Vol. 163, No. 1, 1989 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
August 30, 1989 Pages 84-92

HEPARIN AND ENDOTHELIAL CELL GROWTH FACTOR MODULATE COLLAGEN AND PROTEOGLYCAN PRODUCTION IN HUMAN SMOOTH MUSCLE CELLS

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Received July 14, 1989

The effects of heparin and endothelial cell growth factor (ECGF) on extracellular matrix production were examined in human iliac smooth muscle cells. The cells were grown in (a) medium supplemented with heparin (100 μg/ml) and ECGF (75 μg/ml), (b) medium supplemented with ECGF (75 μg/ml) alone, or (c) unsupplemented medium. In the presence of heparin and ECGF, collagen production was inhibited 91-95% as compared to cultures incubated with ECGF alone or without both supplemental factors. In contrast, the production of proteoglycans was elevated 2.5 fold in the presence of heparin and ECGF. Enzymatic digestion of the proteoglycans indicated that both large and small molecular weight chondroitin sulfate proteoglycans were markedly elevated, while dermatan sulfate and heparan sulfate proteoglycans were increased to a lesser extent. The results suggest that the combination of heparin and ECGF elicits potent modulation of extracellular matrix production, with divergent effects on collagen and proteoglycan synthesis. © 1989 Academic Press, Inc.

Heparin and endothelial cell growth factors influence cellular proliferation and a variety of other biologic activities of many cell types (1,2). Heparin, a highly negatively-charged glycosaminoglycan, has been employed in combination with ECGF in the cloning and long-term culture of human endothelial cells (3,4) and adult human vascular smooth muscle cells (5). In medium unsupplemented with ECGF, heparin exerts antiproliferative effects on vascular smooth muscle cells (6-8). Other biologic effects of heparin include promotion of angiogenesis (9,10), modulation of the synthesis of collagen by chondrocytes (11) and noncollagenous proteins by smooth muscle cells (12), as well as increased synthesis of fibronectin and thrombospondin by human smooth muscle cells (13). Following experimental arterial injury in vivo, heparin suppresses smooth muscle cell proliferation

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0006-291X/89 $1.50
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(14) and inhibits intimal hyperplasia (15). Recently, heparin has been found to influence the deposition of extracellular matrix in vivo in blood vessels following injury (16).

Endothelial cell growth factor or acidic fibroblast growth factor exhibits a variety of biologic effects in cell culture (17,18). ECGF is mitogenic for various cell types, including endothelial cells. Membrane receptors specific for ECGF have been localized on both endothelial (19) and smooth muscle cells (20). Interestingly, the proliferative effect of ECGF is potentiated by heparin (3,5). This effect may arise from the ability of heparin to either stabilize the tertiary structure of ECGF and protect it from proteolytic degradation and acid inactivation (1,21), or increase the affinity of ECGF for cell surface receptors, by inducing conformational changes in the ECGF peptide (22).

The principal goal of the present study was to examine the effects of heparin and ECGF on collagen and proteoglycan synthesis in human iliac arterial smooth muscle cells. The results show that, in combination, heparin and ECGF inhibit collagen production, while stimulating proteoglycan synthesis in confluent smooth muscle cell cultures.

MATERIALS AND METHODS

Cell Culture. Adult human iliac arteries were obtained from brain-dead, heart-beating cadaver renal donors. After harvesting the endothelial cells by collagenase treatment (23), the adventitia was removed and the medial tissue was minced. The minces were placed in gelatin-coated flasks and incubated in Medium 199 supplemented with 2 mM glutamine, 15% fetal bovine serum and either (a) a combination of heparin (100 μg/ml) and ECGF (75 μg/ml), or (b) ECGF alone (75 μg/ml) or (c) without both supplements (control). Explants maintained in heparin (100 μg/ml) alone exhibited negligible outgrowth of cells and, therefore, this condition was not included in the present study. The explants were re-fed weekly and harvested at confluency, usually after three weeks. Thereafter, the cells were harvested weekly with 0.25% trypsin and were seeded at 10^4 cells/cm^2 in medium containing the respective supplements. Cells from two donors (HIAS 101 and HIAS 117), were used at population doubling levels of 26-28. Quadruplicate or triplicate flasks were analyzed in parallel for each experimental growth condition.

