamine into both fractions of SMC cultures was stimulated >10-fold. [^{3}H]Glucosamine incorporation was stimulated to a greater extent than that of [^{35}S]sulfate; this was due to a concurrent stimulation of the synthesis of hexosamine-containing glycoproteins by the TGF-β-treated cells (data not shown). The incorporation of [^{35}S]sulfate and [^{3}H]glucosamine by EC was not comparably affected by TGF-β treatment under identical culture conditions (Table 1). TGF-β stimulated the synthesis of medium- and SMC-associated proteoglycans in a dose-dependent manner with half-maximal stimulation at about 0.2 ng/ml (Fig. 1*A*). Time-course studies revealed that TGF-β-stimulated proteoglycan synthesis in SMC cultures occurred between 3 and 24 hr of continual exposure to the factor (Fig. 1 *B* and *C*). During this period, the secretion of TGF-β-stimulated proteoglycan increased 19-fold, and cell-associated proteoglycan increased 6-fold. The synthesis of medium- and cell-associated proteoglycans by control SMC cultures increased by only 4.5-fold over the same period. The amount of [^{35}S]sulfate incorporated per mg of cell protein was found to vary inversely with population doubling level number of the cells, and their ability to respond to TGF-β stimulation appeared to be reduced with increasing population doubling level. When EC cultures were subjected to similar time-course and dose–response experiments, no obvious stimulation of [^{35}S]sulfate and [^{3}H]glucosamine incorporation by TGF-β was observed.

TGF-β Stimulation of Proteoglycan Is Independent of SMC Proliferation. Proteoglycan biosynthesis by animal SMC and

Table 1. Effect of TGF-β on the incorporation of [^{3}H]glucosamine and sodium [^{35}S]sulfate by human arterial SMC and EC

		Radioactivity incorporated, cpm/mg of cell protein			
		Sodium [^{35}S]sulfate		[^{3}H]Glucosamine	
Cell	Addition	Medium	Cell layer	Medium	Cell layer
SMC	None	109,380 ± 15,030	73,930 ± 13,070	114,420 ± 27,980	40,060 ± 9,100
	TGF-β	443,520 ± 30,830	189,050 ± 7,360	1,396,430 ± 209,290	424,660 ± 61,330
	EGF/TGF-β	499,170 ± 17,910	250,810 ± 3,060	—	—
	HBGF-1/TGF-β	482,350 ± 10,400	170,040 ± 30,290	—	—
	PDGF/TGF-β	472,160 ± 30,600	278,010 ± 25,230	—	—
	EGF	127,270 ± 4,000	92,470 ± 5,080	—	—
	EGF/HBGF-1	122,610 ± 13,990	89,910 ± 11,520	—	—
	EGF/PDGF	155,960 ± 17,600	106,350 ± 2,490	—	—
	EGF/HBGF-1/PDGF	146,520 ± 14,650	100,080 ± 780	—	—
EC	None	79,300 ± 6,780	24,990 ± 1,120	372,700 ± 39,110	336,750 ± 3,750
	TGF-β	60,040 ± 19,730	19,260 ± 660	324,310 ± 79,760	252,570 ± 2,170

SMC and EC were plated at 2 × 10^4 cells per 35-mm collagen-coated Petri dish in 1.5 ml of nutrient medium MCDB 107 containing 2% (vol/vol) FBS, EGF (10 ng/ml), and a growth factor-enriched fraction from bovine brain extract (5 μg/ml). When cells were 70–80% confluent, the medium was replaced with medium MCDB 107 containing 2% (vol/vol) FBS and were further incubated for 48 hr to make cells quiescent. The medium was then replaced for 48 hr with the same medium containing [^{3}H]glucosamine (2 μCi/ml) and/or sodium [^{35}S]sulfate (10 μCi/ml) containing TGF-β (1 ng/ml), EGF (10 ng/ml), HBGF-1 (10 ng/ml), and/or PDGF (20 ng/ml). Medium and cell layer proteoglycans were isolated and quantitated. Cellular protein content was measured by the method of Markwell *et al.* (24). Data are the mean ± SD (n = 4 and 3 for SMC and EC, respectively).

