

PROTEOGLYCANS IN PRIMATE ARTERIES

II. Synthesis and Secretion of Glycosaminoglycans

by Arterial Smooth Muscle Cells In Culture

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ABSTRACT

Glycosaminoglycan synthesis and secretion by primate arterial smooth muscle have been examined in cell culture. Mass cultures of diploid primate arterial smooth muscle cells were either double labeled with [³⁵S]sulfate and [³H]acetate or single labeled with [³H]glucosamine for 24 h and glycosaminoglycans were extracted and isolated from the culture medium. Incorporation of labeled precursors into glycosaminoglycan was maximal during stationary phase of smooth muscle cell growth in culture and reduced, but not eliminated during logarithmic growth. The glycosaminoglycans synthesized and secreted into the culture medium were characterized by differential susceptibility to glycosaminoglycan-degradative enzymes and by cellulose acetate electrophoresis. Both assay procedures indicate that cultured primate arterial smooth muscle cells synthesize principally dermatan sulfate (60%–80% of total), chondroitin sulfate A and/or C (10%–20% of total) and little or no hyaluronic acid (0%–5% of total). This pattern of glycosaminoglycan formation differed significantly from that exhibited by isologous skin fibroblasts cultured under identical conditions. Dermal fibroblasts synthesize and secrete primarily hyaluronic acid (50%–60% of total) with lesser amounts of dermatan sulfate (10%–20% of total) and chondroitin sulfate A and/or C (10%–20% of total). These results indicate that differences exist in proteoglycan metabolism between these two connective tissue-producing cells in vitro, and suggest that the observed pattern of in vitro glycosaminoglycan synthesis by primate arterial smooth muscle cells may be characteristic for this cell type and not a general response to conditions of cell culture.

Although proteoglycans are known to be important constituents of the connective tissues of artery walls (3, 4, 12, 25, 26, 43, 63, 64), knowledge of their cellular source and metabolism is limited. In the accompanying study (63), we demonstrate that the smooth muscle of the intima and media of primate arteries is surrounded by numerous proteoglycan granules and suggest that the smooth muscle cell is responsible for the synthesis

and secretion of these connective tissue components. Other studies have demonstrated that arterial smooth muscle cells are capable of synthesizing and secreting collagen and elastic fibers in vivo (51) and in vitro (48, 49) confirming their connective tissue-synthetic capacity, but this cell's ability to form proteoglycans has not been definitively established.

In the present study, we have examined the

ability of the primate arterial smooth muscle cell to synthesize and secrete glycosaminoglycans¹ in vitro by addressing the following questions: (a) do primate arterial smooth muscle cells synthesize and secrete glycosaminoglycans in vitro? (b) if so, what types of glycosaminoglycan are formed, and in what relative amounts? (c) does the rate of synthesis of these macromolecules change during different stages of growth in culture of the arterial smooth muscle? and (d) is the in vitro pattern of glycosaminoglycan synthesis by primate arterial smooth muscle cells similar to or different from other connective tissue-producing mesenchymal cells in culture, such as skin fibroblasts?

MATERIALS AND METHODS

Preparation of Smooth Muscle and Fibroblast Cell Cultures

Arterial smooth muscle cell cultures were established by the method of Ross (48, 49). Strips of intimal medial tissue from the thoracic aorta of 1-2-yr old pigtail monkeys (*Macaca nemestrina*) were explanted in the Dulbecco-Vogt modification of Eagle's minimal medium supplemented with either 5% or 10% homologous serum. Cells were allowed to grow to confluency, at which time they were trypsinized and passed. Cells were replated at a density of 7.0×10^4 cells/ml in 250-ml Falcon flasks in 10 ml of medium supplemented with 5% pooled homologous serum and allowed to grow to confluency in an atmosphere of 5% CO₂ and 95% air at 37°C. Cultures in the second to sixth passages were used in all experiments. Previous electron microscope studies of cultures prepared in this manner at saturation density have revealed that all the cells have the ultrastructural characteristics of smooth muscle (49).

Arterial smooth muscle cell growth curves were established by maintaining parallel cultures in different concentrations of serum as previously described (49, 50). Equal numbers (9×10^4 cells) of trypsin-treated smooth muscle cells were plated, with a Cornwall syringe, into 60-mm Falcon plastic petri dishes (Falcon Plastics, Div. of B.-D. Laboratories, Los Angeles, Calif.) and maintained in medium containing 1% pooled homologous serum for 7 days (medium changed three times a week). On day 7, one-half of the cultures received medium containing 5% pooled homologous serum while the other half were transferred to a medium containing no serum.

¹ This study uses extraction procedures which remove and isolate the glycosaminoglycan portion of the proteoglycan for analysis. Although the protein moiety of the proteoglycan was not analyzed, it can be assumed that analysis of glycosaminoglycan synthesis reflects proteoglycan synthesis by these cells.