Collagen Synthesis. Confluent cultures of human smooth muscle cells grown under the three experimental conditions were preincubated with 40 μg/ml of ascorbic acid 2 h prior to the addition of 20 μCi/ml of L-[2,3,4,5-^{3}H]proline (108.6 Ci/mmol; New England Nuclear, Boston, MA). After 16 h, the incubation was terminated by separating the medium from cells and cooling the fractions to 4°C. Various protease inhibitors were added to the medium samples. The cells were rinsed with PBS, extracted with 0.4 M NaCl-Tris buffer (pH 7.5) containing the protease inhibitors, and sonicated at 60 Hz for 30 sec. To quantitate the synthesis of [^{3}H]hydroxyproline, aliquots of medium and homogenized cells were dialyzed against tap water, hydrolyzed in 6 N HCl in sealed tubes at 115°C for 24 h and assayed for nondialyzable [^{3}H]hydroxyproline using a specific radiochemical method (24). The values were normalized for cellular protein (25) and DNA(26).

Proteoglycan Synthesis. Confluent cultures were radiolabeled with 15 μCi/ml of (D-[6-^{3}H(N)]glucosamine HCl (29.5 Ci/mmol) and 50 μCi/ml of carrier-free $Na_2{}^{35}SO_4$ (1200 Ci/mmol), or 75 μCi/ml of carrier-free $Na_2{}^{35}SO_4$ alone (all isotopes from NEN, Boston, MA) for 20 or 24 hours at 37°C. The medium was separated from cells and protease inhibitors were added to the medium. The cells were extracted with 4 M guanidine-HCl, 0.1 M sodium acetate, pH 5.8, containing 2% Triton X-100 and various protease inhibitors (27). Aliquots of samples were subjected to gel filtration chromatography on Sephadex G-50 equilibrated with 8 M urea, 0.1 M Tris-HCl, pH 7.0, containing 0.1 M NaCl, 20 mM EDTA and 0.2% Triton X-100 (28). Aliquots of radiolabeled macromolecules were precipitated overnight at -20°C by the addition of 5 volumes of ethanol/1.3% potassium acetate (w/v). Recovery of radioactivity following precipitation exceeded 90%. Aliquots of

the samples were run on analytical Sepharose CL-2B column (0.7 x 90 cm) in 4 M guanidine HCl, 0.1 M Na acetate, pH 7.0, and 0.2% Triton X-100. Parallel samples were also analyzed by agarose gel electrophoresis, using a modified (Pacifici, M. personal communication) method of Thornton et al. (29). Briefly 1% agarose in 40 mM Tris, 1 mM Na_2SO_4 and 0.01% Na dodecylsulfate, pH 6.8, was poured at 56°C and allowed to stand overnight at 4°C. The vertical gels were pre-run for 1 h at 10°C, and 40 ul samples were electrophoresed at 50 mA for 4 h. The gels were fixed in 50% methanol and 10% acetic acid overnight, dried at 55°C under vacuum and exposed to x-ray film for 48 h. Glycosaminoglycans were identified by specific degradation with protease-free chondroitinase ABC (EC 4.2.2.4) or chondroitinase AC (30) or by nitrous acid at low pH (31). All values were corrected for cellular protein or DNA.

RESULTS AND DISCUSSION

The incorporation of [^{3}H]proline into [^{3}H]hydroxyproline was markedly reduced by a combination of heparin and ECGF, while ECGF alone did not elicit alteration in [^{3}H]hydroxyproline synthesis (Fig. 1). The inhibitory effect, despite an increase in cell number, was evident whether the values were normalized for cellular protein (Fig. 1A) or

