An Antiproliferative Heparan Sulfate Species Produced by Postconfluent Smooth Muscle Cells

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ABSTRACT Heparan sulfate was isolated from the cell surface, cell pellet, and culture medium of exponentially growing as well as postconfluent bovine aortic smooth muscle cells (SMCs). After chromatography on DEAE-Sephadex and Sepharose 4B, the various mucopolysaccharides were examined for their ability to cause growth inhibition in a SMC bioassay. The heparan sulfate isolated from the surface of postconfluent SMCs possessed approximately eight times the antiproliferative potency per cell of the heparan sulfate obtained from the surface of exponentially growing SMCs. Heparan sulfate isolated from other fractions of exponentially growing or postconfluent SMCs possesses little growth inhibitory activity. The difference in the antiproliferative activities of heparan sulfate obtained from the surface of SMCs in the two growth states could not be attributed to the synthesis of a greater mass of mucopolysaccharide by postconfluent SMCs. Indeed, heparan sulfate isolated from the surface of the postconfluent SMCs exhibits a specific antiproliferative activity which is 13-fold greater than mucopolysaccharide obtained from the surface of exponentially growing SMCs and more than 40-fold greater than commercially available heparin. In addition, exponentially growing SMCs did not exhibit an enhanced ability to degrade the complex carbohydrate. Furthermore, other investigations indicate that the small amount of growth inhibitory activity intrinsic to heparan sulfate isolated from the surface of exponentially growing SMCs is due to residual, biologically active, mucopolysaccharide produced by the primary postconfluent SMCs from which the exponentially growing SMCs were derived. These studies suggest that bovine aortic SMCs are capable of controlling their own growth by the synthesis of a specific form of heparan sulfate with antiproliferative potency.

Most connective tissues contain glycosaminoglycans as major components of their extracellular matrix. These high molecular weight, negatively charged sulfated mucopolysaccharides have been implicated in determining certain general overall properties of tissues such as hydration, elasticity, and permeability (1). It has also been suggested that glycosaminoglycans that are known to be associated with the surface of the cell could be involved in basic cellular functions, such as adhesion and motility (2, 3). However, little is known about whether specific structural elements on the glycosaminoglycans are responsible for the biologic properties of these components.

Several investigators have demonstrated that the mucopolysaccharide, heparin, must satisfy unique structure-function relationships to interact with its protein co-factor, antithrombin, to alter allosterically the conformation of this protease inhibitor, and thereby accelerate neutralization of coagulation system enzymes (4). Our laboratory has recently shown that anticoagulantly active heparan sulfate molecules are present on the surface of endothelial cells and are, in part, responsible for maintaining the nonthrombogenic properties of the vessel wall (5, 6). These findings suggest that various types of cells may be able to synthesize heparan sulfate moieties of unique structure that could regulate other biologic systems via highly specific interactions. The available data indicate that there may be different structural requirements for the various functions of these mucopolysaccharides.

Guyton et al. (7) have provided evidence that the administration of either anticoagulantly active or anticoagulantly inactive heparin to rats after endothelial injury suppresses the subsequent in vivo proliferation of smooth muscle cells

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(SMCs).[1] Castellot and co-workers (8–10) and Hoover et al. (11) have also shown that SMC growth under in vitro conditions can be inhibited by both forms of heparin, as well as by a heparin-like product from endothelial cells. Here we describe the isolation and growth inhibitory potencies of heparan sulfates obtained from bovine aortic SMCs at two stages of cell growth. We present evidence that postconfluent as well as exponentially growing SMCs synthesize heparan sulfate proteoglycans, but that only postconfluent SMCs produce a cell surface-associated heparan sulfate with potent antiproliferative activity. These results suggest a mechanism by which SMCs may play a part in the regulation of their own growth via the production of this specific form of heparan sulfate.

MATERIALS AND METHODS

Enzymes: *Flavobacterium* heparinase was purified from *Flavobacterium heparinum*, provided by Dr. R. Langer (Massachusetts Institute of Technology, Cambridge, MA) as previously described (5). The final preparation of *Flavobacterium* heparinase was homogeneous as judged by nonequilibrium pH gradient electrophoresis (pH 3.5–10) using 4% acrylamide gels containing 8 M urea. The heparin cleaving activity of the purified bacterial enzyme was 3,650 U/mg as determined by Azure A hyperchromicity. An analysis of the *Flavobacterium* heparinase revealed no significant proteolytic activity as quantitated by hydrolysis of ^{125}I-labeled alpha-casein. Chondroitinase activity was not observed in the purified enzyme preparation as measured by degradation of chondroitin 4-S and 6-S or dermatan sulfate.

Platelet heparitinase, an endoglycosidase, was isolated from human platelets as previously described (12). The final preparation was physically homogeneous as judged by disk gel electrophoresis at acidic pH as well as gel filtration chromatography and exhibited a specific activity of 11 U/mg. This enzyme hydrolyzes glucuronsyl-glucosamine linkages within heparin or heparan sulfate and has no effect on other glycosaminoglycans or proteins (12).

Papain pronase (protease, type XIV), and chondroitin ABC lyase were obtained from Sigma Chemical Co. (St. Louis, MO). Chondroitin ABC lyase completely degraded chondroitin 4- and 6-sulfates (Sigma Chemical Co.) and had no activity against commerically available heparin (Diosynth, Inc., Chicago, IL) or National Institutes of Health standard heparan sulfate. Trypsin-EDTA solution was obtained from Flow Laboratories (McLean, VA).

All other chemicals were obtained from Fisher Scientific Co. (Medford, MA) unless otherwise noted.

Cell Culture: SMCs were grown from explants of bovine aortas as previously described (13). After removing the endothelium, small pieces of media were carefully stripped from the vessel wall. Fragments of the media with average dimensions of 5 × 5 mm were placed with their lumen side up in 100-mm-diameter tissue culture dishes (Costar, Cambridge, MA). Each dish contained 5 ml of Dulbecco's modification of Eagle's minimal essential medium (Flow Laboratories), bovine calf serum (Flow Laboratories) at a final concentration of 10%, 100 U/ml of penicillin (E. R. Squibb & Sons, Inc., Princeton, NJ), and 100 μg/ml of streptomycin sulfate (Eli Lilly, Co., Indianapolis, IN) (10% CS-DME). The explants were incubated in the 10% CS-DME for 7 d in a humidified 5% CO_2 atmosphere. Thereafter, the medium was changed every 3-4 d. After 4–5 wk of growth, the resultant cells were examined by electron microscopy and appeared identical to vascular SMCs described by others (14). In particular, numerous myofilament bundles were noted in the cytoplasm and vesicles were observed near surface membranes.

