Proteoglycans in Primate Arteries. III. Characterization of the Proteoglycans Synthesized by Arterial Smooth Muscle Cells in Culture

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ABSTRACT Near confluent monolayers of arterial smooth muscle cells derived from *Macaca nemestrina* were labeled with $Na_2[^{35}S]O_4$ and the newly synthesized proteoglycans present in the culture medium and cell layer were extracted with either 4 M guanidine HCl (dissociative solvent) or 0.5 M guanidine HCl (associative solvent) in the presence of protease inhibitors. The proteoglycans in both compartments were further purified by cesium chloride density gradient ultracentrifugation. Two size classes of proteoglycans were observed in the medium as determined by chromatography on Sepharose CL-2B. The large population (K_{av} = 0.31) contained predominantly chondroitin sulfate chains with M_r = ~40,000. The smaller population (K_{av} = 0.61) contained dermatan sulfate chains of similar M_r (~40,000). When tested for their ability to aggregate, only proteoglycans in the large-sized population were able to aggregate. A chondroitin sulfate containing proteoglycan with identical properties was isolated from the cell layer. In addition, the cell layer contained a dermatan sulfate component which eluted later on Sepharose CL-2B (K_{av} = 0.78) than the dermatan sulfate proteoglycan present in the medium. Electron microscopy of the purified proteoglycans revealed a bottlebrush structure containing a central core averaging 140 nm in length with an average of 8 to 10 side projections. The length of the side projections varied but averaged between 70 and 75 nm. Similar bottlebrush structures were observed in the intercellular matrix of the smooth muscle cell cultures after staining with Safranin O. This culture system provides a model to investigate parameters involved in the regulation of synthesis and degradation of arterial proteoglycans.

Proteoglycans and their constituent glycosaminoglycans are present in blood vessel walls (1–14) and form important structural links between the fibrous components of the intercellular matrix (collagen and elastic fibers) and the arterial endothelial and smooth muscle cells (15). Recently, considerable attention has focused on these macromolecules since they have been shown to influence the structural integrity of the vascular wall and to affect several arterial properties such as viscoelasticity, permeability, lipid metabolism, hemostasis, and thrombosis (15, 16). Furthermore, their participation in such cell processes as proliferation (17), migration (18), and adhesion (19) highlight their importance in many events that are common to both blood vessel development and disease.

Although several studies have investigated changes in the glycosaminoglycan component of arterial proteoglycans during development and disease (10, 11, 15, 20), only recently have attempts been made to define the physical-chemical nature of entire proteoglycan molecules isolated from arteries. This information is important since it is thought that the structure of the whole molecule, and not merely the component parts, dictates the functional properties of the proteoglycans. Employing methods used to isolate proteoglycans from cartilage, Oegema et al. (5) found two size classes of proteoglycans in bovine aorta: a polydisperse population of chondroitin sulfate and dermatan sulfate and a smaller population containing heparan sulfate. Gardell et al. (8) demonstrated that some of the aortic proteoglycans shared antigenic determinants with those from cartilage and were able to form high molecular weight aggregates. The ability of arterial proteoglycans to aggregate was later confirmed by McMurtrey (7) who isolated

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proteoglycan-hyaluronate complexes from bovine aorta. Most recently, Salisbury and Wagner (6) isolated two major size classes of proteoglycans from human aorta and found that the larger population contained predominantly chondroitin sulfate while the smaller population contained dermatan sulfate. Heparan sulfate proteoglycan appeared to be distributed between these two populations when separated by molecular sieve chromatography. These findings indicate that there are at least three distinct species of proteoglycans present in the arterial wall.

Cell culture techniques have allowed a closer examination of the cell types responsible for the synthesis of the arterial proteoglycans. To date, most studies have concentrated on defining the types of glycosaminoglycans that both endothelial and smooth muscle cells synthesize and secrete (21–28). Endothelial cells synthesize less total glycosaminoglycans but markedly more heparan sulfate than smooth muscle cells (27). On the other hand, smooth muscle cells synthesize significant amounts of chondroitin sulfate and dermatan sulfate (21–25) and, like the situation in vivo, the relative amounts of each of these isomers produced by these cells depend on the donor species as well as the portion of the vasculature that was used to isolate the cells. Although these studies in vitro have identified the cell types responsible for synthesizing particular types of arterial glycosaminoglycans, little is known about the classes of proteoglycans to which these glycosaminoglycans belong. Furthermore, it is not known how proteoglycans synthesized by arterial cells in vitro compare to those proteoglycans present in intact arteries.

The objective of the present study was to investigate biochemical and morphological characteristics of the proteoglycans synthesized by nonhuman primate arterial smooth muscle cells in culture to develop a model system for studying parameters involved in the regulation of synthesis and degradation of arterial proteoglycans in vivo. The results demonstrate that arterial smooth muscle cells derived from subhuman primates synthesize at least two distinctly different proteoglycans which are very similar to those found in human aorta and in part resemble proteoglycans present in some cartilagenous tissues.

MATERIALS AND METHODS

Materials: Guanidine HCl, cesium chloride, and papain were all purchased from Sigma Chemical Co., St. Louis, MO; 6-aminohexanoic acid and benzamidine HCl from Eastman Kodak Co., Rochester, NY; chondroitinase ABC (*Proteus vulgaris*) and chondroitinase AC II (*Arthrobacter aurescens*) from Seikagaku, Kogyo, Tokyo, Japan, through Miles Laboratories, Inc., Elkhart, IN; Sephadex and Sepharose from Pharmacia, Inc., Piscataway, NJ; $Na_2[^{35}S]O_4$ (~10 mCi/ml: 106 mCi/mmol), D-[6-^{3}H]-glucosamine (~1.0 mCi/ml: 31 Ci/mmol) and Aquasol from New England Nuclear, Boston, MA; Dulbecco Vogt medium, Gibco Laboratories, Grand Island Biological Co., Grand Island, NY; flasks and petri dishes, Falcon Plastics, Oxnard, CA. The monomer, D1, and aggregate, aA1[1], were prepared from the Swarm rat chondrosarcoma (29).[2]

