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 has attempted 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
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-aminoheptonic acid and benzamidine HCl from Eastman Kodak Co., Rochester, NY; chondroitinase ABC (Proteus vulgaris) and chondroitinase AC II (Arthrobacter aureus) from Seikagaku, Kogyo, Tokyo, Japan, through Miles Laboratories, Inc., Elkhart, IN; Sephadex and Sepharose from Pharmacia, Inc., Piscataway, NJ; Na$_2$SO$_4$ ($10$ mg/ml; $10$ mg/ml; $10$ mg/ml; $10$ mg/ml), $0.6$-$0.7$ g/ml, 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, D$_1$, and aggregate, A$_1$, were prepared from the Swarn rat chondrosarcoma (29).

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 pigs (Macaca nemestrina) were explanted in Dulbecco Vogt modification of Eagle’s minimal medium supplemented with $5\%$ homologous serum. Cells were allowed to grow to confluence, 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$^2$ 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 $37^\circ C$.

Radioisotope Labeling of Cultures and Extraction of Proteoglycans: Near confluent cultures were labeled by the addition of fresh medium containing Na$_2$SO$_4$ ($50$ $\mu$Ci/ml) for $48$ h unless otherwise specified. Some experiments involved double labeling with Na$_2$SO$_4$ ($50$ $\mu$Ci/ml) and $[^3]H$-$\beta$-glucosamine ($2.5$ $\mu$Ci/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 $\sim 4$ M or $\sim 0.5$ M, respectively (31). The resulting solutions were immediately frozen and stored at $\sim 70^\circ C$ until subsequent analysis. The remaining cell layers were washed twice with phosphate-buffered saline at $0^\circ C$ 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 (associative solvent) or $0.5$ M guanidine HCl (associative solvent) containing $0.1$ M $6$-aminoheptonic 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 $\sim 70^\circ C$ until analyzed.

Relative Hydrodynamic Sizes of the Proteoglycan Monomers: Aliquots ($200$ $\mu$l) of the $4$ M guanidine HCl extracts of the medium and cell layer were mixed with $30$ $\mu$l of nonmonomer proteoglycan carrier ($10$ mg/ml in $4$ M guanidine HCl) and the mixture was applied directly to an analytical Sephacryl CL-2B gel filtration column ($100$ x $0.7$ 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 $4$-liter liquid scintillation pressure, $50$ $\mu$Ci/ml. The fractions were dialyzed against $4$ M guanidine HCl in $0.05$ M sodium acetate buffer, pH $5.8$.

Equilibrium Density Gradient Centrifugation: Dissociative density gradient centrifugation. Aliquots ($1$ ml of the medium and cell layer extracts, were mixed with $100$ $\mu$l of nonmonomer 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 $10^\circ C$ at $37,000$ rpm in a Beckman SW $50.1$ Rotor (33) (Beckman Instruments, Inc., Fullerton, CA). The gradients were divided into four approximately equal fractions, D$_1$ through D$_4$ (29), by means of a tube slicer or gradient separator (Buchler Inst., Chicago, IL). The specific gravity of each fraction was determined by weighing $50$-$\mu$l aliquots on a Mettler microbalance. The amounts of macromolecular $[^3]H$-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 Sephacryl CL-2B analytical column eluted with $4$ M guanidine HCl in $0.05$ M sodium acetate buffer, pH $5.8$.

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 (D$_1$ and D$_2$) by preparative Sephacryl CL-2B chromatography ($0.9$ x $60$ 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 ($30$ $\mu$g of papain in $0.1$ M sodium acetate, pH $7.0$ for $4$ h at $65^\circ C$) (34) or by alkaline degradation (35). Aliquots of the released glycosaminoglycans were chromatographed on a Sephacryl CL-6B column ($0.7$ x $30$ cm), eluted with $0.2$ M NaCl in $20$ mm Tris HCl, pH $7.5$. Fractions of $0.2$ ml were collected and $0.10$ ml of each fraction was taken for measurement of radioactivity. The excluded ($V_x$) and total ($V_t$) volumes of the column were determined by using blue dextran and free $[^3]H$-label, respectively. The average molecular weight, $M_w$, of the released glycosaminoglycans was estimated by comparing the elution position (partition coefficient = $K_p$) 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 vol of $0.5$ M potassium acetate. Precipitates were cooled to $0^\circ C$ and isolated by centrifugation at $10,000$ rpm. The precipitates were
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 aAl 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 x 110 cm) with the same buffer that contained the sample. The amount of [35S]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 DI fractions to aggregate was also tested. Aliquots of the DI 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, 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.