Radiolabeling and Isolation of SMC Glycosaminoglycans: Primary cultures of bovine SMCs 4–5 wk after explant, were treated with 0.05% trypsin-0.02% EDTA solution for 5 min at 37°C, and were then subcultured in 10% CS-DME at a cell density of 0.25×10^4 cells/cm^2. When cells had reached the desired growth stage, 24 h or 10 d later, the culture medium was removed and replaced with fresh 10% CS-DME containing 50 μCi/ml of $Na_2{}^{35}SO_4$ (~200 mCi/mmol) (New England Nuclear, Boston, MA) and incubation was continued for 48 h. At the end of the radiolabeling period, the culture medium was aspirated and saved, the cells were washed five times with phosphate-buffered saline (NaCl, 0.137 M, KCl 0.0027 M, Na_2HPO_4, 0.0081 M, KH_2PO_4, 0.0015 M, pH 7.5) (PBS), and cell surface-associated glycosaminoglycans were released by treatment with 0.05% trypsin–0.02% EDTA solution for 20 min at 37°C. The cell density at the end of the radiolabeling period was ~7.5×10^4 cells/cm^2. The cell pellet was harvested by centrifugation at 100 *g* for 5 min at 4°C. The supernatant, which contained the cell surface associated glycosaminoglycans, was boiled for 10 min and then saved for further processing. The cell pellet was resuspended in 1–2 ml of distilled, deionized water and the cells were homogenized with a Dounce homogenizer. Pronase, 1 mg/ml, was added to the homogenate and digestion was carried out at 37°C for 24 h. Papain, 1 mg/ml, was then admixed and incubation continued for an additional 24 h. The cell digest was centrifuged at 4,000 *g* for 10 min at 4°C to remove debris, and the supernatant was saved. Culture medium from radiolabeled exponentially growing or postconfluent SMC cultures was concentrated by lyophilization and chromatographed at a flow rate of 8 ml/h on a column of Sephadex G50 (1.2 cm × 45 cm) (Pharmacia Fine Chemicals, Piscataway, NJ) equilibrated in 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5, to remove unincorporated radioactivity. The macromolecular material was pooled and lyophilized. Cell surface, cell pellet, and culture medium samples were extensively dialyzed against 0.4 M NaCl, 0.01 M Tris-HCl, pH 7.5 (DEAE equilibration buffer) in preparation for DEAE-Sephadex chromatography.

Isolation of Radiolabeled SMC Heparan Sulfate: Cell surface, cell pellet, and medium fractions from exponentially growing or postconfluent SMC cultures were chromatographed on a column of DEAE-Sephadex A50 (1.9 cm × 185 cm) (Pharmacia Fine Chemicals). After washing with two column volumes of equilibration buffer, glycosaminoglycans were eluted with a linear salt gradient that used a mixing chamber containing 1,000 ml of 0.4 M NaCl in 0.01 M Tris-HCl, pH 7.5, and a reservoir containing 1,000 ml of 2.0 M NaCl in 0.01 M Tris-HCl, pH 7.5. The flow rate of the column was 25 ml/h and 7-ml fractions were collected. Fractions were monitored for ^{35}S by counting an aliquot representing ~1% of the fraction volume. All scintillation counting employed Ultrafluor scintillation fluid (National Diagnostics, Somerville, NJ). Peak fractions were pooled and concentrated by rotary evaporation. The heparan sulfate pool obtained by DEAE-Sephadex chromatography was filtered at a flow rate of 5 ml/h on a column of Sepharose 4B (1.5 cm × 110 cm) (Pharmacia Fine Chemicals) equilibrated with 0.1 M NaCl, 0.01 M Tris-HCl, pH 7.5. Fractions of 2 ml were collected and radioactivity determined as described above. The pooled glycosaminoglycans were digested with Pronase, 1 mg/ml, in 0.01 M calcium acetate, 0.01 M Tris-HCl, pH 8.0, for 24 h at 37°C, and rechromatographed on the same column. Peak fractions were pooled, concentrated by rotary evaporation and dialyzed extensively against PBS for enzymatic analysis or bioassay, or against distilled, deionized water for glucosamine analysis.

Quantitation of Mucopolysaccharides: SMC heparan sulfate or porcine mucosal standard heparin (Diosynth, Inc., Chicago IL, lot number LP 041681) was hydrolyzed in 6 M HCl for 3 h *in vacuo* at 100°C (15). The glucosamine content of the hydrolysates was determined by ion exchange chromatography with a Durrum D-500 amino acid analyzer. The values obtained were multiplied by appropriate conversion factors to provide estimates of the amount of heparan sulfate or heparin within a given fraction. The conversion factors were derived by examining known amounts (dry weight) of the National Institutes of Health standard mucopolysaccharides.

Identification of SMC Glycosaminoglycans: Radiolabeled glycosaminoglycans isolated by ion-exchange chromatography and gel filtration were identified by specific enzymatic degradation. To this end, ^{35}S-labeled mucopolysaccharides were incubated with 30 U of *Flavobacterium* heparinase or 50 ng of platelet heparitinase or 0.1 U of chondroitin ABC lyase at 37°C for 24 h and were boiled for 10 min. Samples were obtained both before as well as immediately after treatment with the above enzymes, and were then analyzed by high performance liquid chromatography at a flow rate of 1 ml/min using a Waters Liquid Chromatography System (Waters Associates, Milford, MA) and a Toya Soda TSK G2000 SW column (Toya Soda Mfg., Tokyo, Japan) (HPLC/G2000 SW) equilibrated with either 1 M NaCl or 1 M ammonium acetate, pH 6.5. Fractions were collected at 0.4-min intervals and peaks of radioactivity were located as described above. Degradation of mucopolysaccharides was ascertained by comparing the levels of complex carbohydrate present in the excluded volume of the column (polysaccharides) vis a vis those located in the included volume of the column (oligosaccharides).

Bioassay of the Growth Inhibitory Activity of SMC Glycosaminoglycans: First passage SMCs in 10% CS-DME were seeded at 6×10^3 cells per 16-mm well in cluster-24 plates (Costar, Cambridge, MA) and allowed to attach for 24 h at 37°C in humidified 5% CO_2. On day 1, the SMCs were arrested in G_0 (G_1) by removing the growth medium, washing the cell layers twice with PBS, and adding 1 ml of 0.1% platelet-poor human plasma-DME to each well. Cells were incubated at 37°C in humidified 5% CO_2 for an additional 72 h. On day 4, the cells were released from G_0 block and exposed to the antiproliferative action of mucopolysaccharides. This was accomplished by removing the growth arrest medium, and adding either 10% CS-DME

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[1] *Abbreviations used in this paper:* 10% CS-DME, Dulbecco's modification of Eagle's minimal essential medium, bovine calf serum at a final concentration of 10%, 100 U/ml of penicillin, and 100 μg/ml of streptomycin sulfate; SMC, smooth muscle cell.