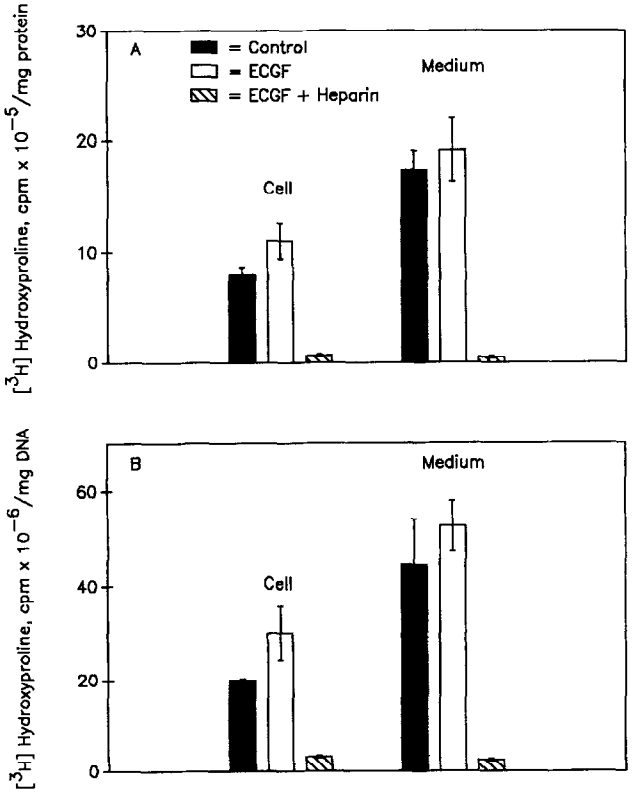

Figure 1: Modulation of collagen production by human smooth muscle cells incubated with ECGF and heparin. Smooth muscle cells were grown in Medium 199 supplemented with either a combination of heparin (100 μg/ml) and ECGF (75 μg/ml) (hatched bars), or ECGF alone (75 μg/ml) (unfilled bars), or without heparin and ECGF (control, filled bars). At confluency, the cells were incubated with 40 μg/ml of ascorbic acid and two h later were radiolabeled with [^{3}H]proline for 16 h at 37°C. The incorporation of [^{3}H]proline into [^{3}H]hydroxyproline in the cellular and medium fractions was determined separately, using a radiochemical assay (see Methods). The values represent the average ± S.D. of four parallel flasks in each experimental condition and are normalized for cellular protein (Panel A) and DNA (Panel B) contents.

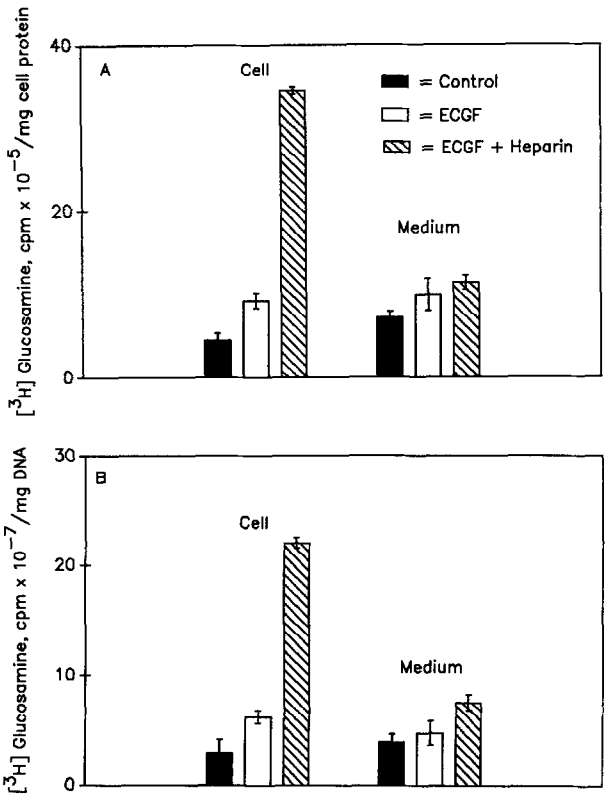

Figure 2: Synthesis of [^{3}H]glucosamine-labeled proteoglycans and glycoproteins by human smooth muscle cells incubated with heparin and ECGF (hatched bars), ECGF alone (unfilled bars), or without heparin and ECGF (control, filled bars). Confluent cultures of human smooth muscle cells grown in the three experimental conditions were radiolabeled with [^{3}H]glucosamine for 20 h at 37^{0}C. The medium and cellular fractions were subjected to gel filtration chromatography on Sephadex G-50 equilibrated with 8 M urea, 0.1 M Tris-HCl, pH 7.0, containing 0.1 M NaCl, 20 mM EDTA, and 0.2% Triton X-100. Aliquots of the eluted fractions were assayed for radioactivity. The values are the mean $\pm$ S.D. of four parallel flasks in each experimental condition and are normalized for cellular protein (Panel A) and DNA (Panel B).