Sufficient numbers of dishes of cells were maintained to determine a growth curve for a period of at least 16 days. Cell numbers were determined by counting in a Fuchs-Rosenthal chamber. Mean and standard errors were calculated for each point in the growth curve.

Skin fibroblast cultures were established from abdominal skin from the same donor animal. Pieces of skin were minced into 2-mm squares and treated in the same manner as the smooth muscle explants. Subsequent to trypsinization, fibroblasts were grown in either 5% (M42) or 4% (M52) homologous serum.

Measurement of Glycosaminoglycan

Synthesis and Secretion

Confluent cultures were either single labeled with 20 μ Ci [³H]glucosamine per flask or double labeled with 20 μ Ci [³⁵S]sulfate, carrier free, and 20 μ Ci [³H]acetate per flask (New England Nuclear, Boston, Mass.) for 24 h. Eight flasks comprised each experiment and yielded between 12 and 30×10^6 cells. After labeling, the medium was removed and cell counts were determined as previously described. The labeled medium was extracted for glycosaminoglycans by the procedure of Nameroff and Holtzer (44). Briefly, this procedure consists of boiling the samples for 5 min, followed by pronase digestion (0.5 mg/ml in 0.2 M Tris-HCl buffer, pH 8.0) for 18 h at 60°C. Ice-cold trichloroacetic acid (TCA) was added to a final concentration of 10% and the precipitates were removed by centrifugation. The supernates were exhaustively dialyzed at 4°C, initially against 0.01 M solutions of the appropriately unlabeled compounds and finally against distilled H₂O. The isotopically labeled material was concentrated by contact desiccation (Aquacide, Calbiochem, San Diego, Calif.) and portions were taken for enzyme-subtraction digestion assays and for electrophoresis.

To eliminate the possibility of nonspecific trapping of the isotope(s) in the culture medium, as well as to check for possible cell-free synthesis (i.e. sulfation), parallel flasks containing culture medium with no cells were labeled and the medium was extracted for glycosaminoglycans as previously described.

Measurement of the Rate of Glycosaminoglycan Synthesis During Log and Stationary Phase of Growth

To determine whether glycosaminoglycan synthesis occurred in both dividing and nondividing cell populations, culture dishes were double labeled with [³⁵S]sulfate and [³H]acetate (20 μ Ci/dish) for 48 h on days 5, 7, 10, 12, and 17 of the growth curve. The culture medium from each of these time intervals was extracted for glycosaminoglycans as previously described and specific activities were determined (dpm/10⁴ cells).

Identification of Isolated Glycosaminoglycans

ENZYME DIGESTION-SUBTRACTION METHOD: To examine the types of glycosaminoglycan synthesized and secreted by arterial smooth muscle cells in culture, we employed an assay system based upon the differential susceptibility of different glycosaminoglycans to specific polysaccharidases according to the scheme of Toole and Gross (60). Equal portions (1.0 ml) of isotopically labeled TCA-soluble, nondialyzable material isolated from the medium were incubated with each of the following enzymes at 37°C: (a) 40 µg of leech hyaluronidase (Biotrics, Inc., Boston, Mass.) in McIlvaine's standard buffer (14), pH 5.4, each hour for 5 h; (b) 1,000 IU of testicular hyaluronidase (Miles Laboratories, Inc., Kankakee, Ill.) in sodium acetate buffer (52) pH 5.4, for 24 h followed by another 1,000 IU of fresh enzyme for 48 h; (c) 0.5 U of chondroitinase ABC (Miles) in enriched Tris buffer (52), pH 8.0, for 24 h followed by another 0.5 U of fresh enzyme for 48 h. Controls consisted of samples plus appropriate buffers without enzymes.

After incubation in all experiments, equal aliquots (1.0 ml) of the digestion products were separated on a column of Sephadex G-25, fine (2.5 × 90 cm), collected as 3.0-ml fractions, and assayed for radioactivity with Aquasol (New England Nuclear) in a Packard Tri-Carb scintillation counter. By determining the percentage of activity retarded on G-25 as a function of total activity applied to the column, it is possible to obtain an estimate of the types of glycosaminoglycans present in the sample (A. Dorfman, personal communication). Using [³H]glucosamine or [³H]acetate as precursors, the percentage of activity retarded in the leech hyaluronidase digests serves as an estimate of the amount of hyaluronic acid present since this enzyme specifically degrades hyaluronic acid (A. Balazs, personal communication). By using [³⁵S]sulfate as a precursor, the retarded fraction from the testicular hyaluronidase digest provides an estimate of the amount of chondroitin sulfate A and C in the sample (52). Subtraction of the amount of ³⁵S activity retarded in the testicular hyaluronidase digests from the ³⁵S activity retarded in the chondroitinase ABC digests provides an estimate of the amount of dermatan sulfate present, since both of these enzymes degrade chondroitin sulfate A and C but only chondroitinase ABC fully degrades dermatan sulfate (52). The activities of these enzymes were checked by incubating them with glycosaminoglycan standards (Seikagaku Fine Biochemicals, Tokyo, Japan) and by performing uronic acid determinations (10) on the digestion products collected as 3.0-ml fractions from Sephadex G25.