(control cultures) or 10% CS-DME with heparin at levels that ranged from 0.01 to 10 μg/ml (heparin standard curve) or 10% CS-DME with heparan sulfate samples at given concentrations of mucopolysaccharide. The SMCs were incubated at 37°C in humidified 5% CO_2 for an additional 7 d. At no time during this incubation did microscopic examination reveal evidence of cells detaching from the surface of the wells. The cell numbers on days 4 and 11 were determined in duplicate by trypsinizing the SMCs and counting the dislodged cells in a Coulter counter (Coulter Electronics, Inc., Hialeah, FL, model Z_F). To ascertain that the procedure had not lysed the SMCs and to ensure that all cells were removed from the cluster-24 plates, we routinely checked trypsinized multiwells by direct microscopic examination. The net growth of SMCs in the control cultures was obtained by subtracting the starting cell number (day 4) from the cell number on day 11. The net growth of SMCs in various concentrations of heparin standards or heparan sulfate samples were computed in similar fashion. The degree of inhibition for a given level of mucopolysaccharide was calculated from the following relationship: % inhibition = 1 − (net growth in culture medium containing mucopolysaccharide/net growth in control culture medium) × 100. The antiproliferative potencies of heparan sulfate fractions were computed by comparing the percent inhibition of cell growth obtained at a given level of the sample with that induced by the heparin standards. For this purpose, we have assumed that one inhibitory unit of biological activity is equivalent to the percent inhibition of cell growth produced by 1 μg/ml of the heparin standard. The specific antiproliferative potency of a given fraction of heparan sulfate is calculated by dividing the biological activity of the sample by the mass of the sample as determined by glucosamine analysis. By definition, the specific antiproliferative potency of the heparin standard is set at a value of 1.0.

The standard error of the mean in percent for the measurement of the inhibitory activity of heparan sulfate or the specific antiproliferative potency of heparan sulfate within a single experiment or between different experiments in which the same source of mucopolysaccharide, SMCs, and serum was used is ~7%. The standard error of the mean for the measurement of the inhibitory activity of heparan sulfate or specific antiproliferative potency of heparan sulfate among experiments in which different sources of mucopolysaccharide, SMCs, and serum were used is ~6%.

RESULTS

Isolation of SMC Heparan Sulfate

Primary cultures of bovine SMCs were treated with 0.05% trypsin–0.02% EDTA solution for 5 min at 37°C, and were then split at a 1:10 ratio into culture dishes with 10% CS-DME. We initially examined glycosaminoglycans from two stages of SMC growth. The first stage was chosen 1 d after plating, when the SMCs had doubled approximately 0.5 time (exponentially growing SMCs). The second stage was selected 10 d after plating, when the SMCs had been confluent for ~5 d (postconfluent SMCs). The SMCs, at either stage of growth, were incubated for 48 h in fresh medium containing $Na_2{}^{35}SO_4$. The medium was saved, the cells were washed with PBS, and cell surface glycosaminoglycans were released by treatment with 0.05% trypsin–0.02% EDTA solution for 20 min at 37°C. After this procedure, ~95 percent of the SMCs were viable as evidenced by trypan blue exclusion. The cell pellet was harvested by centrifugation, homogenized, and extensively proteolyzed with pronase and papain. Medium samples were concentrated and desalted by chromatography on Sephadex G50 (see Materials and Methods for additional experimental details). Cell surface, cell pellet, and culture medium samples were then dialyzed against 0.4 M NaCl, 0.01 M Tris-HCl, pH 7.5.

The radiolabeled glycosaminoglycans from the cell surface, cell pellet, and culture medium of exponentially growing and postconfluent SMCs were isolated by chromatography on DEAE-Sephadex A50 and Sepharose 4B. DEAE-Sephadex chromatography resolved the ^{35}S-labeled material obtained from the surface of exponentially growing and postconfluent SMCs into two peaks (Fig. 1). Peak I eluted at a salt concentration of 0.65 to 0.80 M NaCl, and was identified as heparan sulfate by its sensitivity to digestion with *Flavobacterium* heparinase as well as its resistance to digestion with chondroitin ABC lyase. Peak II eluted at a salt concentration of 0.88 to 1.08 M NaCl and was identified as chondroitin sulfate by its sensitivity to digestion with chondroitin ABC lyase as well as its resistance to digestion with *Flavobacterium* heparinase. The exponentially growing and postconfluent SMCs exhibited approximately equivalent amounts of cell surface heparan sulfate (38.7 ± 9.3% vs. 47.2 ± 4.9%) and chondroitin sulfate (55.1 ± 10.2% vs. 46.4 ± 1.5%) based upon the relative levels of ^{35}S counts within the two peaks. Peaks I and II were tested with respect to their ability to inhibit the proliferation of smooth muscle cells in a biological assay as outlined in Materials and Methods. The assay results indicate that the antiproliferative potency of peak I material is more than 500-fold greater per 10^5 cpm than that of peak II material. The inhibitory activity of peak I was completely destroyed by digestion with *Flavobacterium* heparinase and was unchanged following treatment with chondroitin ABC lyase.

Glycosaminoglycans from cell pellet or medium of exponentially growing and postconfluent SMCs were also chromatographed on DEAE-Sephadex (not shown). Two major peaks were observed which eluted at ionic strengths similar to those of peaks I and II and were degraded by *Flavobacterium* heparinase and chondroitin sulfate in a fashion identical to peaks I and II. However, the exponentially growing and postconfluent SMCs exhibited only small amounts of cell pellet or medium heparan sulfate (10.3 ± 2.1% vs. 8.3 ± 1.3%) as compared to chondroitin sulfate (85.7 ± 5.4% vs. 87.5 ± 3.8%) based upon the relative ^{35}S counts within the two peaks. Peaks I and II from cell surface, cell pellet, or

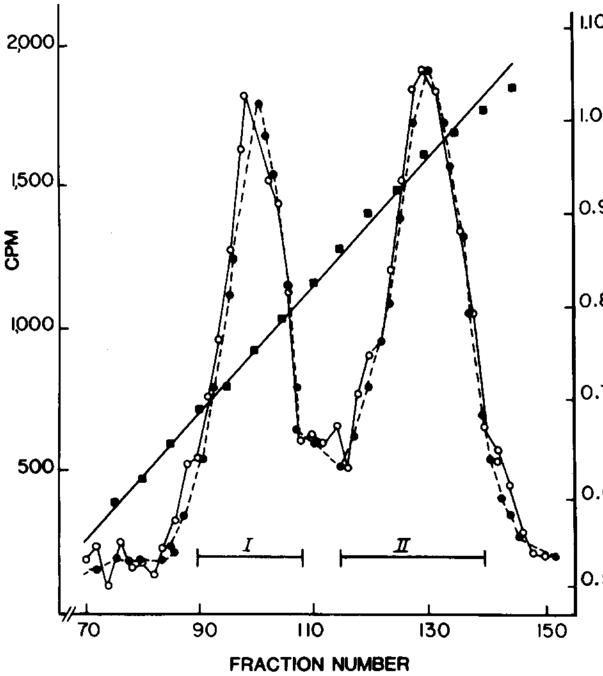

FIGURE 1 DEAE-Sephadex chromatography of radiolabeled glycosaminoglycans from the surface of postconfluent and exponentially growing SMCs. The ^{35}S-labeled glycosaminoglycans obtained from the surface of postconfluent (- - -) or exponentially growing (——) SMC by treatment with trypsin-EDTA were chromatographed a column of DEAE-Sephadex A50. Concentrations of NaCl were determined based on conductance measurements. See Materials and Methods for additional experimental detail.

culture medium contained no detectable absorbance at 280 nm.