Cell Culture: Arterial smooth muscle cells were established by the method of Ross (30). Strips of intimal-medial tissue from the thoracic aorta of 1–2-year-old pigtail monkeys (*Macaca nemestrina*) were explanted in Dulbecco-Vogt modification of Eagle's minimal medium supplemented with 5% homologous serum. Cells were allowed to grow to confluency, at which time they were trypsinized and passed. The passed cells were then plated in either 60-mm petri dishes in 4.0 ml of medium or in 75-cm^3 T-flasks in 10 ml of medium and allowed to grow to confluency in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Radioisotope Labeling of Cultures and Extraction of Proteoglycans: Near confluent cultures were labeled by the addition of fresh medium containing $Na_2[^{35}S]O_4$ (50 μCi/ml) for 48 h unless otherwise specified. Some experiments involved double labeling with $Na_2[^{35}S]O_4$ (50 μCi/ml) and [^{3}H] D-glucosamine (2.5 μCi/ml). After labeling, the incubation medium was removed and either 0.8 g/ml or 0.05 g/ml of solid guanidine HCl was added to make the medium ~4 M or ~0.5 M, respectively (31). The resulting solutions were immediately frozen and stored at −70°C until subsequent analysis. The remaining cell layers were washed twice with phosphate-buffered saline at 0°C for 1–2 min. The washes did not contain significant amounts of macromolecular radiolabeled material and were discarded. The cell layers were then extracted with either 1 ml (60-mm dishes) or 5 ml (75-cm^2 flask) of 4 M guanidine HCl (dissociative solvent) or 0.5 M guanidine HCl (associative solvent) containing 0.1 M 6-aminohexanoic acid, 5 mM benzamidine HCl, 50 mM sodium EDTA, and 50 mM sodium acetate, pH 5. 8. The cell layers were disrupted by scraping with a rubber policeman, extracted in the cold with constant stirring for 1–2 h, centrifuged briefly at 12,000 rpm for 2 min to remove insoluble residue, and stored at −70°C until analyzed.

Relative Hydrodynamic Sizes of the Proteoglycan Monomers: Aliquots (200 μl) of the 4 M guanidine HCl extracts of the medium and cell layer were mixed with 30 μl of monomer proteoglycan carrier (10 mg/ml in 4 M guanidine HCl) and the mixture was applied directly to an analytical Sepharose CL-2 B gel filtration column (110 × 0.7 cm) eluted with 4 M guanidine HCl in 0.05 M sodium acetate buffer, pH 5.8 at room temperature. Fractions (0.6 ml) were collected under 40–50 cm hydrostatic pressure at a flow rate of ~2.0 ml/h. Each fraction was mixed with 1 ml of 70% ethanol and 10 ml of Aquasol and analyzed for radioactivity in a Packard Tri Carb Scintillation Counter.

Equilibrium Density Gradient Centrifugation

DISSOCIATIVE DENSITY GRADIENTS: Aliquots, 1 ml of the medium and cell layer extracts, were mixed with 100 μl of monomer proteoglycan carrier (10 mg/ml in 4 M guanidine HCl) and chromatographed on PD-10 columns in the presence of 4 M guanidine HCl to remove unincorporated isotope (32). Excluded volume fractions were pooled and 0.5 g of cesium chloride was added per gram of solution to yield a starting density of 1.45 g/ml. The gradients were centrifuged for 60 h at 10°C at 37,000 rpm in a Beckman SW 50.1 Rotor (33) (Beckman Instuments, Inc., Fullerton, CA). Gradients were divided into four approximately equal fractions, D1 through D4[1] (29), by means of a tube slicer or gradient separator (Buchler Int., Chicago, IL). The specific gravity of each fraction was determined by weighing 50-μl aliquots on a Mettler microbalance. The amounts of macromolecular [^{35}S]activity were determined by liquid scintillation counting. Fractions were dialyzed extensively against distilled water, and portions were lyophilized. An aliquot of each fraction was chromatographed on a Sepharose CL-2B analytical column eluted with 4 M guanidine HCl in 0.05 M sodium acetate buffer, pH 5. 8.

ASSOCIATIVE DENSITY GRADIENT: 0.5 M guanidine HCl extracts of the medium and cell layer were prepared for associative gradients directly (i.e. no carrier added and no PD-10 column used to remove unincorporated isotope) by adding 0.9 g of cesium chloride per gram of solution to yield a starting density of 1.53 g/ml. Centrifugation was done as described above. The gradients were divided into four equal fractions, aA1 through aA4[1] (29). The aA1 fraction was dialyzed exhaustively against distilled water to remove unincorporated isotope and used for the electron microscopy and the aggregation experiments described below.

Characterization of the Glycosaminoglycans: The major proteoglycan populations in the medium and cell layer extract were isolated from the bottom half of the dissociative density gradient (D1 and D2) by preparative Sepharose CL-2B chromatography (0.9 × 60 cm) eluted with 4 M guanidine HCl in 0.05 M sodium acetate buffer, pH 5.8. Fractions were pooled to recover the major peaks (see Fig. 4), dialyzed, and lyophilized. Glycosaminoglycans in each proteoglycan population were released by papain digestion (30 μg/ml in 0.1 M sodium acetate, pH 7.0 for 4 h at 65°C) (34) or by alkaline degradation (35). Aliquots of the released glycosaminoglycans were chromatographed on a Sepharose CL-6B column (0.7 × 30 cm), eluted with 0.2 M NaCl, 20 mm Tris HCl, pH 7.5. Fractions of 0.22 ml were collected and 0.10 ml of each fraction was taken for measurement of radioactivity. The excluded (V_o) and total (V_t) volumes of the column were determined by using blue dextran and free [^{35}S]sulfate, respectively. The average molecular weight, M_r, of the released glycosaminoglycans was estimated by comparing the elution position (partition coefficient = K_{av}) of the sample fractions to known chondroitin sulfate standards as described by Wasteson (36). The remainder of each fraction in the glycosaminoglycan peak for each sample was pooled and the glycosaminoglycans present were precipitated by adding 3 vol of 95% ethanol:1.3% (wt/vol) potassium acetate. Precipitates were cooled to 0°C and isolated by centrifugation at 10,000 rpm. The precipitates were

[1] Nomenclature: D1, D2, D3, and D4 indicate fractions recovered from the bottom to top of dissociative density gradients and aA1, aA2, aA3 and aA4 fractions from bottom to top of associative density gradients prepared directly from associative extracts as described in reference 29.

[2] The tumor was originally kindly provided by Dr. Theodore Oegema, University of Minnesota, Minneapolis, MN; and was subsequently maintained by transplantation (29).

dissolved in distilled water and then digested either with 0.03 ml of chondroitinase ABC (10 U/ml in 0.3 M Tris) or with 0.03 ml of chondroitinase AC II (10 U/ml in 0.3 M Tris). The relative amounts of the isomeric sulfated disaccharide digestion products were determined by paper chromatography (37).