CELLULOSE ACETATE ELECTROPHORESIS: To confirm the results obtained by using the enzyme digestion subtraction method, additional aliquots of isotopically labeled nondialyzable, TCA-soluble material isolated from the culture medium were mixed with 50 µg of

standard hyaluronic acid, chondroitin sulfate C, and dermatan sulfate, and electrophoresed on cellulose acetate paper (Gelman Instrument Co., Ann Arbor, Mich.) in 0.1 M pyridine-formate buffer, pH 3.0, at 100 V for 60 min at room temperature (44). After drying, the strips were stained with 0.1% toluidine blue in 30% methanol to identify the position of the carriers. The strips were air dried, cut into 0.5-cm fractions, and assayed for radioactivity using Aquasol (New England Nuclear) in a Packard Tri-Carb liquid scintillation counter.

RESULTS

Proteoglycan Synthesis and Secretion by Primate Arterial Smooth Muscle Cells in Vitro

Mass cultures of primate arterial smooth muscle cells incorporated [³⁵S]sulfate, [³H]acetate, and [³H]glucosamine into TCA-soluble, nondialyzable material at saturation density (Table I). Since the majority of the activity was present in the medium (~85%) as compared to the cell layer (~15%), only the labeled medium was analyzed for newly synthesized glycosaminoglycans. Virtually all the activity present in the TCA-soluble material isolated from control flasks (medium incubated with isotopic precursors in the absence of cells) was dialyzable.

To obtain an estimate of the types of glycosaminoglycans present in these medium isolates, parallel aliquots were digested with glycosaminoglycan-specific degradative enzymes and the digestion products separated on Sephadex G-25 columns. Approximately 95% of the activity present in the [³H]acetate- and [³H]glucosamine-labeled isolates emerged in the void volume after leech hyaluronidase digestion, indicating minimal degradation (Fig. 1). 10–20% of the [³⁵S]sulfate activity and slightly less [³H]acetate or [³H]glucosamine activity emerged slightly behind the void volume in the testicular hyaluronidase digests, indicating partial degradation (Fig. 2). 80–90% of the [³⁵S]sulfate activity and 60–80% of the [³H]acetate or [³H]glucosamine activity in the medium isolates emerged as a distinct second peak behind the void volume after chondroitinase ABC digestion, indicating extensive degradation (Fig. 3).

By calculating the percentage of activity retarded on Sephadex G-25 columns in each digest as a function of total activity applied to the column and using the enzyme digestion subtraction method, we are able to make the following esti-

TABLE I
 Incorporation of [³⁵S]Sulfate, [³H]Acetate, and [³H]Glucosamine into TCA-Soluble, Nondialyzable Material Isolated from the Medium of Primate Arterial Smooth Muscle Cell Cultures

Experiment no.	[³⁵ S]Sulfate*		[³ H]Acetate*		[³ H]Glucosamine*	
	Medium	Cell Layer	Medium	Cell Layer	Medium	Cell Layer
M ₂₅ T ₂	63,700	2,100	—	—	—	—
M ₂₆ T ₃	45,145	2,369	—	—	—	—
M ₂₇ T ₃	46,938	2,367	—	—	—	—
M ₃₄ T ₃	45,016	7,290	44,925	5,705	—	—
M ₃₅ T ₃	14,574	1,807	37,198	9,101	—	—
M ₃₆ T ₂	28,197	1,458	239,572	30,851	—	—
M ₃₆ T ₅	25,558	1,473	130,833	13,456	—	—
M ₃₈ T ₃	—	—	—	—	48,071	4,015
M ₃₈ T ₆	—	—	—	—	239,335	17,553

* dpm/10⁶ cells.

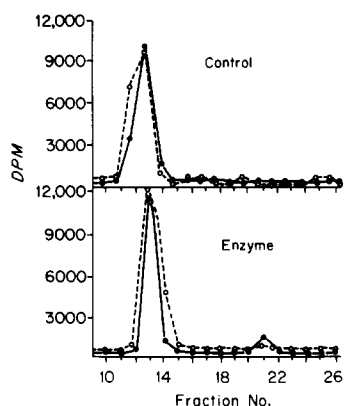


FIGURE 1 Elution pattern from Sephadex G-25 of the products from the digestion with leech hyaluronidase of [³H]glucosamine- (●—●) and [³H]acetate- (○-----○) labeled TCA-soluble, nondialyzable material isolated from the medium of primate arterial smooth muscle cells in vitro. Approximately 95% of the activity present in the ³H-labeled isolates emerged in the void volume, indicating minimal degradation and small amounts of hyaluronic acid present in the sample.