DNA (Fig. 1B). The decrease in total cell and medium was 95% and 91% based on cell protein and DNA, respectively. Total [^{3}H]proline incorporation into protein was decreased by 80% and 62% following normalization based on cell protein and DNA, respectively (not shown). These results suggest that other proline-containing proteins may be influenced by the addition of heparin.

Marked stimulation of [^{3}H]glucosamine incorporation into glycoproteins and proteoglycans was noted in cultures incubated with heparin and ECGF, after normalization for cellular protein (Fig. 2A) or DNA (Fig. 2B). Heparin with ECGF enhanced the incorporation of [^{3}H]glucosamine by 7-8 fold in the cell-matrix layer and 1.5-2 fold in the medium (Fig. 2A & B). ECGF alone showed modest stimulation of [^{3}H]glucosamine-labeled macromolecules in the cell-matrix layer relative to the supplement-free control cultures (Fig. 2A & B). Proteoglycan synthesis was clearly increased in the medium of cultures incubated with heparin and ECGF (Fig. 3). The level was 2.5 times greater than

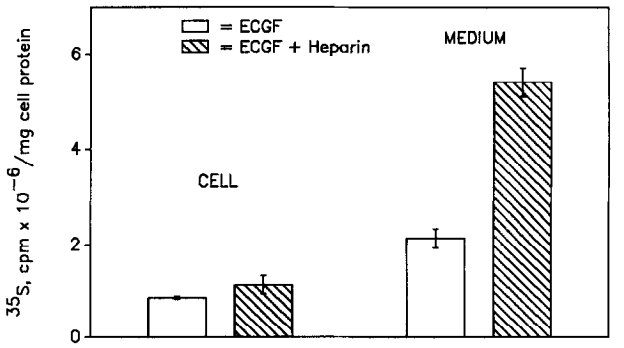

Figure 3: Stimulation of proteoglycan production by heparin and ECGF in smooth muscle cell cultures. Smooth muscle cells were grown to confluence in 75 μg/ml of ECGF (unfilled bars), or a combination of 100 μg/ml heparin and 75 μg/ml ECGF (hatched bars). The cells were radiolabeled with $Na_2{}^{35}SO_4$ for 24 hours and the medium and cellular samples were separately analyzed by gel filtration chromatography on Sephadex G-50. Aliquots of the eluted fractions were assayed for radioactivity. The amount of ^{35}S-labeled proteoglycans was normalized for cellular protein content. The values represent the mean ± S.D. of triplicate parallel flasks under the two experimental conditions.

that of the cultures incubated with ECGF alone. The overall stimulation of total ^{35}S-labeled proteoglycans in the medium and cells following incubation with the combination of heparin and ECGF was more than twice that of the cultures with ECGF alone.

Analytical Sepharose CL-2B chromatography (Fig. 4A & B) showed that the human smooth muscle cells produced both large and small molecular weight proteoglycans with profiles similar to those described in primate aortic (32) and human colon (33) smooth muscle cells. No significant difference was seen in the peaks of the cell fractions treated with ECGF alone vs. those with both heparin and ECGF (Fig. 4A), but a prominent increase of large molecular weight proteoglycans in the medium was induced by the combination of heparin and ECGF (Fig. 4B). The small molecular weight proteoglycans in the medium compartment were also elevated by both factors (Fig. 4B).

Agarose gel electrophoresis of identical aliquots run on Sepharose CL-2B showed similar changes in the medium, primarily an increase in the level of large chondroitin sulfate proteoglycans (Fig. 5A & B). Enhanced level of the small proteoglycans was also evident in the medium of cultures incubated with heparin and ECGF (Fig. 5A & B). Examination of the electrophoretic profile indicated a lesser increase in the large proteoglycans of the cellular fractions of cultures incubated with the combination of heparin and ECGF as compared to those with ECGF alone (Fig. 5A). Selective enzymatic digestion (Fig. 5B) showed that both large and small proteoglycans were sensitive to chondroitinase ABC, indicating that the predominant proteoglycan was chondroitin sulfate proteoglycan and that its synthesis was stimulated in the presence of heparin.

