Distinct Synthetic and Structural Characteristics of Proteoglycans Produced by Cultured Artery Smooth Muscle Cells of Atherosclerosis-susceptible Pigeons*

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Proteoglycan (PG) metabolism by aortic smooth muscle cell cultures derived from atherosclerosis-susceptible White Carneau (WC) and -resistant Show Racer (SR) pigeons was compared using [35S]sodium sulfate and [3H]serine or [3H]glucosamine as labeling precursors. Chondroitin sulfate (CS) PG and dermatan sulfate (DS) PG were the major PG secreted into the medium by both cell types. Total PG production, whether measured by incorporation of radiolabel into either core protein or glycosaminoglycan chains, was consistently lower in WC compared to SR cultures at several time points. This difference was due in part to lower (30–37%) PG synthesis in WC cells, but degradation of newly synthesized PG was an important contributor. A pulse-chase study indicated that of the total radiolabeled PG present at time 0, only 47% was present at 24 h in WC cultures compared to 88% in SR cultures. The large CS-PG appeared to be the primary target for degradation in WC cells, and this selective processing resulted in a higher DS-PG:CS-PG ratio in these cultures. Structural studies indicated similar core protein and glycosaminoglycan chain sizes within a PG type for both cell types. PG monomer composition differed, however, by a higher sulfation of WC CS-PG compared to SR CS-PG and by a disaccharide sulfation position favoring 6-sulfation in WC PG and 4-sulfation in SR PG.

Pigeon models for the study of human atherosclerosis allow a comparison between a relatively resistant breed (SR) and one with enhanced atherosclerosis susceptibility (WC). These divergent responses are seen in both naturally occurring (1) and cholesterol-induced (2–4) atherosclerosis. Since plasma lipoprotein concentrations and distributions are similar for the two breeds (5), factors at the level of the artery wall have been proposed to account for the genetic predisposition to the disease. One such factor may be the structural or metabolic properties of the pericellular and intercellular matrix proteoglycans (PG).

We have previously compared the in vitro synthesis of PG by SR and WC pigeon artery segments and have shown a lower PG production in WC cultures at several different ages ranging from 6 months to 10 years (6). This finding suggested reduced numbers of PG monomers in the extracellular space in WC versus SR cultures, a situation which would favor greater tissue permeability. We have also shown that WC artery-derived PG have a higher binding affinity for plasma low density lipoproteins (LDL) than analogous PG isolated from SR arteries (7). Since the negative sulfate groups of PG participate in binding to LDL (8,9), this finding suggests that WC PG may contain more sulfation or PG with distributions favoring increased contents of the more electronegative glycosaminoglycans (GAG).

Smooth muscle cells are the major cell type in the artery wall and are responsible for the synthesis of intercellular matrix components including PG. Cultured smooth muscle cells from monkey (10, 11), bovine (12, 13), rat (14, 15), and pig (16) aortas have been used to study PG synthesis. These studies have indicated that the PG types produced in culture are similar to those found in aortic tissue (see Ref. 17 for review). This study, utilizing WC and SR smooth muscle cells in culture, was undertaken to examine proteoglycan structure and metabolism to define further the nascent properties of chondroitin sulfate PG and dermatan sulfate PG that may be implicated in an altered functional property of the artery wall of the atherosclerosis-susceptible WC pigeon.

**EXPERIMENTAL PROCEDURES**

**Materials**

All tissue culture reagents were purchased from Flow Laboratories, Inc. (Rockville, MD), and plasticware was from Corning Glass Works (Corning, NY). [35S]Sodium sulfate was from Amersham Corp.; [3H]serine was from ICN Biomedicals (Irvine, CA); [3H]glucosamine and ENHANCE® were from Du Pont-New England Nuclear; Liquiscint was from National Diagnostics, Inc. (Somerville, NJ); ultrapure GdnHCl and urea were from Schwarz/Mann; benzamidine hydrochloride, β-aminohexanoic acid, and Alcian blue were from Eastman; 1-Hexadecylpyridinium chloride (CPC), also from Eastman, was twice-purified by recrystallization. Tryptamine hydrochloride, 1.10-phenanthroline, and papain were from Sigma; chondroitin ABC lyase (Proteus vulgaris), chondroitin AC lyase (Arthrobacter aurescens), and hyaluronidase (Streptococcus haemolyticus) were from Miles Laboratories Inc.; chondroitin ABC lyase was from Du Pont-New England Nuclear; Licilucint was from National Diagnostics, Inc. (Somerville, NJ); ultrapure GdnHCl and urea were from Schwarz/Mann; and benzamidine hydrochloride, β-aminohexanoic acid, and Alcian blue were from Eastman.