Proteoglycan Aggregation: Aliquots (250 μl) of the aA1 fractions prepared directly from both the medium and cell layer without carrier as described above were mixed with 30 μl of carrier proteoglycan aggregate, aA1 (29) (10 mg/ml in 0.5 M sodium acetate, 2.5 mM EDTA, pH 7.0). The mixture was eluted on an analytical Sepharose CL-2B column (0.7 × 110 cm) with the same buffer that contained the sample. The amount of [^{35}S]activity eluting in the void volume of the column provides an estimate of the amount of proteoglycan present as aggregate in the samples (38).

The ability of proteoglycans in D1 fractions to aggregate was also tested. Aliquots of the D1 fractions in 4 M guanidine HCl (300 μl) were mixed with 100 μl of carrier proteoglycan aggregate, aA1 from rat chondrosarcoma (10 mg/ml in 4 M guanidine HCl). The mixture was dialyzed overnight against 0.5 M sodium acetate, 2.5 mM EDTA, pH 7.0 and applied to an analytical associative Sepharose CL-2B column eluted with the same buffer. The percentage of radioactivity eluting in the void volume of the column provides an estimate of labeled proteoglycan monomer that can interact with hyaluronic acid to form aggregates.

Electron Microscopy of Isolated Proteoglycans: Aliquots of the aA1 fractions prepared directly without added carrier as described above were spread on electron microscopic grids by the DNA spreading technique as modified by Rosenberg et al. (39) and, more recently, by Kimura et al. (40) and Hascall (41). For each sample, ~75 μl of a 1:5 dilution of aA1 in 0.3 M ammonium acetate was mixed with 25 μl of cytochrome *c* solution (1 mg/ml in 1 mM Tris, 1 mM EDTA, pH 8.5) and left at room temperature for 1 h. A 25-μl aliquot was then layered on a 0.3 M ammonium acetate hypophase, pH 5.0, via a wet glass ramp. The thin film produced was transferred to Formvar-coated grids and stained for 30 s in diluted uranyl acetate in ethanol. The grids were rotary shadowed with carbon platinum-palladium at low angles (6–10°) and observed in a JEOL 100-B electron microscope at 60 kV and a magnification of 70,000 times. The monomer lengths and widths were obtained by tracing contours of well-spread monomers on photographs printed at 70,000 times with a Hewlett-Packard digitizer (Model 48309 A) coupled to a computer program capable of providing size frequency and distribution and basic statistics.

Electron Microscopy of Proteoglycans within the Matrix of Arterial Smooth Muscle Cell Cultures: Representative monolayers were fixed in 2% glutaraldehyde in 0.1 M Sorenson's sodium phosphate, pH 7.4, containing 0.1% Safranin O (42) for 1 h at room temperature. Subsequently, the cultures were rinsed thoroughly in phosphate buffer containing 0.2 M sucrose and 0.05% Safranin O before postfixation in 2% osmium tetroxide in 0.1 M sodium phosphate plus 0.025% Safranin O. After a rinse in phosphate buffer, the monolayers were dehydrated and embedded in epoxy resin as previously described (25). Thin sections were cut with a diamond knife on an LKB Ultratome III, double-stained with uranyl acetate and lead citrate, and examined in a JEOL 100-B electron microscope at 60 kV.

The specificity of this staining procedure for identifying proteoglycans was confirmed by incubating representative monolayers with chondroitinase ABC (0.5 U/ml) in 0.2 M enriched Tris buffer for 1 h at 37°C (25). Controls were incubated with buffer without enzyme. After enzyme treatment, the monolayers were rinsed and processed as described above.

RESULTS

The amount of [^{35}S]-labeled macromolecules released into the medium by arterial smooth muscle cells and present in 4 M guanidine HCl extracts of the cell layer at various times after beginning continuous labeling are shown in Fig. 1. The release of the [^{35}S]-labeled macromolecules into the medium was linear for at least 48 h, while the amount of incorporated radioactivity present in the 4 M guanidine HCl extracts of the cell layer first increased linearly for ~16 h and then reached a plateau, staying fairly constant up to 48 h. After 48 h, uptake of $Na_2[^{35}S]O_4$, ~70–80% of the [^{35}S]-labeled macromolecules were present in the medium.

Samples of the medium and cell layer from cultures labeled for 48 h were chromatographed on Sepharose CL-2B with 4 M guanidine HCl as the elutant. Two major peaks of [^{35}S]activity were observed for both the medium and cell layer (Fig. 2). The first peak, with the largest proteoglycan species (K_{av} = 0.31), eluted similarly for both the medium and cell layer samples. The second labeled peak eluted later for the sample from the

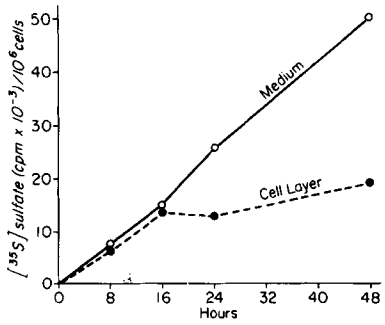

FIGURE 1 Accumulation of ^{35}S-labeled macromolecules in the culture medium (open circles) and cell layer (solid circles) as the cells approach confluence.

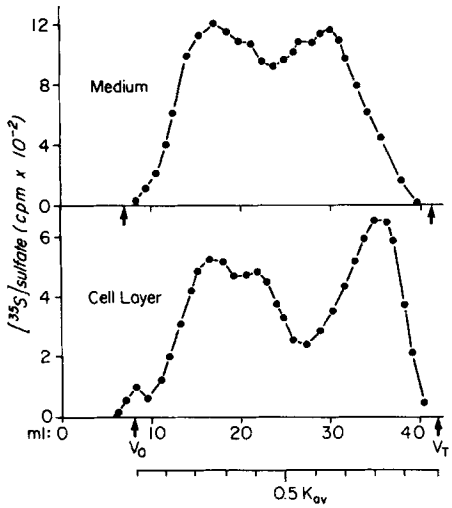

FIGURE 2 Sepharose CL-2B elution profile in 4 M guanidine HCl of ^{35}S-labeled macromolecules extracted from the medium (top panel) and cell layer (bottom panel).

cell layer (K_{av} = 0.78) than for the medium (K_{av} = 0.61). In addition, a small amount of macromolecular radioactivity (5–10% of the total) always eluted in the excluded volume of the column in extracts of the cell layer, but this [^{35}S]-labeled peak was not observed in the medium sample.

The smaller proteoglycans in the second peaks were not derived from degradation of proteoglycans in the first peaks. This was shown by eluting aliquots of 4 M guanidine HCl extracts of the medium and cell layer on Sepharose CL-2B obtained from cultures labeled for 8, 16, and 24 h (Fig. 3 *A*). At 8 h, the second peak present in the medium sample was the predominant peak, while by 16 h and later, the first peak became predominant. These data indicate that the first peak, containing the large proteoglycans, increased relative to the second peak with time of labeling and argues against the smaller proteoglycans being a breakdown product of the larger. The ratio of label in the two populations was nearly constant with time for proteoglycans extracted from the cell layer (Fig. 3 *B*).