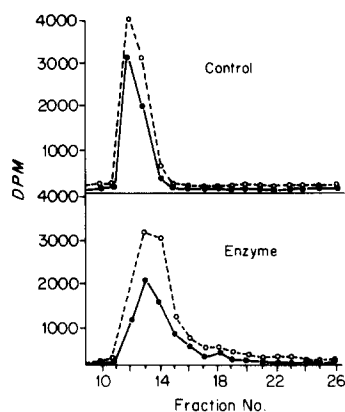


FIGURE 2 Elution pattern from Sephadex G-25 of the products from the digestion with testicular hyaluronidase of [³⁵S]sulfate- (●—●) and [³H]acetate- (○-----○) labeled TCA-soluble, nondialyzable material isolated from the medium of primate arterial smooth muscle cells in vitro. 10–20% of the [³⁵S]sulfate activity and slightly less [³H]acetate activity emerged slightly behind the void volume, indicating that there are moderate amounts of chondroitin sulfate A and/or C (10–20% of total) present in the sample.

mates of the types of glycosaminoglycans synthesized and secreted by primate arterial smooth muscle cells in vitro: hyaluronic acid (0–5%); chondroitin sulfate A and/or C (10–20%); dermatan sulfate (60–80%); other sulfated glycosaminoglycans (10% or less). These values represent mean ranges in four separate experiments.

To verify this assay for glycosaminoglycan identification, parallel portions of labeled TCA-soluble, nondialyzable material isolated from the smooth muscle cell culture medium were charac-

terized by cellulose acetate electrophoresis against known glycosaminoglycan standards. By spotting approximately 20 μg of each standard, it was possible to obtain clear separation of hyaluronic acid, dermatan sulfate, and chondroitin sulfates A and/or C (chondroitin sulfate A migrates together with chondroitin sulfate C and it is not possible to separate these isomers by using the conditions of electrophoresis in this study). The major peak of radioactivity in all electrophoresed portions corre-

sponded to the dermatan sulfate standard (60–70% of the total activity), with lesser peaks associated with the chondroitin sulfate C standard (10–40%) and several minor peaks which migrated behind dermatan sulfate (0–18%) (Fig. 4).

Although there is slight variation in the relative amounts of glycosaminoglycans synthesized and secreted by arterial smooth muscle cells in vitro as determined by these two assay systems, both assays were reproducible in several different experiments and indicate that the major glycosaminoglycan synthesized and secreted by these cells is dermatan sulfate (at least 60% of total). Furthermore, these results indicate that these cells make

little or no hyaluronic acid under the culture conditions used in these experiments.

Synthesis and Secretion of Glycosaminoglycans by Primate Arterial Smooth Muscle Cells During Log and Stationary Phases of Growth

To determine whether glycosaminoglycan synthesis could occur in both actively growing and quiescent cell populations, arterial smooth muscle

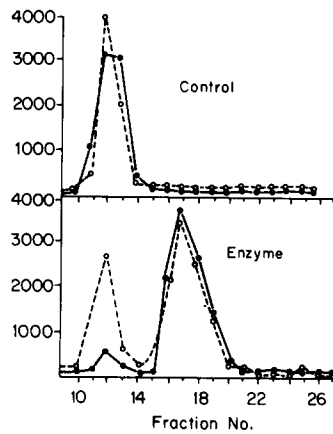


FIGURE 3 Elution pattern from Sephadex G-25 of the products from the digestion with chondroitinase ABC of [³⁵S]sulfate- (●—●) and [³H]acetate (○-----○) labeled TCA-soluble, nondialyzable material isolated from the medium of primate arterial smooth muscle cells in vitro. 80–90% of the [³⁵S]sulfate activity and 60–80% of the [³H]acetate activity emerged in the retarded fraction, indicating large amounts of dermatan sulfate present in the sample.

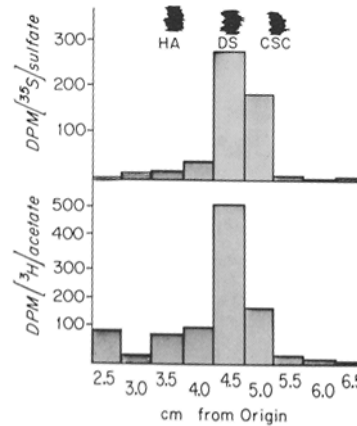


FIGURE 4 Cellulose acetate electrophoresis of [³⁵S]sulfate- and [³H]acetate-labeled TCA-soluble, nondialyzable material isolated from the medium of primate arterial smooth muscle cells in vitro. Electrophoresis of standard glycosaminoglycans (HA = hyaluronic acid; DS = dermatan sulfate; CSC = chondroitin sulfate C) is superimposed at the top of the figure. The major peaks of radioactivity in all electrophoresed aliquots corresponded to the dermatan sulfate standard with less activity associated with the chondroitin sulfate C standard. Minimal [³H]acetate activity coelectrophoresed with the hyaluronic acid standard.