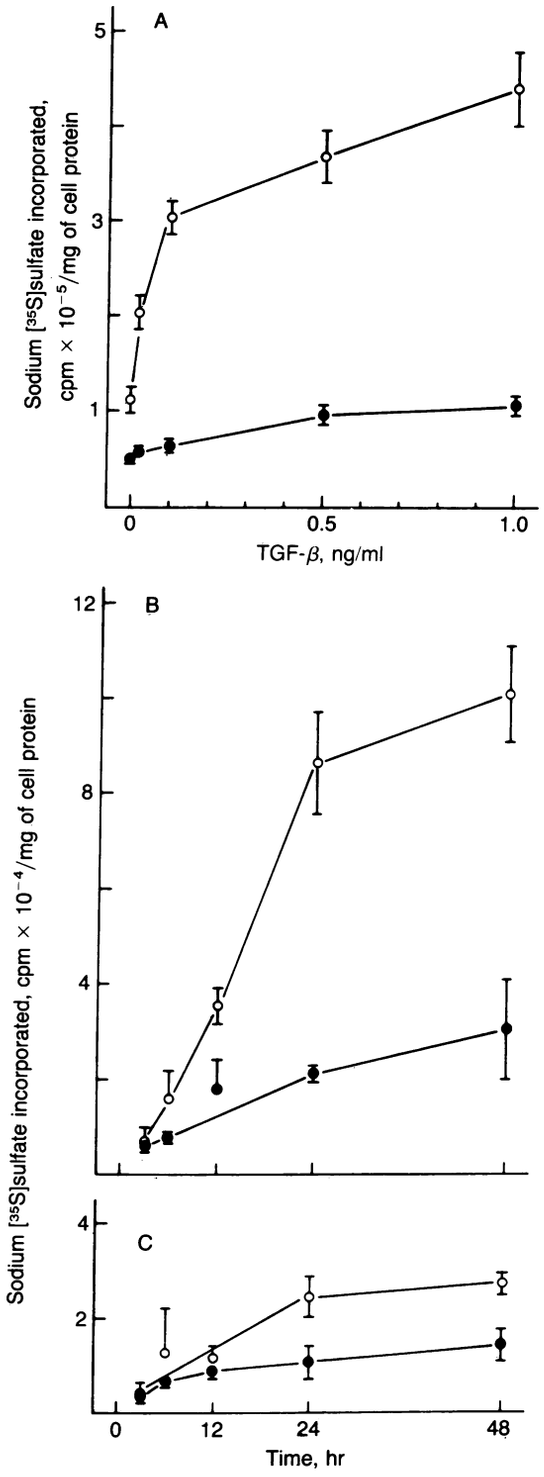

FIG. 1. Dose-dependent and time-course stimulation of SMC proteoglycan synthesis by TGF-β. (*A*) Test cultures were prepared as described in Table 1, exposed to TGF-β, and immediately labeled with sodium [^{35}S]sulfate (10 μCi/ml) for 48 hr. After the removal of the growth medium, medium- (○) and cell- (●) associated proteoglycans were isolated and quantitated. (*B* and *C*) Subconfluent cultures were incubated in medium MCDB 107 containing 2% (vol/vol) FBS for 2 days and labeled with sodium [^{35}S]sulfate (10 μCi/ml) in the absence (●) or presence (○) of TGF-β (1 ng/ml). At the times indicated, two dishes from each group were removed, and the medium- (*B*) and cell- (*C*) associated proteoglycans were quantitated.

EC has been postulated to be regulated coordinately with cell growth (21). We, therefore, examined the effect of SMC growth promoters at optimal concentrations for growth stimulation determined from separate dose–response studies. Epidermal growth factor (EGF), PDGF, and brain-derived heparin-binding growth factor (HBGF-1) were tested. HBGF-1 is identical to and was isolated from bovine brain by methods described for "prostatropin" (25). HBGF-1 is identical in biological activity to various acidic forms of brain-derived heparin-binding growth factors, reported by a number of laboratories (26), and probably to basic forms of the same class of polypeptide growth factor (27). All SMC mitogens, tested alone or in combinations, elicited a <50% increase in [^{35}S]sulfate incorporation of that observed in nonstimulated cells. The stimulation of proteoglycan synthesis elicited by SMC growth promoters was <20% of that elicited by TGF-β over the same period (Table 1). EGF, HBGF-1, and PDGF slightly potentiated the stimulatory effect of TGF-β. TGF-β stimulated proteoglycan biosynthesis at similar rates in nonproliferating SMC and SMC that were stimulated to proliferate in the presence of EGF, PDGF, and HBGF-1 (Table 1). Under conditions where TGF-β markedly stimulated proteoglycan synthesis, TGF-β had only small effects on SMC proliferation. TGF-β in medium MCDB 107 and 2% (vol/vol) FBS had no effect on cell growth (Table 2) and exerted only a slight stimulation of SMC proliferation in the presence of PDGF and/or HBGF-1. In contrast to effects on SMC, TGF-β reversibly inhibited HBGF-1-stimulated proliferation of EC (Table 2; H.H., M. Kan, J.-K.C., and W.L.McK., unpublished data), had no significant effect on medium proteoglycans, and slightly inhibited cell-layer proteoglycan synthesis in EC cultures (Table 1).