The heparan sulfate–containing fractions from the cell surface, cell pellet, and culture medium of exponentially growing as well as postconfluent SMCs were individually chromatographed on Sepharose 4B as described in Materials and Methods. Heparan sulfate from the surface of postconfluent SMCs separated into two peaks (Fig. 2*A*). Proteolytic digestion of peak I with pronase and subsequent rechromatography of the material on Sepharose 4B revealed a heparan sulfate species with an elution position identical to that of peak II (Fig. 2*A*). Similar treatment of peak II resulted in no detectable change in the elution position of this component. Given the above results, heparan sulfate from peaks I and II were pooled for further processing. Heparan sulfate from the surface of exponentially growing cells displayed only one peak after chromatography on Sepharose 4B and the position of the peak did not change upon digestion with pronase (Fig. 2*B*). The elution position of this latter peak coincided with that of the protease treated heparan sulfate from the surface of postconfluent SMCs (Fig. 2*A*, peak II). The approximate molecular weights of the two cell surface mucopolysaccharide are 35,000–40,000, as determined by the elution position of glycosaminoglycan standards of similar charge density. Sepharose 4B chromatography of heparan sulfate from the cell pellet or culture medium of exponentially growing or postconfluent SMCs revealed a single major peak with an average molecular weight of 35,000–40,000 (data not shown). Proteolytic digestion of heparan sulfate from cell pellet, or culture medium of exponentially growing and postconfluent SMCs did not change the elution position of these components.

Growth Inhibitory Activity of SMC Heparan Sulfate

The antiproliferative activity of heparan sulfate isolated from the cell surface, cell pellet, and culture medium of exponentially growing and postconfluent SMCs was quantitated with a SMC growth inhibition assay. The data show that heparan sulfate obtained from the surface of a given number of postconfluent SMCs possesses approximately eight times the amount of antiproliferative activity of the heparan sulfate present on the surface of the same number of exponentially growing SMCs (Table I). The data also indicate that the heparan sulfates from the cell pellet, and culture medium of similar numbers of exponentially growing and postconfluent SMCs contain only minimal amounts of this biologic activity (Table I). It should be noted that chondroitin sulfate derived from the cell surface, cell pellet, or culture medium of exponentially growing or postconfluent SMCs has no growth inhibitory activity when assayed at levels 10 to 100 times higher than that used with heparan sulfate (data not shown).

The chemical masses of heparan sulfate obtained from the cell surface, cell pellet, and culture medium of exponentially growing and postconfluent SMCs were determined by direct measurement of glucosamine. It is apparent from these data

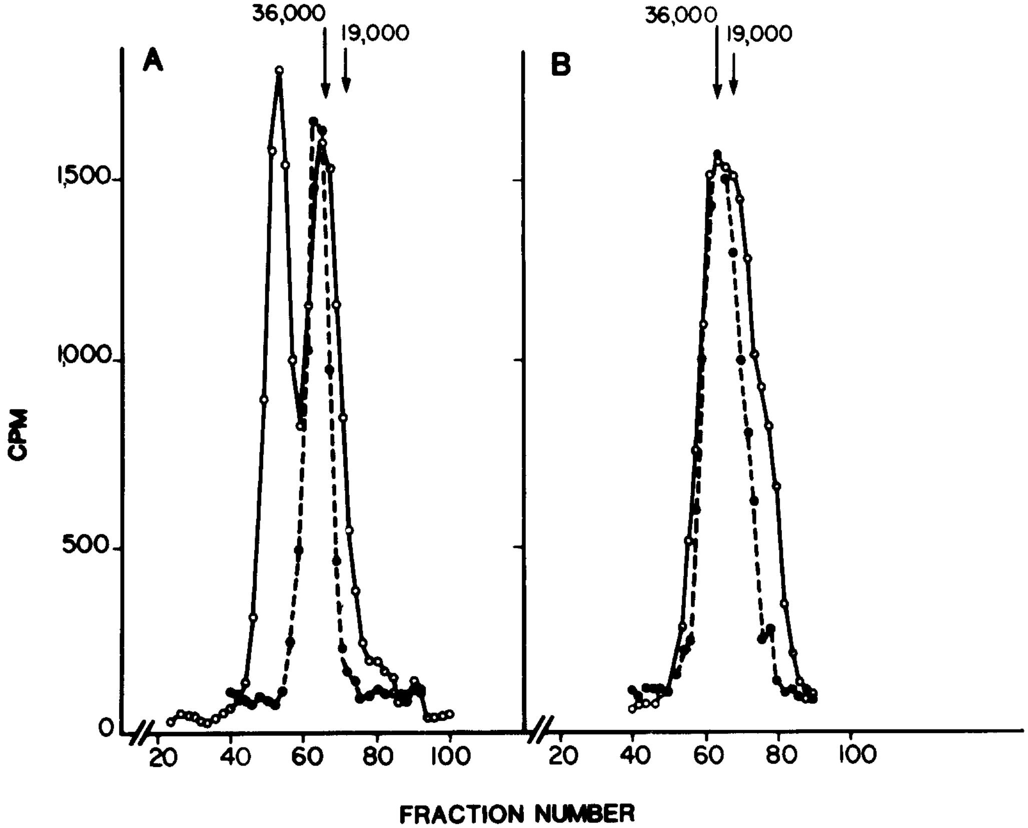

FIGURE 2 Sepharose 4B chromatography of heparan sulfate from the surface of postconfluent and exponentially growing SMCs. The heparan sulfate pool obtained by DEAE-Sephadex chromatography was gel filtered on a Sepharose 4B column. Elution profiles are provided for heparan sulfate isolated from the surface of postconfluent SMCs (*A*) and exponentially growing SMCs (*B*) before (——) and immediately after (- - -) protease digestion. Proteolytic digestion was accomplished by incubation of the heparan sulfate with pronase. Chondroitin sulfate molecular weight standards were generously provided by Dr. L. Rosenberg (Montefiore Hospital, Bronx, NY). Additional experimental details are provided in Materials and Methods.

that a given number of exponentially growing SMCs produce 1.5 to 3.0 times the mass of heparan sulfate found on the cell surface, cell pellet, or culture medium of the same number of postconfluent SMCs cells (Table I). The specific antiproliferative potency of heparan sulfate derived from the cell surface, cell pellet, or culture medium of exponentially growing and postconfluent SMCs was calculated by dividing the biological activity of a given sample by the chemical mass of that same sample. The specific antiproliferative activity of the heparin standard is set at 1.0. The results show that heparan sulfate isolated from the surface of postconfluent SMCs possesses the highest specific antiproliferative activity of any fraction examined, with a potency approximately 13-fold greater than that of heparan sulfate obtained from the similarly designated compartment of exponentially growing cells and more than 40-fold greater than that of the heparin standard (Table I). Indeed, this growth inhibitory cell surface heparan sulfate can function at the nanogram level in suppressing the proliferation of SMCs (Fig. 3). The specific antiproliferative activities of heparan sulfate derived from the cell pellet, and medium of exponentially growing and postconfluent SMCs were considerably lower with potencies closely approximating that of the heparin standard (Table I).