Representative monolayers were fixed in 2% glutaraldehyde in 0.1 M Sorensen’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 [35S]-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 [35S]-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 Na2[35S]O4, ~70-80% of the [35S]-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 eluant. Two major peaks of [35S]activity were observed for both the medium and cell layer (Fig. 2). The first peak, with the largest proteoglycan species (K_M = 0.31), eluted similarly for both the medium and cell layer samples. The second labeled peak eluted later for the sample from the cell layer (K_M = 0.78) than for the medium (K_M = 0.61). In addition, a small amount of macromolecular radioactivity (5-10% of the total) was eluted in the excluded volume of the column in extracts of the cell layer, but this [35S]-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. 3A). 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. 3B).

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 [35S]-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 [35S]-labeled macromolecules (12%). On the other hand, considerably more [35S]activity (27%) was present in the D4 fraction of the cell layer. The results indicate that the
proteoglycans present in the two culture compartments separate somewhat differently in the density gradient.

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

<table>
<thead>
<tr>
<th></th>
<th>Medium</th>
<th>Cell layer</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sp gravity</td>
<td>(a) SPM</td>
</tr>
<tr>
<td>D1</td>
<td>1.37</td>
<td>5.5 (12%)</td>
</tr>
<tr>
<td>D2</td>
<td>1.40</td>
<td>9.3 (13%)</td>
</tr>
<tr>
<td>D3</td>
<td>1.48</td>
<td>14.2 (32%)</td>
</tr>
<tr>
<td>D4</td>
<td>1.66</td>
<td>19.1 (43%)</td>
</tr>
</tbody>
</table>

* These samples were prepared from ~6 x 10^9 cells incubated with 50 μCi/ml of Na_2[^35]SO_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.

**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.

**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 chains. It is possible that all of the 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 chains. It is possible that all of the 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. 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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. 4A), 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 proteoglycan and the species that eluted in the excluded volume were confined to the denser fractions (Fig. 4B). 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_m = 0.66 and 0.80, respectively. The D4 fraction was not further analyzed.

[^35]S in the chondroitin 4-sulfate position after digestion with chondroitinase ABC or chondroitinase AC II, although it should be recognized that this method can overestimate the iduronic acid in a heteropolymer. See reference 31.
amino sugars 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 [35S]-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 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. 6a, b). Both samples contained a small percentage of the [35S] 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 [35S] sulfate-labeled large proteoglycan (Kd = 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
The number of side branches varied with filament length, and varied between 57 and 242 nm with an average of 140 ± 31 nm. Monomers aggregating into larger structures was observed. Approximately 33% of the structures were >100 nm in length, but averaged eight for those monomers whose length was <100 nm. It has been noticed the presence of dermatan sulfate proteoglycans in the small "ubiquitous" component produced by other mesenchymal cells in vitro (49, 50, 52, 53) and that recently isolated dermatan sulfate. To some extent, this population resembles the large proteoglycan population recently isolated from bovine aorta and cartilage by demonstrating that the major proteoglycan isolated from bovine aorta reacted with antisera raised against that from cartilage. In addition, Mangkornkaok-Nark 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 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 (Khv = 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 noncartilaginous 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, the small 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...
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 [35S]-labeled population that eluted at the V_e 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. X 78,000.

Electron microscopy of the αA1 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 its tissue function with respect to proteoglycan synthesis in vitro.

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