Portions of the 4 M guanidine HCl solution of the medium and of the extract of the cell layer were fractionated in a dissociative cesium chloride equilibrium density gradient after removing unincorporated radioactive precursors by chromatography on PD-10 columns. As shown in Table I, the majority of the [^{35}S]-labeled macromolecules present in both samples were recovered in the high density fractions (D1 and D2). The lowest density fraction (D4) from the medium contained the least amount of [^{35}S]-labeled macromolecules (12%). On the other hand, considerably more [^{35}S]activity (27%) was present in the D4 fraction of the cell layer. The results indicate that the

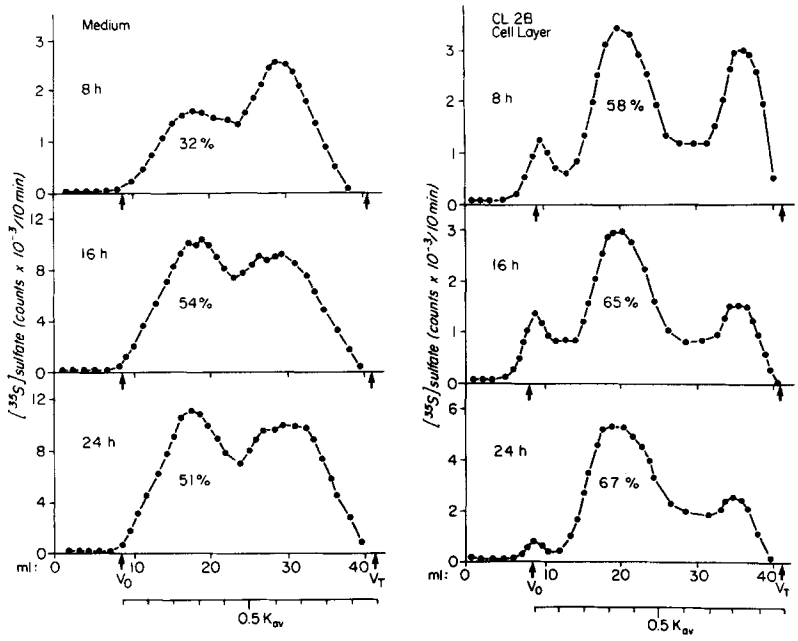

FIGURE 3 Sepharose CL-2B elution profiles in 4 M guanidine HCl of ^{35}S-labeled macromolecules in the (*a*) culture medium and (*b*) cell layer after 8 h (top panel), after 16 h (middle panel), and after 24 h (bottom panel) of continuous labeling. The percent values indicate the proportion of ^{35}S radioactivity in the first peak.

TABLE I

*Distribution of Radioactivity in a Dissociative CsCl Density Gradient Centrifugation**

	Medium		Cell layer	
	Sp gravity	[^{35}S]-DPM	Sp gravity	[^{35}S]-DPM
	g/ml	× *10*^{4}	*g/ml*	× *10*^{4}
D4	1.37	5.5 (12%)	1.39	5.1 (27%)
D3	1.40	5.9 (13%)	1.44	2.4 (13%)
D2	1.48	14.2 (32%)	1.51	5.2 (28%)
D1	1.66	19.1 (43%)	1.61	6.2 (33%)

* These samples were prepared from ~6 × 10^6 cells incubated with 50 μCi/ml of $Na_2[^{35}S]O_4$ for 48 h. ~70% of the total [^{35}S] macromolecular activity was secreted into the medium while 30% of the [^{35}S] macromolecular material was associated with the cell layer. Of the material present in the medium, ~80% of it was recovered in the gradient. Similarly, 70% of the macromolecular [^{35}S] activity was recovered from the cell layer in the gradient. The percent unextracted (i.e., left after extraction of the cell layer) accounted for <5% of the total radioactivity.

proteoglycans present in the two culture compartments separate somewhat differently in the density gradient.

Sepharose CL-2B profiles of each fraction of the dissociative gradient (D1 to D4) from the medium revealed that the large proteoglycans were predominantly present in the denser fractions (D1 and D2) (Fig. 4*A*), while the smaller proteoglycans were predominant in the less dense fractions (D3 and D4). However, the denser fractions also contained a significant amount of the smaller proteoglycan population and no single population was restricted to only one fraction. Gel chromatography of the D1 to D4 fractions of the cell layer extract revealed that the smaller of the [^{35}S]-labeled peaks was present throughout the gradient, but that the larger proteoglycans and the species that eluted in the excluded volume were confined to the denser fractions (Fig. 4*B*). The D4 fraction of the cell layer is enriched in the smaller [^{35}S]-labeled populations which contains two size classes of [^{35}S]-labeled macromolecules with K_{av} = 0.66 and 0.80, respectively. The D4 fraction was not further analyzed.

Characterization of the Glycosaminoglycans

Combined portions of D1 and D2 from the medium and cell layer were eluted on a preparative CL-2B column and peaks I and II equivalent to those indicated in Fig. 4 recovered. Aliquots of each peak were either digested with papain or alkaline degraded to release glycosaminoglycan chains. Papain digestion and alkaline degradation gave identical profiles when chromatographed on Sepharose CL-6B (Fig. 5). The glycosaminoglycans isolated from peak I present in the medium eluted at a position which corresponds to a molecular weight of ~40,000 (Fig. 5). This value was obtained by comparing the elution position of the isolated glycosaminoglycans to standard fractions of chondroitin sulfate of known molecular weight from bovine nasal cartilage as described by Wasteson (36). The glycosaminoglycan chains isolated from the smaller proteoglycan population, peak II, from the medium were identical to those present in the large population, indicating that the size of the glycosaminoglycan chain does not contribute to differences in the hydrodynamic sizes of the two populations of proteoglycans produced by the cultured cells. Released glycosaminoglycans from peaks I and II in the cell layer presented similar profiles when chromatographed on Sepharose CL-6B (Fig. 5). The isolated glycosaminoglycan chains were treated with either chondroitinase ABC or chondroitinase AC II and the digests analyzed by paper chromatography as described in Materials and Methods. Approximately 90% of peak I in the medium and cell layer was chondroitinase ABC:chondroitinase II-sensitive, indicating that the majority of peak I contained chondroitin sulfate. On the other hand, peak II contained material that was 90% susceptible to chondroitinase ABC, but 20–30% insensitive to chondroitinase AC II, indicating the presence of dermatan sulfate[3] chains. It is possible that all of the glycos-

[3] Dermatan sulfate was calculated by comparison of the amount of [^{35}S]- in the chondroitin 4-sulfate position after digestion with chondroitinase ABC and chondroitinase AC II, although it should be recognized that this method can overestimate the iduronic acid in a heteropolymer. See reference 31.