The results cited above were obtained with an assay that employs SMCs that have been growth arrested by exposure to low concentrations of platelet-poor plasma before admixture with heparan sulfate samples or heparin standards and 10% CS-DME. However, exponentially growing SMCs, cultured in 5% CS-DME, without previous growth arrest are also sensitive to the action of heparin and heparan sulfate. Data virtually identical to those outlined above were obtained when exponentially growing SMCs were employed in the growth inhibition assay. Indeed, the extent of growth suppression induced by heparin or heparan sulfate in the exponentially growing cell assay is approximately equal to 70% of that observed in the growth arrested cell assay (data not shown).

TABLE I

Chemical Mass and Antiproliferative Activities of Heparin Sulfate Isolated from Exponentially Growing and Postconfluent SMCs

Fraction growth stage	Antiproliferative activity	Heparin sulfate	Specific antiproliferative activity
	Inhibitory units/10^6 cells	*$\mu g/10^6$ cells*	
Cell surface (exponentially growing)	2.97	0.818	3.63
Cell surface (postconfluent)	22.35	0.499	44.82
Cell pellet (exponentially growing)	1.27	0.640	1.98
Cell pellet (postconfluent)	1.21	0.464	2.61
Culture medium (exponentially growing)	1.80	0.680	2.63
Culture medium (postconfluent)	0.63	0.236	2.65

The chemical mass of heparin sulfate obtained from the cell surface, cell pellet, and medium of exponentially growing as well as postconfluent SMCs was determined by glucosamine assay. The antiproliferative activity was calculated by comparing the amount of growth inhibition produced by each sample to that produced by known amounts of the heparin standard. Under these conditions, 1 μg of heparin per milliliter produced a 30–40% inhibition of growth relative to control cells which underwent four to five doublings during the experiment. Cell numbers in control wells typically reached 0.8–1.0 × 10^5 cells per centimeter squared. One inhibitory unit is equivalent to the amount of inhibition caused by 1 μg of heparin per milliliter. The specific antiproliferative activity of a given heparin sulfate is computed by dividing the growth inhibitory activity of the sample by its chemical mass. The specific antiproliferative activity of heparin is equal to 1. Additional experimental detail is provided in Materials and Methods.

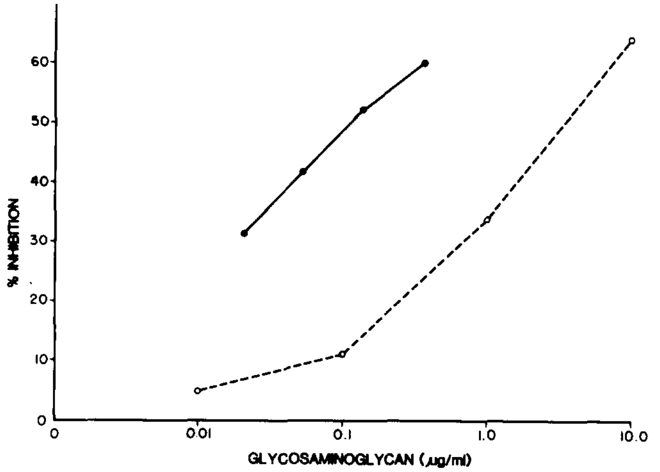

FIGURE 3 The dose response curves for heparin and heparan sulfate in the SMC growth inhibition assay. The growth inhibitory activities of the heparin standard and heparan sulfate isolated from the surface of postconfluent SMCs were determined as outlined in Materials and Methods. Each point in the heparin curve (- - -) represents the average of at least 10 determinations in experiments using the same heparin standard. Each point in the heparan sulfate curve (——) represents the average of at least four determinations in experiments using three different preparations of heparan sulfate isolated from the surface of postconfluent SMCs.

Characterization of Heparan Sulfate Isolated from the Surface of Postconfluent SMCs

Heparan sulfate obtained from the surface of postconfluent SMCs was digested with platelet heparitinase or *Flavobacterium* heparinase and the resultant species were assayed for their antiproliferative potency. Platelet heparitinase, an endoglycosidase, cleaves heparan sulfate at glucuronsylglucosamine linkages producing fragments with a wide range of molecular sizes (12). *Flavobacterium* heparinase, an exoglycosidase, scissions heparan sulfate at sulfated glucosaminyl-iduronic acid linkages generating disaccharides and tetrasaccharides (16). Neither enzyme displays any activity against other glycosaminoglycans or proteins.

Platelet heparitinase cleaved the cell surface heparan sulfate of 35,000 to 40,000 mol wt (Fig. 4*A*) into fragments of molecular weight that range from 35,000 to 1,300 (Fig. 4*B*). The undigested heparan sulfate and the resultant fragments were tested for antiproliferative activity in the growth inhibition assay. Undigested heparan sulfate was designated pool I (35,000 to 40,000 mol wt) (Fig. 4*A*); the heparan sulfate fragments were arbitrarily divided into pool II (35,000 to 13,000 mol wt), pool III (12,000 to 7,500 mol wt), pool IV (6,500 to 4,500 mol wt), and pool V (2,600 to 1,300 mol wt) (Fig. 4*B*). Undigested heparan sulfate and fragment pools II through IV exhibited approximately 25 inhibitory units per 10^5 cpm, whereas pool V possessed essentially no antiproliferative potency. *Flavobacterium* heparinase scissioned the cell surface glycosaminoglycan into tetrasaccharides and disaccharides of molecular weight 1,300 and 650, respectively (Fig. 4*C*). *Flavobacterium* heparinase-digested heparan sulfate was divided into pools VI (35,000 to 2,600 mol wt), pool VII (2,600 to 900 mol wt) and pool VIII (<900 mol wt) (Fig. 4*C*). The degraded heparan sulfate exhibited no inhibitory activity before column chromatography and pools VI and VII, as well as VIII, possessed no antiproliferative potency.