TABLE II

Incorporation of [³H]Acetate and [³⁵S]Sulfate into TCA-Soluble, Nondialyzable Material by Cultured Primate Arterial Smooth Muscle Cells during Stationary and Log Growth

Labeling period	1% Serum		0% Serum		5% Serum	
	[³⁵ S]Sulfate*	[³ H]Acetate*	[³⁵ S]Sulfate*	[³ H]Acetate*	[³⁵ S]Sulfate*	[³ H]Acetate*
5–7	1,510	20,900	—	—	—	—
7–9	—	—	1,880	25,100	1,010	20,0660
10–12	—	—	1,930	19,990	660	7,540
12–14	—	—	2,230	26,790	700	10,580
17–19	—	—	1,850	17,530	430	3,820

* dpm/10⁴ cells.

cell growth curve cultures were double labeled with [³⁵S]sulfate and [³H]acetate during log and stationary phases of growth, and the specific activity (dpm/10⁴ cells) of the TCA-soluble, nondialyzable material in the medium isolates was determined. Data in Table II demonstrate that the synthesis and secretion is maximal during stationary phases of growth and decreases to approximately one-third the normal value by 3–4 days after the cells have been stimulated to divide by the addition of 5% serum (Fig. 5). This reduced rate of synthesis was evident throughout the culture period as long as the cells grew logarithmically.

The types of glycosaminoglycans synthesized during these different phases of growth were not determined because the radioactivity in the medium isolates was too low for analysis. Additional experiments using a greater number of cells are currently in progress to investigate the pattern of glycosaminoglycan synthesis by arterial smooth

muscle cells during their different growth phases in culture.

Pattern of Glycosaminoglycan Synthesis by Arterial Smooth Muscle Cells vs.

Skin Fibroblasts

To determine whether the observed pattern of glycosaminoglycan synthesis was characteristic of arterial smooth muscle cells or represented a general response displayed by other types of connective tissue cells in culture, we compared the pattern of glycosaminoglycan synthesis between arterial smooth muscle cells and dermal fibroblasts derived from the same donor under the identical culture conditions.

Using [³H]acetate and [³⁵S]sulfate as precursors, the TCA-soluble, nondialyzable material isolated from the medium of skin fibroblast cultures contained considerably more [³H]acetate activity but less [³⁵S]sulfate activity than similar material isolated from the smooth muscle cell culture medium (Table III). Approximately 60% of the [³H]acetate activity in the skin fibroblast culture medium was retarded on Sephadex G-25 after leech hyaluronidase digestion (Fig. 6). Testicular hyaluronidase caused approximately 72% of the [³H]acetate activity in the medium isolates to elute in the retarded fractions (Fig. 7), while 85% of the [³H]acetate activity was retarded after chondroitinase ABC digestion (Fig. 8). It should be noted that testicular hyaluronidase and chondroitinase ABC degrade hyaluronic acid in addition to their specific chondroitin sulfate isomers (52). Using the enzyme digestion subtraction method, we are able to make the following estimates as to the types of glycosaminoglycans synthesized and secreted by skin fibroblasts in vitro: hyaluronic acid (50–60%); chondroitin sulfate A and/or C (10–20%); dermatan sulfate (10–20%); other (10–20%). This pattern of in vitro glycosaminoglycan synthesis by skin

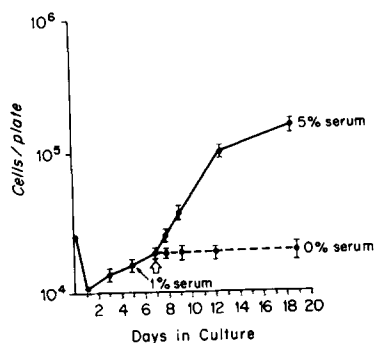


FIGURE 5 Growth curve of primate arterial smooth muscle cells in vitro. Cultures were initially maintained in a 1% homologous serum containing medium and then transferred on day 7 (arrow) to medium containing either no serum (-----) or 5% homologous serum (—). Cultures were double labeled with [³⁵S]sulfate and [³H]acetate for 48 h on days 5, 7, 10, 12, and 17 and the medium was extracted for glycosaminoglycans at each of these times (see Table II).

TABLE III

Comparison of the Incorporation of [³⁵S]Sulfate and [³H]Acetate into TCA-Soluble, Nondialyzable Material by Primate Arterial Smooth Muscle Cells and Skin Fibroblasts In Vitro

Experiment no.	Smooth muscle		Skin fibroblast	
	[³⁵ S]Sulfate*	[³ H]Acetate*	[³⁵ S]Sulfate*	[³ H]Acetate*
M ₄₂ T ₁	7,026	66,029	1,768	184,335
M ₄₂ T ₁	4,342	34,209	2,820	197,514
M ₄₂ T ₂	14,574	37,198	10,752	120,540

* dpm/10⁴ cells.