Characterization of TGF-β-Stimulated Proteoglycans. Medium- and cell-layer associated [^{35}S]sulfate-containing macromolecules produced by SMC in the absence and presence of TGF-β were digested with Pronase, and the digests were analyzed by Sepharose CL-6B column chromatography. Medium- and cell layer-derived ^{35}S-labeled materials synthesized in the absence and presence of TGF-β eluted as a sharp peak at the void volume (partition coefficient, $K_{av} = 0$) with a trailing tail extending to a K_{av} of ≈0.4 (Fig. 2). After Pronase digestion, all ^{35}S-labeled materials were eluted as bell-shaped peaks at K_{av}s of 0.32 and 0.25 for medium- and cell layer-derived materials, respectively (Fig. 2). TGF-β-stimulated SMC proteoglycan was further characterized by DEAE-Sephacel chromatography. The medium-associated proteoglycan fractionated into two major peaks that eluted at ≈0.3

Table 2. Effect of TGF-β on the growth of human adult arterial SMC and EC

Cell	Addition	Cells, no. per well
SMC	None	15,640 ± 1960
	TGF-β	16,500 ± 240
	EGF	27,340 ± 1800
	EGF/TGF-β	24,540 ± 240
	HBGF-1	40,840 ± 1420
	HBGF-1/TGF-β	43,620 ± 980
	PDGF	37,130 ± 940
	PDGF/TGF-β	39,820 ± 2630
	EGF/HBGF-1/PDGF	54,920 ± 200
	EGF/HBGF-1/PDGF/TGF-β	60,360 ± 1580
EC	None	24,280 ± 490
	HBGF-1	58,260 ± 500
	HBGF-1/TGF-β	41,020 ± 680

Cells were plated at 10^4 cells per well in 16-mm collagen-coated multi-well tissue culture dishes in 1 ml of medium MCDB 107 containing 2% (vol/vol) FBS, and the indicated growth factors were added 3 hr later. Cell number was determined on day 5. The concentrations of the growth factors were indicated in Table 1. Data are the average of duplicate wells.

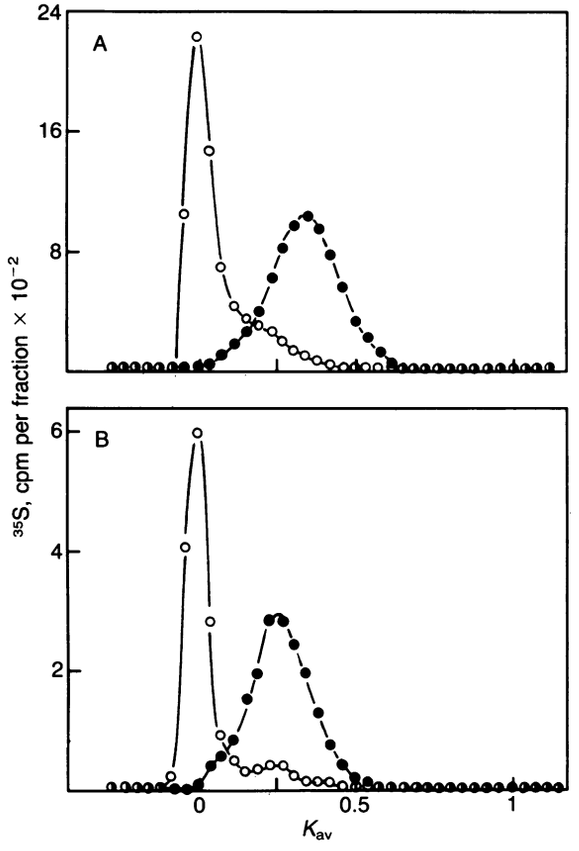

FIG. 2. Sepharose CL-6B column elution of [^{35}S]sulfate-labeled macromolecules isolated from TGF-β-treated SMC culture medium (*A*) and cell layer (*B*) before (○) and after (●) Pronase digestion. Each 1-ml fraction was mixed with 5 ml of Ecoscint (National Diagnostic, Somerville, NJ), and radioactivity was measured in a scintillation counter.