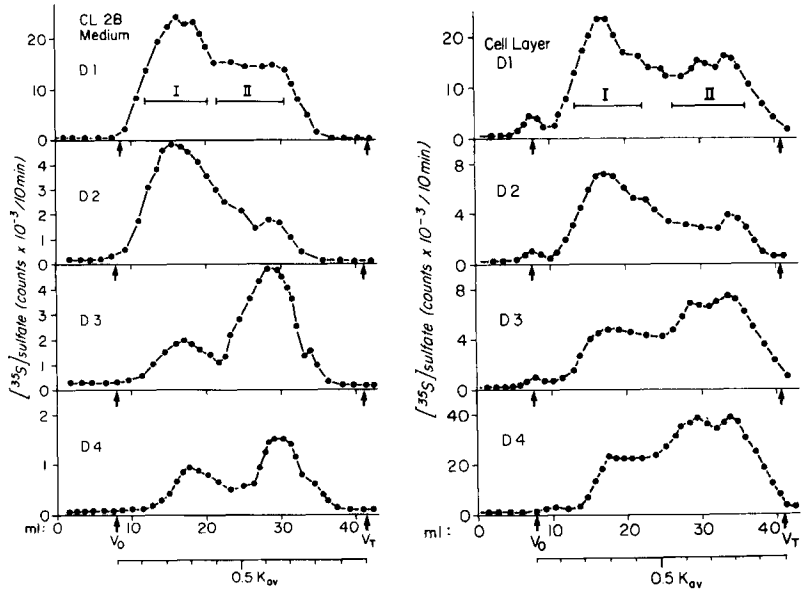

FIGURE 4 Sepharose CL-2B elution profiles in 4 M guanidine HCl of D1-D4 fractions from a dissociative CsCl density gradient of ^{35}S-labeled macromolecules in the (*a*) medium and (*b*) cell layer. The solid bar indicates the fractions pooled for subsequent glycosaminoglycan analysis. V_o, void volume; V_t, total volume.

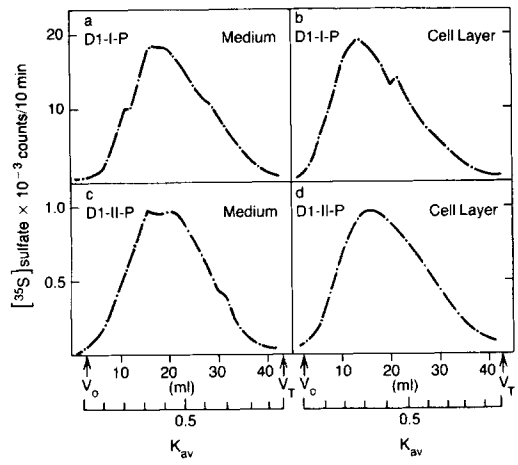

FIGURE 5 Sepharose CL-6B elution profiles of released glycosaminoglycans from the major proteoglycan populations present in the D1 fraction in the medium and cell layer after papain digestion. (*a*) peak I medium, (*b*) peak I cell layer, (*c*) peak II medium, and (*d*) peak II cell layer. Fractions of 0.22 ml were collected. V_o, void volume, V_t, total volume.

aminoglycans in this fraction contain a small proportion of iduronic acid and would be considered to be dermatan sulfate with a low percentage of iduronic acid residues. Alternatively, there may be a mixture of chondroitin sulfate chains (with no iduronic acid) and dermatan sulfate chains with a higher iduronic acid content. Detailed analysis of glycosaminoglycans in each peak is the subject of a following manuscript (Chang et al. Manuscript in preparation.).

Proteoglycan Aggregation

Samples of the medium in 0.5 M guanidine HCl and of an associative extract of the cell layer were subjected to cesium chloride centrifugation under association conditions. As shown in Table II, the majority of the [^{35}S]-labeled macromolecules in the medium and cell layer samples were recovered in the denser, aA1 fraction (sp gr 1.6–1.7 g/ml). An aliquot of each aA1 fraction was subsequently mixed with carrier aA1 in

TABLE II

*Distribution of Radioactivity in an Associative CsCl Density Gradient Centrifugation**

	Medium		Cell layer	
	Sp gravity	[^{35}S]-DPM	Sp gravity	[^{35}S]-DPM
	g/ml	*× 10⁴*	*g/ml*	*× 10⁴*
aA4	1.33	15.7 (26%)	1.32	6.8 (6%)
aA3	1.40	8.2 (14%)	1.42	6.1 (6%)
aA2	1.47	5.9 (10%)	1.50	17.3 (17%)
aA1	1.60	30.1 (50%)	1.68	74.6 (71%)

* These samples were prepared from ~2 × 10^6 cells incubated with 50 μCi/ml of $Na_2[^{35}S]O_4$ for 48 h. A portion of the labeled medium (~3.5 ml of 10 ml) was taken for density gradients while the entire associative extract of the cell layer (~5 ml) was used for the density gradient. Of the material present in the medium, ~78% was recovered from the gradient. Likewise, ~53% of the [^{35}S] macromolecular material associated with the cell layer was recovered from the gradient.

associative conditions, dialyzed to removed CsCl and chromatographed on an analytical Sepharose CL-2B column in an associative solvent as described in Materials and Methods (Fig. 6 *a*, *b*). Both samples contained a small percentage of the [^{35}S]activity (5–10%) eluting in the excluded volume where aggregate would be recovered. These results indicate that the majority of proteoglycans synthesized and secreted into the medium or extracted from the cell layer with associative solvents are not already bound in aggregate structures.