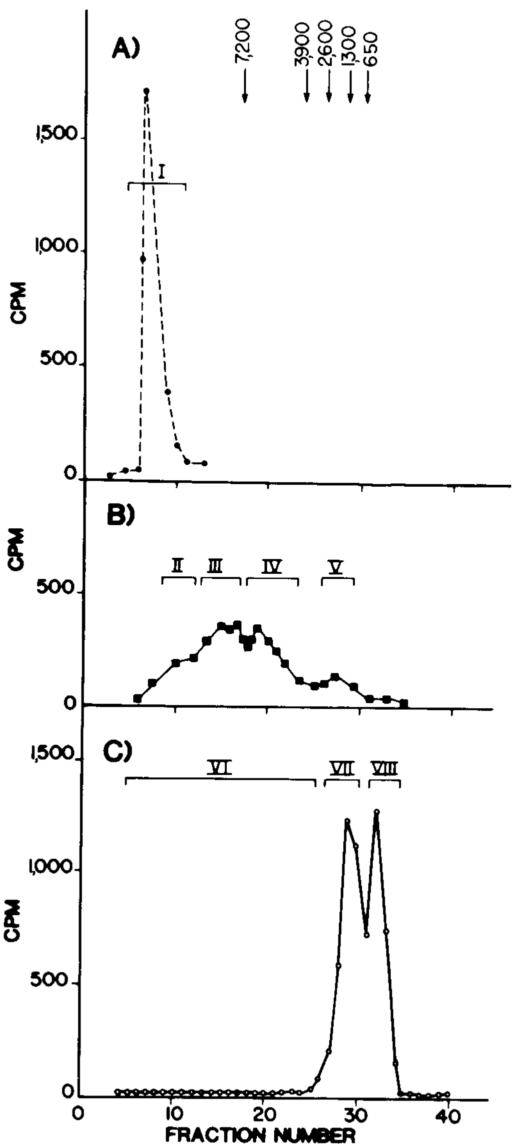

Figure 4 The sensitivity of heparan sulfate isolated from the surface of postconfluent SMCs to enzymatic cleavage. Heparan sulfate obtained from the surface of postconfluent SMCs was incubated for 24 h at 37°C with PBS buffer control (*A*), 50 ng of platelet heparitinase (*B*), or 30 U of *Flavobacterium* heparinase (*C*). After incubation, the samples were analyzed by HPLC/G2000 SW. Fraction pools, as shown by brackets, were concentrated by lyophilization, dissolved in PBS, and assayed to determine residual growth inhibitory activity. Molecular size markers indicate the elution positions of heparin fragment standards of 22, 12, 8, 4, and 2 saccharide residues. These components were prepared as previously described (10). See Materials and Methods for additional experimental detail.

An Evaluation of the Ability of Exponentially Growing SMCs to Degrade Heparan Sulfate with Growth Inhibitory Activity

The difference in the specific antiproliferative activities of heparan sulfate isolated from the surface of exponentially growing and postconfluent SMC might be due to the ability of exponentially growing cells to preferentially degrade or partially desulfate the mucopolysaccharide with antiproliferative potency. To test this hypothesis, we incubated ^{35}S-labeled heparan sulfate from the surface of postconfluent SMCs for 24 h at 37°C in 10% CS-DME with or without first passage exponentially growing SMCs at a density of 10^4 cells per cm^2. At the end of this time, the radiolabeled material within the culture medium was analyzed by HPLC/G2000 SW. As shown in Fig. 5, heparan sulfate incubated for 24 h in 10% CS-DME exhibited a minor reduction in molecular size which is probably caused by the action of small amounts of platelet heparitinase normally found within calf serum (9). However, no further change in peak profile was detected when heparan sulfate was incubated in the presence of exponentially growing SMCs. These results suggest that extensive scissioning of heparan sulfate by exponentially growing SMCs does not take place. Furthermore, no peak of radioactivity eluting in the included volume of the column was detected that would correspond to free ^{35}S. These findings strongly indicate that exponentially growing SMCs would not be able to reduce the biologic activity of heparan sulfate by desulfating the mucopolysaccharide.

The Source of the Small Amounts of Antiproliferative Heparan Sulfate Isolated from the Surface of Exponentially Growing SMCs

We have standardly obtained exponentially growing SMCs by exposing primary postconfluent SMCs to a 0.05% trypsin-0.02% EDTA solution for 5 min before subculturing. The subsequent harvesting of surface glycosaminoglycans is achieved by a 20-min treatment of the SMCs with the same type solution of trypsin-EDTA. Therefore, it is possible that the small amounts of heparan sulfate with antiproliferative activity isolated from the surface of exponentially growing SMCs represent residual mucopolysaccharide present on the initially seeded postconfluent SMCs, which was not removed

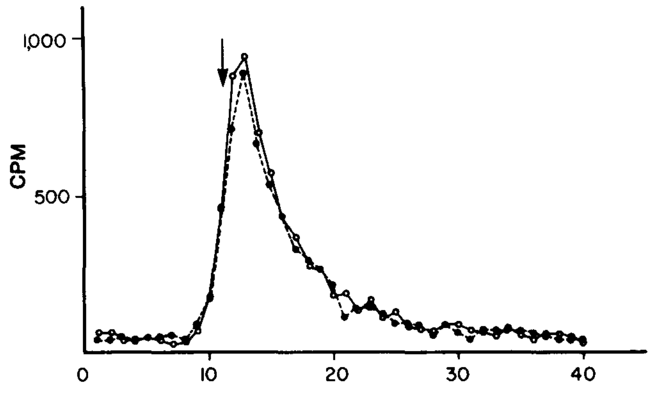

Figure 5 The ability of exponentially growing SMCs to cleave heparan sulfate isolated from the surface of postconfluent SMCs. First passage SMCs were plated in 10% CS-DME at 2.0 × 10^4 cells/ml in cluster-24 plates, 1 ml/well. Wells were also prepared without cells. After 24 h of incubation at 37°C in 5% CO$_2$, ^{35}S-labeled heparan sulfate isolated from the surface of postconfluent SMCs were added to wells with (- - -) or without (——) SMCs, and incubation was continued for an additional 24 h. At the end of the incubation period, the culture medium was removed, filtered, and 0.5 ml was analyzed by HPLC/G2000 SW. Controls consisted of an equal volume of culture medium from wells with or without SMCs to which ^{35}S-labeled heparan sulfate was added immediately before injection. The elution position of the control peak is indicated by an arrow. Additional experimental detail is provided in Materials and Methods.