M and 0.4 M NaCl. TGF-β specifically stimulated the fraction that eluted at 0.4 M NaCl (Fig. 3). The cell layer-associated proteoglycan separated into three major fractions that eluted at ≈0.3 M, 0.4 M, and 0.5 M NaCl (Fig. 3). TGF-β stimulated the amount of cell layer proteoglycan that eluted at 0.5 M NaCl without an obvious change in the peaks at 0.3 M and 0.4 M. Thus, TGF-β specifically stimulated the synthesis of at least two groups of proteoglycan molecules by human arterial SMC: a secreted form that eluted from the DEAE-Sephacel column at 0.4 M NaCl and a cell layer-associated form that eluted at 0.5 M NaCl. The elution pattern of cell layer- and medium-associated proteoglycans synthesized by EC was unchanged by TGF-β (data not shown).

Analysis of SMC glycosaminoglycans by agarose gel electrophoresis revealed that chondroitin sulfate glycosaminoglycan was specifically stimulated by TGF-β in both medium- and cell-layer-associated fractions (Fig. 4). This was further confirmed by the complete digestion of the medium glycosaminoglycans that eluted from DEAE-Sephacel at 0.4 M NaCl and the cell layer fraction that eluted at 0.5 M NaCl by chondroitinase AC and ABC enzymes (data not shown).

DISCUSSION

We report that platelet-derived TGF-β exerts a cell- and factor-specific effect on proteoglycan synthesis by human arterial SMC that is independent of growth promoters and the proliferative state of the SMC. Therefore, vascular SMC proteoglycan synthesis appears to be regulated independently of cell proliferation. Elution profiles on DEAE-Sephacel