1-Hexadecylpyridinium chloride (CPC), also from Eastman, was twice-purified by recrystallization. Tryptamine hydrochloride, 1.10-phenanthroline, and papain were from Sigma; chondroitin ABC lyase (Proteus vulgaris), chondroitin AC lyase (Arthrobacter aurescens), and hyaluronidase (Streptococcus haemolyticus) were from Miles Laboratories Inc.; chondroitin sulfate was from Nutritional Biochemicals Corp. (Cleveland, OH); Sepharose CL-4B and CL-6B and DEAE-Sephaloc were from Pharmacia LKB Biotechnology Inc.; polycrylamide gel electrophoresis reagents were from Bio-Rad; and unsaturated disaccharide standards containing ADI-0S, ADI-4S, ADI-
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6S, and ADi-diSE were from Seikagaku Kogyo Co. Ltd. (Tokyo). All other chemicals used were from Fisher. Heparan sulfate, dermatan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate were National Institutes of Health standards obtained from Drs. M. B. Matthews and J. A. Cifonelli (University of Chicago) (Contract N01-AM-5-2205 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health). Pig PG samples were obtained from ruminants of two breeds 5 of Racer and White Carneau (WC-2) genetically selected for increased susceptibility to atherosclerosis (4).

Cell Culture and Labeling

Medial-intimal explant cultures were prepared from aortas of 1-day-old WC and SR pigeon squabs as previously described (18). When confluent, the cells were passaged in Eagle's minimal essential medium with Earle's base salts supplemented with 10% units/ml penicillin, 100 µg/ml streptomycin sulfate, 200 mM L-glutamine, Eagle's minimal essential medium vitamins, and 10% fetal bovine serum. Cells of the third passage were used in all experiments. They were plated in 35-mm Petri dishes in 2 ml of medium (for pulse-chase studies), or in 75-cm² T-flasks in 10 ml of medium (for structure studies). On day 6, when the cultures were confluent, the culture media were replaced with media containing 50 µCi/ml [3H]serine or 30 µCi/ml [3H]glucosamine. Triplicate cultures were used for all studies.

Isolation of Proteoglycans

Media Fraction—After the cultures were radiolabeled, the culture medium was removed and cleared of cell debris by centrifugation at 450 × g for 10 min to obtain media PG.

Pericellular Fraction—The cell sheet was washed twice with phosphate-buffered saline, pH 7.4, prior to the addition of 0.05% trypsin, 0.02% EDTA in phosphate-buffered saline (0.2 ml for 35-mm² dishes, 0.5 ml for 60-mm dishes, 0.5-ml for 75-cm² flasks). The cells were trypsinized for 5 min at 37°C, at which time they were released from the dish. Complete medium was added to inhibit the trypsin, the cell suspensions were removed to an ice bath, and a media rinse of the culture vessel was pooled with each suspension. A 10-µl aliquot was removed for cell counting by hemocytometry. The cells were pelleted for 5 min at 450 × g, and the supernatant was removed and termed the pericellular PG fraction.

Intracellular Fraction—The pelleted cells were washed once with phosphate-buffered saline and treated with 4 M GdnHCl, pH 5.8, containing protease inhibitors (19) for 24 h at 4°C to extract intracellular PG.

All three fractions were dialyzed exhaustively against 0.15 M NaCl, 0.05 M Na2SO4 at 4°C to remove unincorporated radiolabel prior to further purification of PG.