Further enzymatic digestion identified chondroitin sulfate, dermatan sulfate and heparan sulfate as the major glycosaminoglycans produced by the arterial smooth muscle cells in the cell-matrix layer and medium (Fig. 6A & B). Comparison of the cultures incubated with heparin and ECGF vs. ECGF alone showed that chondroitin sulfate was the predominant glycosaminoglycan and that it increased by approximately 2.5 fold in the

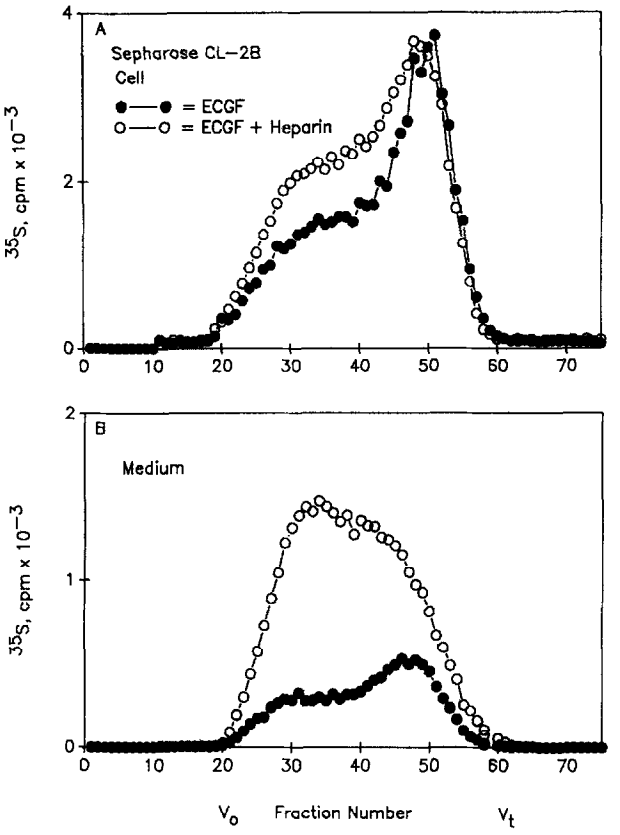

Figure 4: Elution profiles of ^{35}S-labeled proteoglycans from analytical Sepharose CL-2B chromatography of smooth muscle cell cultures incubated with heparin and ECGF or ECGF alone. Following gel filtration chromatography on Sephadex G-50 and precipitation at -20°C with ethanol containing 1.3% (w/v) potassium acetate, aliquots of ^{35}S-labeled proteoglycans from cell matrix layer (Panel A) and medium (Panel B) were run on an analytical Sepharose CL-2B column (0.7x90 cm). The recovery of ^{35}S activity following precipitation exceeded 90%. The eluted fractions were assayed for radioactivity. o---o, heparin and ECGF; ●---●, ECGF alone.

presence of heparin. This increase accounted for most of the elevation in the total [^{35}S]sulfate incorporation. Chondroitin sulfate was enhanced in both cell and medium. Total dermatan sulfate was increased by 64% but was recovered primarily in the medium fraction (Fig. 6B), suggesting that heparin altered the compartmentalization or secretion of this proteoglycan. Heparan sulfate was elevated to a lesser extent in both cell layer and medium. These results indicate that heparin and ECGF caused a substantial stimulation of proteoglycan synthesis, with a predominant effect on chondroitin sulfate proteoglycan.

In this study, heparin in combination with ECGF exerted opposing effects on the biosynthesis of extracellular matrix proteins in human smooth muscle cells. Collagen production was inhibited while proteoglycan synthesis was stimulated. Our data are the first to clearly demonstrate these divergent effects of heparin and ECGF on the matrix production in a single cell type. Previous reports have shown that heparin increases fibronectin and thrombospondin levels in umbilical arterial smooth muscle cells (13), while it decreases the collagen level in chick chondrocyte cell cultures (11).