An experiment was done to determine whether or not the proteoglycans synthesized by arterial smooth muscle cells possess the capacity to form aggregate. An aliquot of each of the D1 fractions from the medium and 4 M guanidine HCl extracts of the cell layer was mixed with excess carrier aA1 in 4 M guanidine HCl. After dialysis to 0.5 M sodium acetate, pH 7.0, to reassociate aggregates, each sample was eluted on Sepharose CL-2B in an associative solvent (Fig. 6 *c*, *d*). In each case, there was a pronounced shift in the [^{35}S]sulfate-labeled large proteoglycan (K_{av} = 0.31) in the medium and cell layer to the excluded volume of the column. A similar shift was not observed for the smaller proteoglycan populations. Thus, the proteoglycans in the aggregate fraction are derived from the

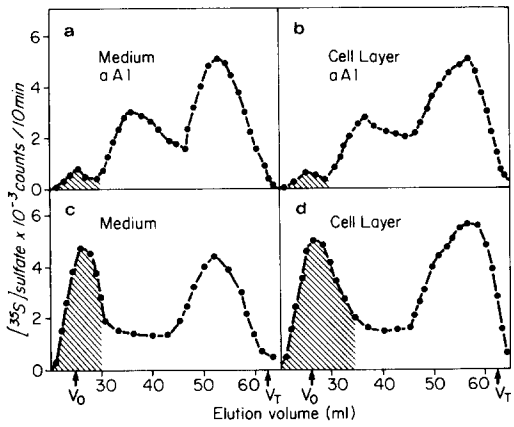

FIGURE 6 Sepharose CL-2B profiles of proteoglycans present in the culture medium and cell layer eluted under associative conditions (0.5 M sodium acetate, pH 7.0). Top panels (*a* and *b*) are profiles obtained by chromatography of the bottom fraction of an associative gradient (aA1) directly on an associative column without added carrier. The bottom panels (*c* and *d*) are profiles obtained when D1 fractions were mixed with excess aA1 carrier, allowed to reaggregate by dialysis against 0.5 M sodium acetate and eluted on an associative column. The amount of radioactivity that elutes in the V_0 of the column serves as an estimate for the percentage aggregation.

largest macromolecules, those which contained predominantly chondroitin sulfate.

Electron Microscopy of the Isolated Proteoglycans

Aliquots of the aA1 fractions prepared without carrier from the medium and cell layer were observed with the electron microscope. Fig. 7 demonstrates a field with several individual monomers, some of which are circled. Individual molecules at a higher magnification demonstrate a bottlebrush structure consisting of a central filament containing side branches. Measurements of 300 individual, well-spread and isolated molecules indicate that the central filament of the bottlebrush structure varied between 57 and 242 nm with an average of 140 ± 31 nm. The number of side branches varied with filament length, but averaged eight for those monomers whose length was <100 nm. Approximately 33% of the structures were >100 nm in length and these longer monomers frequently contained as many as 12–14 side branches. The average length of the side branches was ~73 nm. Occasionally, structures with only one apparent side projection were observed (note dashed circles in Fig. 7) and may represent proteoglycans from the population of smaller macromolecules (peak II). No evidence of individual monomers aggregating into larger structures was observed.

Electron Microscopy of the Intercellular Matrix of the Cultured Arterial Smooth Muscle Cells

Safranin O staining of the cultures (Fig. 8) preserved a matrix which was filled with short "brushlike" structures which in some sections resembled the structures identified in spreads of isolated proteoglycans shown in Fig. 7. There was evidence of filamentous projections emanating from a central core and when these structures existed free, their average length was close to the length of purified monomers (140 nm). These bottlebrush structures were entangled within and among fine fibrils as well as present singularly throughout the intercellular matrix (Fig. 8). In addition, in some sections they were observed to be associated with the plasma membrane of the smooth muscle cells (Fig. 8, *inset*). Chondroitinase ABC digestion was effective in removing these Safranin O preserved bottlebrush-like structures (data not shown).

DISCUSSION

Arterial smooth muscle cells derived from the nonhuman primate, *Macaca nemestrina*, have been shown to synthesize at least two size classes of proteoglycans when cultured in vitro. The largest size class of proteoglycans was recovered primarily in the higher density fractions of CsCl density gradients as has been reported for proteoglycans synthesized by other cell types in vitro (31, 38, 40, 43–47). This large proteoglycan fraction gave similar elution profiles on molecular sizing columns for both the medium and cell layer (K_{av} = 0.31) and was shown to contain predominantly chondroitin sulfate chains with an average weight of ~40,000. A similar large chondroitin sulfate-containing proteoglycan population has been observed in cultures of glial cells (48), skin (49), and lung (50) fibroblasts and indicates that this class of proteoglycan may be synthesized by a large number of noncartilagenous cells. This proteoglycan resembles the major proteoglycan present in cartilage as well as those synthesized by differentiated chondrocytes in vitro in terms of its elution position on Sepharose CL-2B, but differs in possessing chondroitin sulfate chains of higher molecular weight (~40,000 vs. ~20,000) (45–47). Gardell et al. (8) have also emphasized the similarity of the proteoglycan derived from aorta and cartilage by demonstrating that the major proteoglycan isolated from bovine aorta reacted with antisera raised against that from cartilage. In addition, Mangkornkanok-Mark et al. (51) demonstrated immunofluorescent staining of bovine aorta with antisera raised against cartilage proteoglycan. Furthermore, these investigators demonstrated that antisera raised against aortic proteoglycans only stained the pericellular matrix of cartilage while anti-cartilage proteoglycan stained both the pericellular and interterritorial zones of cartilage. These results demonstrate that proteoglycans isolated from these two tissues may possess some immunologic similarities as well as some differences. The large proteoglycans produced by arterial smooth muscle cells appear to be able to interact with hyaluronic acid to form large molecular aggregates as has been demonstrated for cartilage (38, 39). These findings support the concept that the ability of proteoglycans to aggregate is not solely restricted to cartilage. The large proteoglycan synthesized by arterial smooth muscle cells resembles the large proteoglycan population recently isolated from human aorta by Salisbury and Wagner (6) in terms of: (*a*) elution position on Sepharose sizing columns; (*b*) glycosaminoglycan composition; and (*c*) ability to form aggregate.

The smaller proteoglycan present in the medium contains dermatan sulfate. To some extent, this population resembles the small "ubiquitous" component produced by other mesenchymal cells in vitro (49, 50, 52, 53) and that recently isolated and characterized from cartilage (54). Several investigators have noticed the presence of dermatan sulfate proteoglycans in various tissue (55–59) including aorta (1, 3, 4, 5, 55). Most recently, Salisbury and Wagner (6) identified a dermatan sulfate-containing proteoglycan of identical size to the dermatan sulfate proteoglycan synthesized and secreted into the medium by cultured primate arterial smooth muscle cells described in this study. These results suggest that the arterial smooth muscle cells retain some of their differentiated characteristics with respect to proteoglycan biosynthesis. Similar conclusions were