Isolation of Different PG Types

Dialyzed samples were made by the addition of solid guanidine HCl and concentrated when necessary using an Amicon stirred cell with a YM-2 membrane. Concentrated PG were chromatographed on a 60 × 0.9-cm Sepharose CL-4B column eluted with 4 M GdnHCl in 0.05 M sodium acetate buffer, pH 5.8, at 4°C. Fractions (0.5 ml) were collected and analyzed for radioactivity prior to preparation of pools comprising the different PG types. PG from Sepharose CL-4B was dialyzed against 0.05 M NaCl and 0.03 M Na2SO4, and precipitated in 70% ethanol for 2 h at 0°C, followed by centrifugation at 8000 g for 5 min. The ethanol supernatants were air-dried, resuspended in 20 µl of HPLC solvent containing 1 µg each of mixed disaccharide standards, and chromatographed on a 4.6 × 250-mm Whatman Partisol-10 FAC column according to the method of Seldon et al. (24). The HPLC solvent used for elution was 70% acetonitrile/methanol (3:1, v/v) and 30% 0.5 M ammonium acetate, pH 5.3.

Gel Electrophoresis of PG Core Protein—PG were prepared following CPC precipitation from media of SR and WC cells labeled with [35S]sulfate and [3H]serine for 24 or 48 h. PGs were digested with chondroitinase ABC as previously described in order to obtain the core protein free of GAG chains. A sample was adjusted to 0.05 M Tris, pH 8.0, 1% sodium dodecyl sulfate, 1% β-mercaptoethanol and boiled for 2 min. Samples were electrophoresed on a 7.5% polyacrylamide gel by the method of Laemmli (25). Both high and low molecular weight protein standards were used as molecular weight markers. The gel was stained with 0.1% Coomassie Blue in 50% acetic acid and treated with ENHANCE0 for 1 h. After treatment for 1 h with 0.1% Cooamassie Blue in 50% methanol, 10% acetic acid; destained for 18 h in 50% methanol, 10% acetic acid; and treated with ENHANCER® for 1 h. After treatment for 1 h with 0.5% glycerol, the gel was dried for 1.5 h by electrodrying Model 224 gel slab dryer, and autodiagnostics were prepared by exposing the dried gel to Kodak X-Omat AR x-ray film for 96 h at −60°C.

Radioactivity Measurements

All radioactivity measurements were made in a Beckman LS200 scintillation counter using Liquiscint as the fluor.

RESULTS

Smooth muscle cell numbers per dish over the labeling period of the first experiment are presented in Table I. The results demonstrate a higher saturation density for WC cells. However, cell numbers for either WC or SR cultures did not change over time, indicating that both cell types were confluent. The WC cells used in this study reached a saturation density of 2.1 × 10⁴ cells/cm² compared with 1.2 × 10⁴ for SR cells. Cell protein for WC cultures was 206 ± 24 µg/10⁶ cells compared with 275 ± 4.5 µg/10⁶ cells for SR cultures. In an unrelated experiment, four different WC cell lines at similar passage numbers compared with four SR lines had a cell protein:DNA ratio at confluence of 24 ± 3 for WC cells and 30 ± 2 for SR cells. From these observations and assessing the morphology of the cells, it appears that WC smooth muscle cells may be slightly smaller. Since relative differences

3 R. W. St. Clair, personal communication.
TABLE I

Saturation density of cells during radiolabeling period

<table>
<thead>
<tr>
<th>Incubation time (h)</th>
<th>WC culture</th>
<th>SR culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cells/dish ($\times 10^6$)</td>
<td>cells/dish ($\times 10^6$)</td>
</tr>
<tr>
<td>12</td>
<td>6.7 ± 0.80</td>
<td>4.0 ± 1.09</td>
</tr>
<tr>
<td>36</td>
<td>5.5 ± 0.33</td>
<td>3.4 ± 0.46</td>
</tr>
<tr>
<td>60</td>
<td>5.9 ± 0.93</td>
<td>2.7 ± 0.40</td>
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</table>

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Values represent mean ± S.E. Cells were grown to confluence in 60-mm Petri dishes. The media were replaced with labeling media containing 10% fetal bovine serum, 50 µCi/ml [35S]sulfate, and 30 µCi/ml [3H]serine. After radiolabeling, the medium was removed, and the cells were harvested by trypsinization. Cell counts were obtained by hemocytometry using trypan blue exclusion as an indication of cell viability. No difference in cell viability was observed either between cell type or within a cell type over time.