by the 5-min trypsinization employed before plating. To test this hypothesis, we measured the antiproliferative activity of cell surface heparan sulfate per 10^6 cells from the time of plating (day 0) to the stage of postconfluence examined in this communication (day 12). Table II provides a comparison of the observed antiproliferative activity of cell surface heparan sulfate per 10^6 cells during different stages of growth, with theoretical values calculated by assuming that exponentially growing SMCs possess the initially measured amounts of growth inhibitory mucopolysaccharide found on primary postconfluent SMCs but do not synthesize these biologically active complex carbohydrates. In this case, the residual antiproliferative activity of heparan sulfate per 10^6 cells obtained from the surface of SMCs should be diluted as a linear function of the growth of the transferred primary postconfluent SMCs. The data in Table II suggest that most of the residual antiproliferative heparan sulfate present on the surface of exponentially growing and confluent SMCs can be accounted for by this mechanism. However, the level of growth inhibitory heparan sulfate found on the surface of postconfluent SMCs is 38 times what was expected. These results indicate that postconfluent SMCs have the ability to synthesize the antiproliferative heparan sulfate and place this component on their surface while the exponentially growing and confluent SMCs have little, if any, of this capacity. Indeed, a comparison of observed postconfluent and confluent antiproliferative activities suggests a difference of several hundredfold in this parameter. Preliminary data indicate that synthesis of the antiproliferative heparan sulfate begins 4 to 5 d after SMCs reach confluence (data not shown).

DISCUSSION

The SMCs found within the medial region of the arterial wall are normally present in a relatively quiescent growth state (17, 18). However, experimental desquamation of the overlying endothelium permits platelets to adhere to the denuded surface with subsequent release of mitogens such as platelet-derived growth factor (17–20). Macrophages may also be present within this region of the vasculature and may liberate such mitogens as macrophage-derived growth factor (21). These components can bind to specific receptors on the SMC membrane and induce these cellular elements to migrate to the luminal surface of the blood vessel as well as stimulate them to undergo an exuberant proliferative response (22–24). The resultant myointimal lesion bears a striking resemblance to the early atherosclerotic plaque (17, 18). It also appears likely that endothelial cells themselves may be able to synthesize and release mitogens that can induce migration and proliferation of SMCs (25). Hence abnormalities in the generation of these mitogens by endothelial cells as well as platelets and macrophages might also result in the development of the myointimal plaque described above.

TABLE II

Theoretical and Observed Activities of Heparin Sulfate Isolated from the Surface of SMC at Different Stages of Growth

Time in culture	No. of cells/ cm^2	Theoretical activity	Observed activity
Days	*× 10^4*	*Inhibitory units/10^6 cells*	*Inhibitory units/10^6 cells*
0	0.254		17.17
3 (exponentially growing)	2.71	1.61	2.97
5 (confluent)	6.34	0.69	0.14
12 (postconfluent)	7.53	0.58	22.35

Primary cultures of SMCs were labeled with ^{35}S and exposed to trypsin-EDTA solution for 5 min as described in Materials and Methods. This first trypsin-cell suspension was removed from the culture dishes, centrifuged, and the cells were replaced in the culture dishes. Fresh trypsin-EDTA solution was added and incubation was continued for an additional 15 min. The ^{35}S-labeled surface-associated glycosaminoglycans removed by this 5–20 min exposure to trypsin were purified by column chromatography to obtain heparin sulfate. Surface-associated heparin sulfate was also purified from SMC cultures at 3, 5, and 12 d after initiation of the cultures. The antiproliferative potencies of the various samples of heparin sulfate were determined with the SMC growth inhibition assay and the activities are expressed per 10^6 cells. The theoretical values for the antiproliferative activity of exponentially growing, confluent, and postconfluent cells are calculated as described in the text. See Materials and Methods for experimental details.

Several laboratories have provided evidence that natural components may exist within the vessel wall that could oppose the action of the above mitogens and hence prevent the development of smooth muscle proliferation. Clowes and Karnovsky (26) showed in a rat model that exogenously administered commercial heparin markedly suppressed myointimal proliferation following injury to the endothelium. These investigators initially believed that the antiproliferative action of heparin might be due to the ability of the mucopolysaccharide to accelerate antithrombin-dependent inhibition of thrombin. However, a collaborative study conducted by this group and our laboratory revealed that anticoagulant as well as non-anticoagulant heparin, prepared by affinity-fractionation on antithrombin-Sepharose, are equally effective in suppressing SMC proliferation under in vivo conditions (7). Hoover et al. (11) were then able to demonstrate with cultured rat SMCs that both forms of heparin could inhibit the growth of these cellular elements under in vitro conditions. These studies indicated that the structural determinants on the heparin molecule that are responsible for antiproliferative activity are distinct from those which are required for anticoagulant potency.

Given the above findings, we wondered whether endothelial cells might produce heparin-like molecules that could be released by specific enzymes and that might regulate the growth of SMCs. With this hypothesis in mind, Castellot et al. (8) were able to show that conditioned culture medium from bovine aortic endothelial cells inhibits the growth of aortic SMCs and that serum is required for the release of the antiproliferative activity. The molecular species responsible for growth inhibition was initially identified as a heparin-like component since its antiproliferative activity could be completely destroyed when incubated with purified *Flavobacterium* heparinase. Thereafter, Castellot et al. (9) and Oosta et al. (12) provided compelling evidence that the serum component that released the heparin-like species with antiproliferative activity from the surface of bovine endothelial cells was a platelet endoglycosidase that scissions occasional glucuronsyl-glucosamine bonds. This enzyme has also been found within the lysosomes of different cell types including SMCs (Fritze, L., and R. D. Rosenberg, unpublished observation).

However, we have recently isolated a heparan sulfate from the medial region of the bovine aortic wall and have shown that this mucopolysaccharide is able to inhibit the growth of SMCs (Reilly, C. and R. D. Rosenberg, manuscript in preparation). Since SMCs are the predominant cell type within this segment of the vascular tissue, we thought it likely that these cells might also synthesize heparin-like molecules that could regulate their own growth potential. We investigated this possibility by isolating heparan sulfate from the cell surface, cell pellet, and culture medium of exponentially growing and postconfluent SMCs and determining their antiproliferative potencies.

To accomplish this purpose, we incubated primary cultures of exponentially growing as well as postconfluent SMCs with $Na_2{}^{35}SO_4$ and obtained the radiolabeled glycosaminoglycans from the cell surface, cell pellet, as well as culture medium. These molecular species were freed of protein by extensive proteolytic digestion and the resultant mucopolysaccharide chains were isolated by DEAE-Sephadex and Sepharose 4B chromatography. The latter chromatographic techniques suggest that the average size and average charge density of either heparan sulfate or chondroitin sulfate are virtually identical, independent of cellular origin and growth stage. The bioassay of these components demonstrated that heparan sulfate from the cell surface, cell pellet, and culture medium of exponentially growing, as well as postconfluent SMCs, exhibits antiproliferative activity, whereas the similarly designated fractions of chondroitin sulfate possess no such biologic potency. Therefore, the anionic charge of the heparan sulfate cannot be the sole reason for the growth inhibitory potency of this component, since the DEAE-Sephadex chromatographic analyses clearly show that chondroitin sulfate has a higher average charge density than heparan sulfate. Indeed, the above findings indicate that certain specific structural elements of heparan sulfate, such as glycosidic bond configuration, sulfate position, or iduronic acid residues, are required for the observed antiproliferative effect (10). The data in Table I indicate that heparan sulfate isolated from the surface of postconfluent SMCs exhibits about eight times the antiproliferative potency of the corresponding material obtained from the surface of exponentially growing SMCs. Heparan sulfate isolated from the cell pellet or culture medium of SMCs in either growth state possesses only minimal amounts of the growth inhibitory activity.