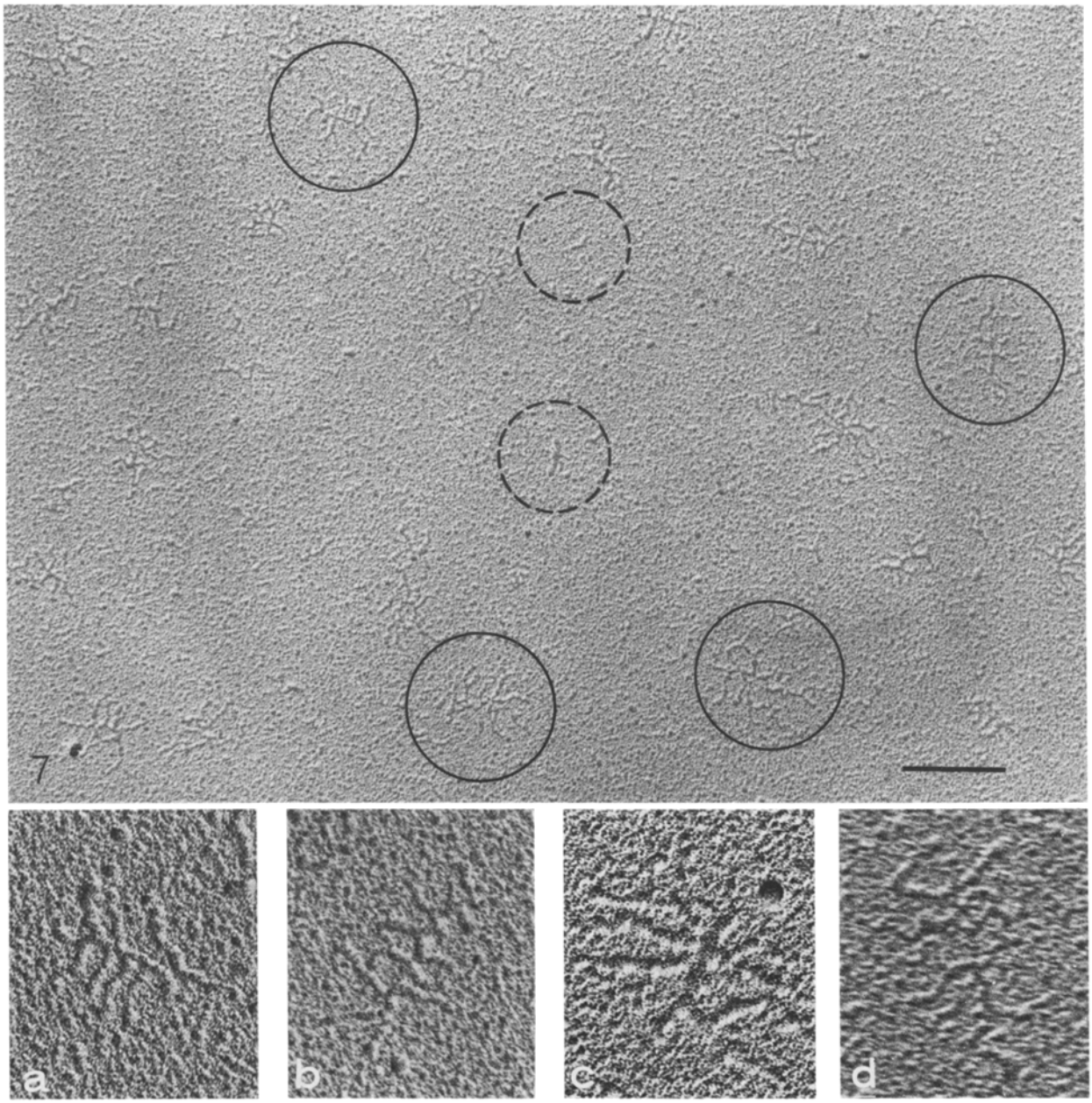

FIGURE 7 Electron micrographs of purified proteoglycans from an associative density gradient (aA1) of culture medium derived from arterial smooth muscle cells. Circles surround individual monomers of proteoglycans. Dotted circles identify monomers containing only one side projection. Bar, 0.25 μm. × 64,000. Higher magnification of individual monomers illustrates their bottlebrush structure and polydispersity. *b* and *d* are typical of monomers whose lengths are >100 nm, while *a* and *c* are typical of monomers whose length is <100 nm, Bar, 0.25 μm. *a*, × 140,000; *b*, × 147,000; *c*, × 165,000; and *d*, × 156,300.

reached regarding collagen biosynthesis by cultured nonhuman primate arterial smooth muscle cells (60). This is in contrast to some other cell types which lose their capacity to synthesize tissue-specific proteoglycans when placed in culture (52).

Although the majority of the glycosaminoglycans in the two proteoglycan peaks produced by arterial smooth muscle cell cultures could be identified as containing chondroitin sulfate or dermatan sulfate, a portion of each sulfate-containing peak resisted degradation with enzymes specific for these glycosaminoglycans. One possibility is that this resistant material represents a heparan sulfate proteoglycan and recent experiments have demonstrated the presence of a small amount of heparan sulfate proteoglycan in these cultures (Chang et al. Manuscript in preparation.). In addition, heparan sulfate may have been present in the [^{35}S]-labeled population that eluted at the V_o from the cell layer extracts since Vogel and Peterson (50) found heparan sulfate proteoglycans from lung fibroblast cultures to elute at this same position. Recently, Salisbury and Wagner (6) found a polydisperse heparan sulfate proteoglycan distributed between the large and small proteoglycans in human aorta. Oegema et al. (5) described the presence of a small proteoglycan in bovine aorta which resisted digestion with chondroitinase ABC and was suggested to contain heparan sulfate. It is unlikely that the smooth muscle cells are a major source of heparan sulfate in the blood vessel since endothelial cells synthesize predominantly heparan sulfate proteoglycans (27, 61).