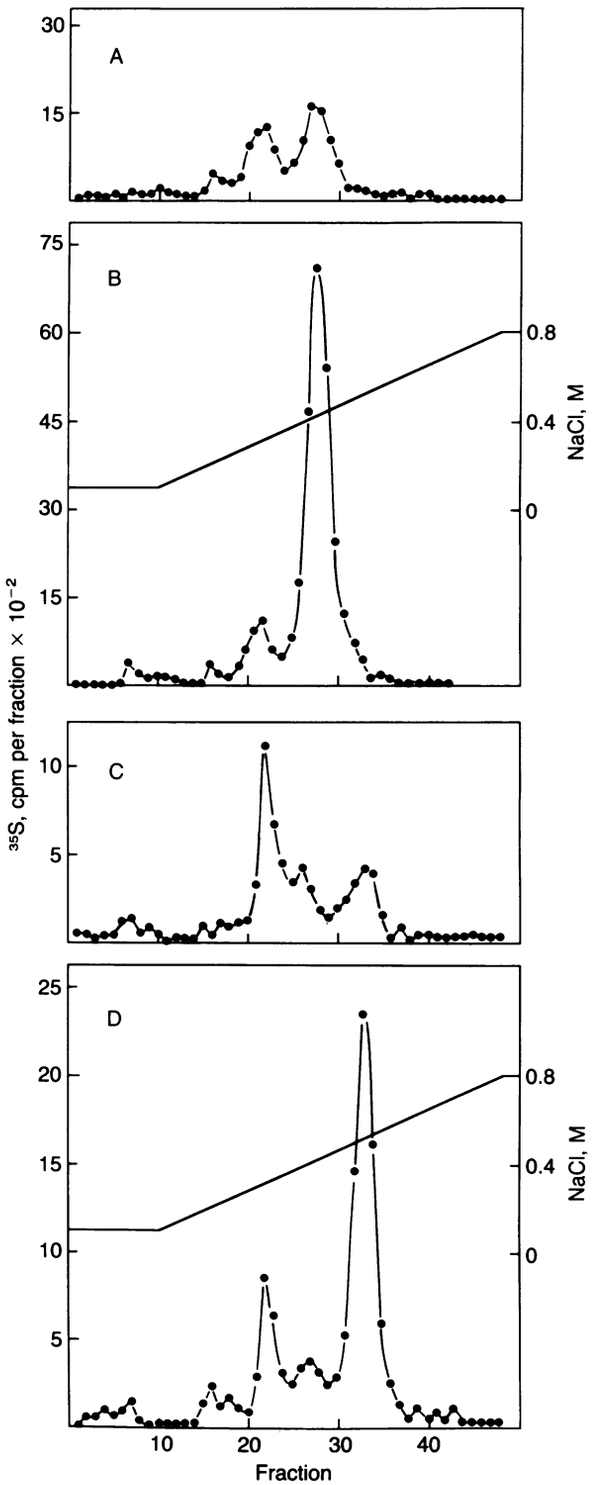

FIG. 3. DEAE-Sephacel column chromatography of medium- and SMC-associated proteoglycans. Medium- (*A* and *B*) and SMC- (*C* and *D*) associated proteoglycans equivalent to 0.1 mg of cellular protein isolated from TGF-β-treated (*B* and *D*) and untreated (*A* and *C*) cells were fractionated on a DEAE-Sephacel column (0.6 × 10 cm), which was equilibrated with 50 mM sodium acetate, pH 6.0/8 M urea/0.5% Triton X-100/0.1 M NaCl. After sample application, the column was washed with 10 ml of the same solution, and then proteoglycan was eluted with 36 ml of a linear 0.1–0.8 M NaCl gradient. Radioactivity of the cold trichloroacetic acid-insoluble material in each fraction was determined in a scintillation counter.

columns suggested that TGF-β stimulated the synthesis of at least two specific species of SMC chondroitin sulfate pro-

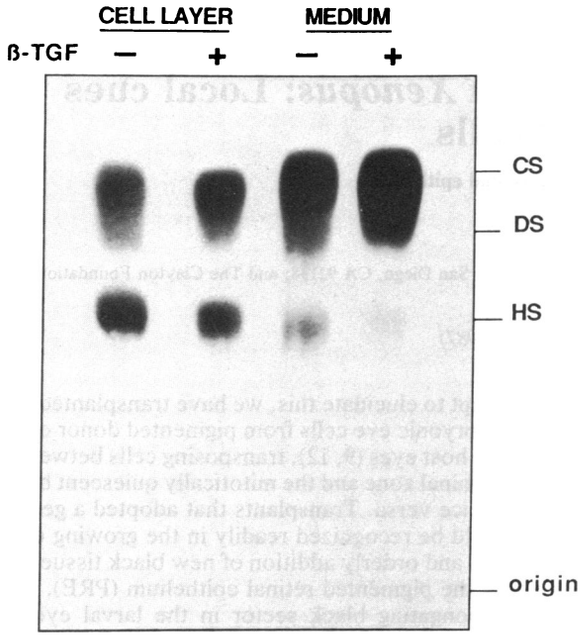

FIG. 4. Agarose gel electrophoresis of glycosaminoglycans synthesized by SMC with and without TGF-β treatment. Glycosaminoglycan samples were subjected to 0.5% agarose gel electrophoresis in 50 mM 1,3-diaminopropane acetate buffer (pH 9.0) for 2 hr at 120 V. Glycosaminoglycan standards were added to each sample and served as an internal standard for the estimation of the recovery. CS, chondroitin sulfate; DS, dermatan sulfate; HS, heparan sulfate.

teoglycans: one was secreted, and another was associated with cells.

Our results support the hypothesis that, similar to PDGF, TGF-β may play a major role in tissue repair (28, 29). Stimulation of specific mesenchymal cell proteoglycan synthesis may be one important mechanism by which TGF-β exerts its effects. Placed in the framework of the Ross hypothesis of formation of atherosclerotic lesions in the human vascular system (4), chronic release of PDGF and TGF-β from platelets and leukocytes, which are concentrated in the atherosclerotic lesion, would retard EC regeneration via TGF-β (refs. 30 and 31; H.H., M. Kan, J.-K.C., and W.L.McK., unpublished data), promote SMC proliferation via PDGF, and promote accumulation of myo-intimal proteoglycan via TGF-β, independent of the proliferative state of the SMC. Myo-intimal accumulation of specific SMC-derived proteoglycan may be an indirect consequence of SMC hyperplasia and a direct consequence of chronic stimulation of SMC by TGF-β.

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