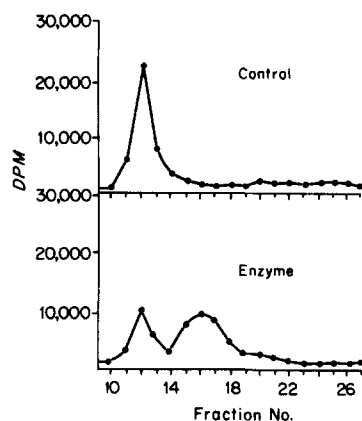


FIGURE 6 Elution pattern from Sephadex G-25 of the products from the digestion with leech hyaluronidase of [^3H]acetate-labeled, TCA-soluble, nondialyzable material isolated from the medium of primate skin fibroblast cultures. Approximately 60% of the [^3H]acetate activity was eluted in the retarded fractions, indicating that a significant amount of the sample contained hyaluronic acid.

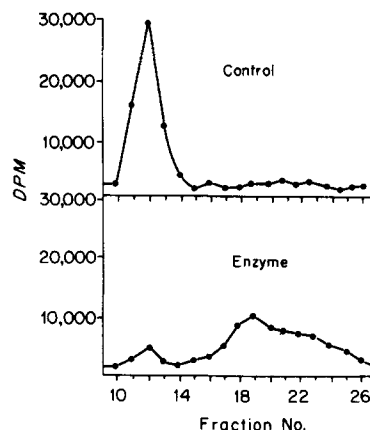


FIGURE 8 Elution pattern from Sephadex G-25 of the products from the digestion with chondroitinase ABC of [^3H]acetate-labeled, TCA-soluble, nondialyzable material isolated from the medium of skin fibroblast cultures. An additional 13% of the [^3H]acetate activity was retarded after this digestion, indicating approximately equal amounts of dermatan sulfate and chondroitin sulfate A and/or C present in the sample.

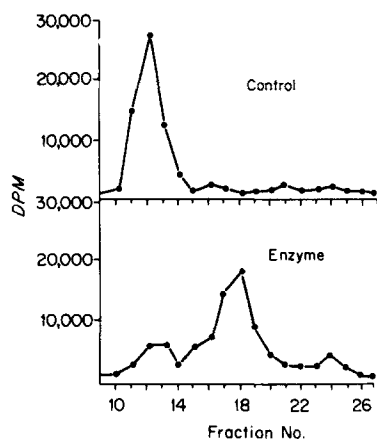


FIGURE 7 Elution pattern from Sephadex G-25 of the products from the digestion with testicular hyaluronidase of [^3H]acetate-labeled TCA-soluble, nondialyzable material isolated from the medium of skin fibroblast cultures. This digestion resulted in retarding 72% of the [^3H]acetate activity, indicating that approximately 12% of the total glycosaminoglycans present in the sample was chondroitin sulfate A and/or C.

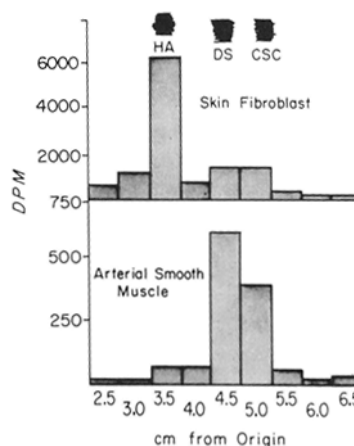


FIGURE 9 Comparison of the cellulose acetate electrophoretic pattern of [^3H]acetate-labeled, TCA-soluble, nondialyzable material isolated from the medium of primate arterial smooth muscle cells and skin fibroblasts in vitro. Electrophoresis of standard glycosaminoglycans (HA = hyaluronic acid; DA = dermatan sulfate; CSC = chondroitin sulfate C) is superimposed at the top of the figure. The patterns for the two cell types are clearly different and confirm the results of the enzyme digestion subtraction assay.

fibroblasts is distinctly different from that of arterial smooth muscle cells.

Cellulose acetate electrophoresis of medium isolated from skin fibroblast and arterial smooth muscle cell cultures confirmed this difference (Fig. 9). Approximately 60–70% of the [^3H]acetate label

was concentrated in the hyaluronic acid band in medium isolates derived from skin fibroblasts, while 0–4% of the label was present in this band in medium isolates from the smooth muscle cell

cultures. On the other hand, only 3–5% of the total [³H]acetate activity present in the medium isolates from skin fibroblast cultures comigrated with the dermatan sulfate band from medium isolates of arterial smooth muscle cultures. Medium isolates from the fibroblast cultures contained only 2–3% of the [³H]acetate activity associated with the chondroitin sulfate A/C standard, while smooth muscle medium isolates contained 10–20% of total [³H]acetate activity associated with this band.