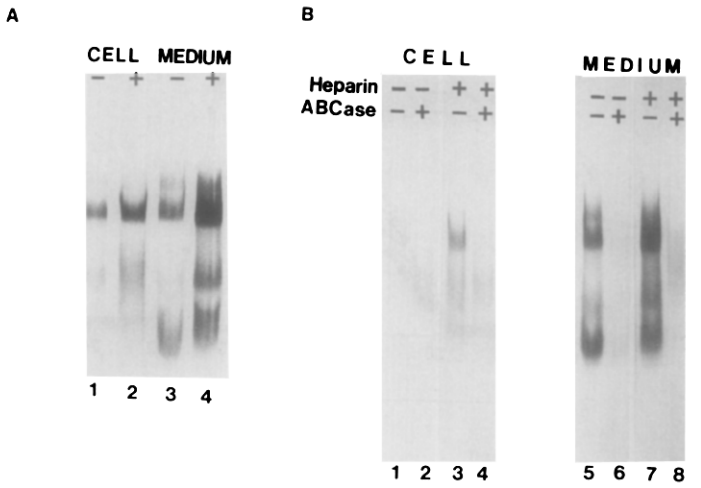

Figure 5: Agarose gel electrophoresis of proteoglycans synthesized by human smooth muscle cells. The samples represent parallel aliquots of those analyzed by Sepharose CL-2B chromatography (see Fig. 4). In panel A, lane 1, cells incubated with ECGF alone; lane 2, cells incubated with heparin and ECGF; lane 3: medium of cultures incubated with ECGF alone; lane 4, medium of cultures incubated with heparin and ECGF. In panel B, samples were digested with protease-free chondroitinase ABC to characterize the proteoglycans. Lanes 1,2 and 5,6 are cultures that were incubated with ECGF alone, while lanes 3,4 and 7,8 are those incubated with both heparin and ECGF. The samples were digested with protease-free chondroitinase ABC (ABCase) in lanes 2,4 and 6,8.

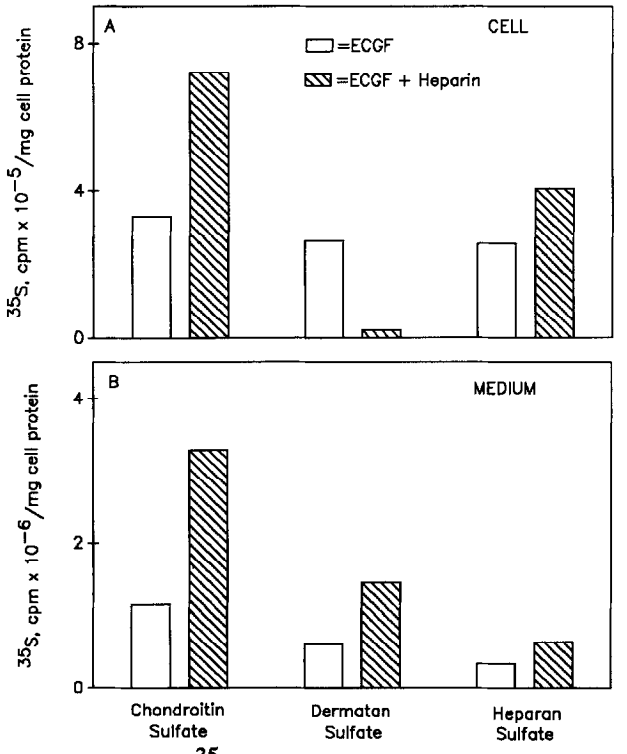

Figure 6: Characterization of ^{35}S-labeled glycosaminoglycans in smooth muscle cell cultures incubated with ECGF alone, or heparin and ECGF. Selective enzymatic digestion of glycosaminoglycans was performed employing degradation with chondroitinase ABC, AC, and nitrous acid at low pH to identify chondroitin sulfates, dermatan sulfate, and heparan sulfate, respectively. The values are normalized for cellular protein in the cell-matrix layer (Panel A) and in the medium (Panel B). unfilled bars, ECGF alone; hatched bars, heparin and ECGF.

In vivo, intravenous infusion of heparin inhibits myointimal hyperplasia (14,15) in experimentally-injured rat carotid artery and elicits divergent effects on extracellular matrix macromolecules (16), i.e. a decrease in collagen and an increase in proteoglycans. These results corroborate our in vitro data and strongly suggest that growth modulators exert profound effects on the extracellular matrix metabolism by vascular cells.

ECGF by itself did not elicit dramatic alterations in the production of collagen and proteoglycans by smooth muscle cells. The alterations in collagen and proteoglycan production by the combination of heparin and ECGF may be explained either by the action of heparin or by the potentiation of ECGF activity by heparin. Since smooth muscle cell growth was not supported by medium supplemented with heparin alone, it is likely that interaction of ECGF and heparin is critical to effect a profound shift in the pattern of synthesis of extracellular matrix macromolecules.

ACKNOWLEDGMENTS

This work was supported in part by NIH grants CA-39481, AG-5707, HL-34153 and AG-04861, and by the American Heart Associate, Southeastern Pennsylvania Chapter.

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