To prove that heparan sulfate itself was responsible for the antiproliferative phenomenon observed above, we exposed the postconfluent SMC mucopolysaccharide to either purified *Flavobacterium* heparinase or platelet endoglycosidase, separated the resultant species by HPLC/G2000 SW, and assayed these components for growth inhibitory activity. On the one hand, our results indicated that degradation of the heparan sulfate to tetrasaccharides and disaccharides completely abolished antiproliferative potency. On the other hand, our data showed that cleavage of the heparan sulfate into larger fragments with molecular weights that ranged from 20,000 to 1,300 had little effect on the growth inhibitory activity per 10^5 ^{35}S counts until a molecular weight of about 4,000 (dodecasaccharide) was attained. Our demonstration that the antiproliferative potency of the heparan sulfate comigrates with the ^{35}S counts of large fragments of the mucopolysaccharide and that the growth inhibitory activity of the heparan sulfate can be eliminated by degradation of the mucopolysaccharide to tetrasaccharides and disaccharides strongly supports our contention that this glycosaminoglycan is responsible for the suppression of SMC proliferation.

The chemical masses of the various fractions of heparan sulfate were also determined by hexosamine analyses and the specific antiproliferative activity of these glycosaminoglycans were calculated. The data showed that exponentially growing SMCs synthesize about 1.5 to 3.0 times the amount of heparan sulfate found in the corresponding fractions of postconfluent SMCs. Thus, the large amounts of antiproliferative activity present on the surface of postconfluent SMCs are not simply due to the augmented production of this glycosaminoglycan. Indeed, the heparan sulfate isolated from the surface of postconfluent SMCs had a specific inhibitory activity which is 13 times that of the similarly designated mucopolysaccharide obtained from exponentially growing SMCs. This highly active heparan sulfate is able to inhibit dramatically SMC proliferation when added at a level as low as 20 ng/ml and hence its potency is more than 40 times greater than that of commercial heparin in suppressing the growth of these cells.

We thought it possible that the reduced specific antiproliferative activity of heparan sulfate obtained from the surface of exponentially growing SMCs might be due to the degradation of the biologically active component by these cellular elements. To examine this hypothesis, we incubated radiolabeled heparan sulfate from the surface of postconfluent SMCs with exponentially growing SMCs for 24 h and showed by HPLC/G2000 SW chromatography that minimal cleavage or desulfation had taken place.

Thus our data indicate that postconfluent SMCs are uniquely able to synthesize a heparan sulfate with remarkably potent antiproliferative activity and place these components on their cell surface. This highly active heparan sulfate is likely to differ structurally to only a very minor extent when compared to mucopolysaccharides isolated from exponentially growing cells since both types of glycosaminoglycans appear to have similar average molecular sizes and average charge densities.

Given that the surface of exponentially growing SMCs possess heparan sulfate with minimal growth inhibitory activity, we wondered whether the levels of this biologically potent mucopolysaccharide could represent residual highly active glycosaminoglycan generated by the primary postconfluent SMCs used to seed our cultures. To test this hypothesis, we harvested the surface glycosaminoglycans present on SMCs from the time of seeding to the period of postconfluence, isolated heparan sulfate by column chromatography, and ascertained the antiproliferative activity of the mucopolysaccharide per 10^6 cells. Our results indicate that exponentially growing SMCs retain small amounts of residual highly active heparan sulfate from the surface of primary postconfluent SMCs but can produce little of the biologically active component. Indeed, we would suggest that the postconfluent SMCs and SMCs at other stages of growth probably differ by as much as several hundred-fold in their ability to synthesize heparan sulfate with growth inhibitory potency and place these components on their surface. It is also of interest to note that the production of the biologically potent glycosaminoglycan appears to be abruptly induced at ~4 d after confluence is attained (data not shown). The molecular signals needed to accomplish this end are unknown but could be similar to those required to express specific receptors on cell surfaces immediately after the cessation of growth (27, 28).

At the present time, it is difficult to explain completely the relative absence of the heparan sulfate with growth inhibitory activity from the culture medium and cell pellet of the postconfluent SMCs. These findings may be due to the differential placement of this component on the surface of the cells with an associated reduction in the sensitivity of this glycosaminoglycan to platelet endoglycosidase in the serum. With respect to the intracellular levels of heparan sulfate with antiproliferative activity, our observations are consistent with the presence of a large pool of minimally active heparan sulfate within the postconfluent SMCs and/or an accelerated destruction of mucopolysaccharide with growth inhibitory potency within the SMCs. In this regard, other investigators have observed that secreted and cell surface heparan sulfate are handled as separate metabolic pools in other cell types (29).

On the basis of the above observations, we would propose a simple model for the possible role of heparin-like components and endoglycosidase in the regulation of SMC growth within the vessel wall. In the normal artery, endothelial cells, macrophages, and/or platelets serve as sources for mitogenic factors necessary for the growth of medial SMCs. However, SMCs also generate a specific type of heparan sulfate with antiproliferative activity that is positioned at the surface of the cell. The endoglycosidase that can liberate the growth inhibitory mucopolysaccharide is also available within the vessel wall. Under normal circumstances, the net effect of the mitogenic factors and the synthesis/release of the above heparan sulfate permits a small amount of SMC growth to compensate for the death of these cellular elements (<0.1% per day).

During damage to the endothelium, platelets and macrophages would appear at the site of injury and release high concentrations of growth factors. The SMCs might be able to respond to these pathologic alterations by augmenting the synthesis/release of the heparan sulfate with antiproliferative activity in a fashion identical to that noted when these cellular elements reach a postconfluent stage of cell growth. In this situation, the net balance between the elevated concentrations of mitogenic factors and the increased levels of free heparan sulfate with growth inhibitory activity would ultimately determine whether SMCs migrate to the luminal surface of the blood vessel wall and mount a proliferative response. Thus, this specific form of heparan sulfate would be positioned to act as a negative control element during the regulation of cell proliferation in a similar manner to that postulated for certain proteins isolated from the surface of 3T3 cells and endothelial cells (30, 31). It should be possible to test the above model once the structure of this unique heparan sulfate has been elucidated and the biosynthetic steps required to generate the mucopolysaccharide have been defined.

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