DISCUSSION

Evidence for Glycosaminoglycan Synthesis and Secretion In Vitro

In the present study, we have demonstrated that cell cultures of primate arterial smooth muscle, prepared from 1–2-yr old pigtail monkey (*M. nemestrina*) thoracic aortas synthesize and secrete significant quantities of sulfated glycosaminoglycans into the culture medium. The finding that approximately 90% of the [³⁵S]sulfate-labeled TCA-soluble, nondialyzable material isolated from the medium of these cultures was degraded by chondroitinase ABC indicates that the majority of the [³⁵S]sulfate was incorporated into newly synthesized glycosaminoglycans. Approximately the same percentage of [³⁵S]sulfate activity was associated with standard sulfated glycosaminoglycans when the medium isolates were subjected to electrophoresis, confirming the results obtained after chondroitinase ABC digestion and indicating that sulfate is a relatively specific precursor for examination of sulfated glycosaminoglycan synthesis by these cells. Whether the remaining 10% of the ³⁵S label was present in chondroitinase ABC-resistant glycosaminoglycans (heparan sulfate and/or keratosulfate), or incorporated into other macromolecules such as sulfated glycoproteins (55), was not determined in this study.

The incorporation of [³H]acetate and [³H]glucosamine into TCA-soluble, nondialyzable material isolated from the medium of these cultures and the subsequent degradation of this labeled material by chondroitinase ABC further indicate that total glycosaminoglycan synthesis occurred, and not merely sulfation of pre-existing nonsulfated glycosaminoglycans (19, 30, 47, 58). The observation that 60–80% of the isolated [³H]acetate and [³H]glucosamine activity in the medium coelectrophoresed with sulfated glycosaminogly-

can standards provides further support for this conclusion.

Our data further demonstrate that cultures in logarithmic growth continue to synthesize glycosaminoglycans, although at a reduced rate. These observations suggest that both dividing and nondividing cells synthesize and secrete glycosaminoglycans and support previous studies of in vitro glycosaminoglycan synthesis by corneal fibroblasts (7) and chondrocytes (6, 28, 29, 46). However, we did not determine the percentage of cells actively dividing, hence it is possible that the lower rate of synthesis during log growth reflects synthesis by quiescent cells that are not synthesizing DNA. Further studies would be necessary to exclude this possibility.

Correlation of In Vitro Pattern of Glycosaminoglycan Synthesis with In Vivo Observations

The observation that cultured primate arterial smooth muscle cells form principally one type of sulfated glycosaminoglycan, dermatan sulfate, and lesser amounts of chondroitin sulfate A and/or C is of interest, because it has been reported that dermatan sulfate comprises only 10–20% of the total glycosaminoglycans present in artery walls, whereas chondroitin sulfate A and/or C are present in much greater amounts (3, 9, 12, 16, 21, 25, 26, 43, 53). There are several possible explanations for this apparent discrepancy. It is conceivable that the in vitro pattern of synthesis may be a response by the smooth muscle cells to the special conditions of cell culture, since a variety of culture conditions are known to exert different effects on glycosaminoglycan synthesis by other cell types (8, 32, 36, 57). Dermatan sulfate increases markedly in arteries that develop intimal lesions resulting from trauma (16) or after extensive lipid infiltration (27). These observations suggest that dermatan sulfate synthesis may be a response by arterial smooth muscle cells to some form of mechanical or chemical stress. On the other hand, since experimentally induced arterial intimal hyperplasia is characterized by extensive focal smooth muscle proliferation, it is also possible that the culture conditions in which cells are rapidly proliferating are analogous to in vivo intimal hyperplasia. Thus, the pattern of in vitro glycosaminoglycan synthesis may be a characteristic of proliferating cells.

It is also possible that this pattern of glycosaminoglycan synthesis reflects the particular state of development of the arterial smooth muscle cells. For example, it is well documented that in some developmental systems, such as cornea (37, 62), cartilage (24, 35, 41, 44, 54, 59), and heart (13, 31), the pattern of *in vitro* and *in vivo* glycosaminoglycan synthesis changes with age and degree of development. In the primate aorta, the amount of dermatan sulfate has been shown to increase with age with a concomitant decrease in chondroitin sulfate A and/or C (25, 53). It is noteworthy that our arterial smooth muscle cell cultures were established from 1–2-yr old monkeys and that the cells had been passed an average of three to four times. Thus, it is possible that dermatan sulfate synthesis by these cells may reflect an age-dependent (number of cell doublings) biosynthetic program of these macromolecules (18). It would be of interest to compare the pattern of glycosaminoglycan synthesis by cultures established from embryonic aortas with that observed by cells from older donors or cells that had gone through a greater number of generations in culture.

Toole and Lowther (61) have demonstrated that dermatan sulfate proteoglycan isolated from skin and heart valves exhibits the ability to form precipitates when mixed with tropocollagen at physiological pH and ionic strengths. These observations have led to the suggestion that one possible function of dermatan sulfate is to participate in the formation and orientation of collagen fibrils. Primate arterial smooth muscle cells do synthesize and secrete collagen *in vitro* (J. Burke, unpublished observations) and it may be that the synthesis of dermatan sulfate by these cells is closely tied to collagen fibrillogenesis in these cultures. Collagen as well as chondromucoprotein are known to influence the synthesis of glycosaminoglycans by other cell types (20, 38, 39, 45).