The marked difference in the relative amount of native

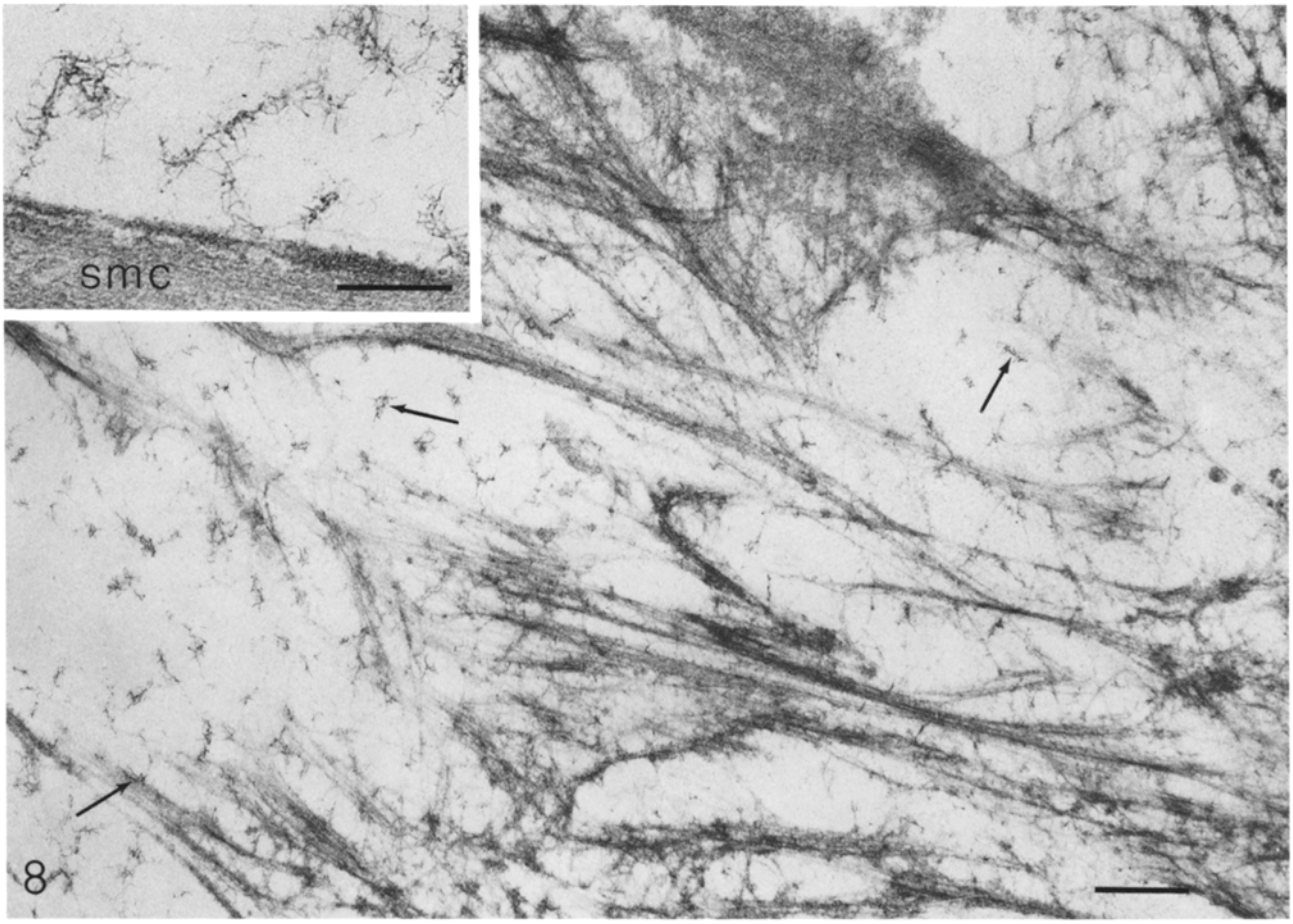

FIGURE 8 Electron micrograph of a portion of intercellular matrix of an arterial smooth muscle cell culture prepared in the presence of Safranin O. Numerous short bottlebrush structures that exhibit an affinity for Safranin O can be recognized throughout the intercellular matrix (arrows) as well as associated with fine intercellular fibrils. *Inset.* Higher magnification of a similar preparation as shown in Fig. 8, demonstrating that some of these "bottlebrush" structures are associated with the surface of arterial smooth muscle cells (*smc*). Bar, 0.2 μm. × 78,000.

proteoglycan aggregate produced by these cells versus the high capacity of the newly synthesized large proteoglycans to form aggregate when reconstituted with carrier cartilage proteoglycan is not understood. Native proteoglycan aggregate has been demonstrated in blood vessels (7, 8). It may be that the culture conditions are incorrect for aggregation to occur. For example, nonhuman primate arterial smooth muscle cells produce very little hyaluronic acid (21) and availability of hyaluronic acid within these cultures may be limiting for aggregation to occur. In addition, no information is yet available regarding the ability of these cells to produce link proteins which are essential for the formation of stable aggregates (33). However, the finding that arterial smooth muscle cells synthesize and secrete two distinct proteoglycans which differ in their capacity to form high molecular weight aggregates emphasizes the possible complexities with which these molecules associate with one another as well as with other components in the arterial wall.

Electron microscopy of the aA1 fraction from both the medium and cell layer of the arterial smooth muscle cell cultures revealed a heterogeneous population of macromolecules which possessed an overall structure of a central core with various numbers of side projections along its length, similar to spread proteoglycan monomers isolated from cartilage (39–41, 62). However, the average central core length was only one-third to one-half as long as the monomers isolated from cartilage, while the side projections were approximately twice as long. The side projections are thought to represent glycosaminoglycans and the longer extended length of the side projections of the proteoglycans produced by arterial smooth muscle cells is consistent with their higher molecular weight compared to cartilage proteoglycan (39–41, 61).

Proteoglycans could also be identified morphologically within the intercellular matrix of the arterial smooth muscle cell cultures using Safranin O (42). The fine, filamentous structures present throughout the matrix resembled the spread proteoglycan monomers and thus may represent the monomers in their more extended state within the intercellular matrix. Similar extended proteoglycan filaments have been observed by Scott (63) in tendon using a cinchomeronic dye. The preservation of bottlebrush structures with Safranin O, which are susceptible to chondroitinase ABC digestion, is in contrast to the granule-like network preserved with ruthenium red in cultures of arterial smooth muscle cells (25) and intact arteries (64, 65). Granules identified in ruthenium red preparations have been interpreted to represent proteoglycan monomers that have collapsed during processing (41). Safranin O appears to prevent collapse of the monomers.

In conclusion, this study has demonstrated that cultured arterial smooth muscle cells produce distinct species of proteoglycans which resemble those present in aortic tissue in vivo

and confirms the ability of this cell type to retain its differentiated function with respect to proteoglycan synthesis in vitro. The finding that distinct size classes are produced by these cells, which differ in their glycosaminoglycan composition and ability to aggregate, emphasizes the need to determine whether or not each of these populations differs in its location as well as its functional properties within blood vessels. Furthermore, it will be important to determine whether or not conditions that influence the synthesis of proteoglycans by arterial smooth muscle cells differentially affect the production of a specific species of proteoglycans. Preliminary studies in our laboratory (66) suggest that rapidly dividing arterial smooth muscle cells synthesize proportionately more of the large chondroitin sulfate proteoglycan than nondividing cells. Identifying such a modulation in proteoglycan synthesis becomes feasible using this culture system and may help explain changing patterns of arterial proteoglycans that occur during development and disease in this tissue.

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