It is also possible that other types of cells present in the artery wall synthesize one class of glycosaminoglycan while the smooth muscle cells synthesize another. Recent experiments have demonstrated that cultured rabbit aortic endothelial cells form significant quantities of chondroitin sulfate A and considerably less dermatan sulfate (5). It remains to be determined whether primate endothelial cells exhibit a similar pattern of glycosaminoglycan synthesis.

To our knowledge, the only other cell type known to synthesize such large quantities of

dermatan sulfate *in vitro* is the skin fibroblast derived from patients with the Hurler's or Hunter's syndrome (33, 34). The large amount of dermatan sulfate associated with these fibroblasts has been shown to be due to the lack of turnover of this macromolecule by these cells since they do not produce the enzyme necessary to degrade dermatan sulfate (L-iduronidase) (1). There are no data on the ability of arterial smooth muscle cells to synthesize glycosaminoglycan-degradative enzymes *in vitro*. In contrast, Hermein et al. (17) recently demonstrated that dermatan sulfate exhibited the most rapid turnover of all of the glycosaminoglycans tested in the rat aorta, a species notably resistant to atherosclerosis. Thus, even though arterial smooth muscle cells synthesize large quantities of dermatan sulfate, this macromolecule may be degraded at a rapid rate in non-injured arteries, preventing its accumulation. However, in species that are susceptible to lesion formation, dermatan sulfate may turn over more slowly, explaining its accumulation in lesions such as the human atherosclerotic fatty streaks (27). There are at present no data on the *in vivo* content of glycosaminoglycans in the nonhuman primate artery (*M. nemestrina*). Further studies are needed to determine whether differences in turnover of glycosaminoglycans are responsible for the differential accumulation of these macromolecules in the artery wall.

The function of dermatan sulfate in the artery wall needs to be clarified. Berenson (2) demonstrated that dermatan sulfate isolated from bovine aorta exhibits antithrombotic activity, as has been demonstrated for dermatan sulfate from other sources (15). Thus, one possible function of this macromolecule in the artery wall may be in the prevention of thrombosis (11).

The importance of dermatan sulfate in the artery wall has recently been emphasized by Iverius (22, 23) who has demonstrated that dermatan sulfate exhibits the greatest affinity for binding with low density lipoproteins at physiological pH and ionic strength of all of the glycosaminoglycans tested. His observations suggest a role for dermatan sulfate in acting as a trap or sink for lipid deposits that form in the lesions of atherosclerosis.

Arterial Smooth Muscle vs. Dermal Fibroblasts

Whatever the reason for dermatan sulfate synthesis by primate arterial smooth muscle cells in

vitro, this pattern of synthesis is clearly different from that exhibited by primate skin fibroblasts cultured under identical conditions. The large amount of hyaluronic acid produced by dermal fibroblasts in this study is consistent with other studies which have shown hyaluronic acid to be the major glycosaminoglycan synthesized by cultures of various types of fibroblasts, including dermal fibroblasts (see, for reviews, references 40 and 42). The large amount of hyaluronic acid and the small amount of dermatan sulfate produced by dermal fibroblasts contrast sharply with the arterial smooth muscle cells which demonstrate an opposite pattern, namely the formation of large quantities of dermatan sulfate and relatively little hyaluronic acid. This difference, coupled with other morphological differences in their pattern of in vitro growth and in their fine structure, provides an additional means to discriminate between these two cell types in culture.

The authors wish to express appreciation to Dr. Mark Nameroff, Department of Biological Structure, University of Washington School of Medicine, for his advice and help in learning to do the glycosaminoglycan analysis. The authors would also like to thank Ms. Beverly Kariya, Ms. Lynn Phillips, and Mrs. Shirley Robie for their excellent technical assistance.

This work was supported in part by a grant from the United States Public Health Service no. HL-14823 and the New Hampshire State Heart Association. Dr. Wight was the recipient of a United States Public Health Postdoctoral Fellowship no. HL-53109.

Received for publication 28 April 1975, and in revised form 31 July 1975.

Note Added in Proof: Since the submission of this paper, Radhakrishnamurthy et al. (1975. Composition of connective tissue in aortas from rhesus monkeys during regression of diet-induced fatty streaks. *Lab. Invest.* 33: 136.) have demonstrated that dermatan sulfate is the major aortic glycosaminoglycan present in the nonhuman primate rhesus monkey unlike other species where chondroitin sulfate A and/or C is the predominant aortic glycosaminoglycan. These findings indicate that the large amount of dermatan sulfate synthesized and secreted by cultured nonhuman primate arterial smooth muscle cells observed in the present study may reflect the pattern of glycosaminoglycan synthesis that occurs in intact arteries of nonhuman primates.

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