**Characterization of Media PG**—The distribution of [35S]sulfate-labeled proteoglycans to media (○) and pericellular (●) and intracellular (▲) fractions over time. Points and lines at each point represent the mean ± S.E. of three 60-mm dishes at each time. PG were isolated and separated on Sepharose CL-4B and precipitated with CPC. Counts were calculated from CPC-precipitated material A, SR cells; B, WC cells.

In cell numbers exceed differences in protein:DNA ratios, other factors such as cell spreading may be important in determining cell density.

**Distribution and Production of Newly Synthesized Proteoglycans**— Cultures were incubated with [35S]sodium sulfate and [3H]serine for 12, 36, and 60 h to determine the relative cellular location of newly synthesized PG over time. PG were isolated from media and pericellular and intracellular compartments following precipitation with CPC (Fig. 1). Secretion of sulfated PG into the media increased over time for 60 h, whereas accumulation in the intracellular and pericellular pools remained relatively constant after 12 h. At 60 h, the percent distribution of [35S] for the media and pericellular and intracellular compartments was 87:11:2 and 85:13:2 in WC and SR cells, respectively. At all time points, [35S]sulfate in newly synthesized PG was lower in WC than in SR cultures, with the greatest difference being in the media fraction.

Whereas greater than 90% (range of 91–100%) of nondialyzable [35S]sulfate was precipitable with CPC, only 7% of the [3H]serine in the media fractions was precipitable, indicating that it was a component of the PG core protein. For the WC cultures, both the newly synthesized total protein and the PG core protein were lower in comparison to SR cultures (Table II). The PG core protein of WC cells represented a lower percentage of the total protein synthesized, with the greatest difference being at later incubation times (6.7% in SR versus 4.6% in WC cells). A lower level of newly synthesized PG in the culture medium of WC compared to SR smooth muscle cells was confirmed in three additional independent experiments (data not shown).

**Proteoglycan Turnover**—To examine the relative contribution of PG synthesis versus degradation to the lower media PG content in WC cultures, a pulse-chase experiment was conducted (Fig. 2). Cultures were prelabelled with [35S]sulfate for 24 h and then chased with unlabeled medium. At time 0, WC cultures contained 1800 dpm of [35S]-PG/10⁶ cells (total of all three compartments), whereas SR cells had 2550 dpm of [35S]-PG/10⁶ cells. This difference of 30% suggests that the production of PG in WC cultures is significantly reduced. For both cell types, radioactivity in intracellular and pericellular PG pools decreased rapidly during the first 2 h of the chase, followed by little additional change in the intracellular fractions and a slower, constant decrease in the pericellular fractions. During the 24-h chase period, 91% of the WC and 80% of the SR intracellular radiolabeled PG were lost, as were 92% of the WC and 89% of the SR pericellular PG. Amounts of radiolabeled PG in the media fraction were lower in WC than in SR cultures by 2 h, and this difference increased over time.

**Characterization of PG**—In order to examine the size distribution of the newly synthesized PG and to isolate different PG types, the media and pericellular and intracellular PG pools were chromatographed on Sepharose CL-4B under dissociative conditions (Fig. 3). Three distinct profiles of [35S] radioactivity were obtained which remained similar over time within each pool. For any given PG pool, no major differences in PG distribution were seen between the two cell types. The profiles for media PG were typical of those obtained using 4 M GdnHCl extracts of pigeon artery tissue (26) and showed a large molecular weight PG species which eluted at the Vₐ of the column and a smaller polydisperse species which was included around a Kᵥ of 0.46. The pericellular PG eluted from Sepharose CL-4B as two peaks of radioactivity with a Kᵥ of 0.46 and 0.83. Since these PG were obtained following trypsin treatment of the cells, partial cleavage of the PG core protein may have occurred. Therefore, the profiles shown may represent monomers smaller than native PG. Pericellular PG is the focus of a subsequent investigation and will be presented in a later report. The intracellular fractions contained a small peak of high molecular weight PG eluting at the Vₐ and an included species which, based upon its similar elution position to chondroitin sulfate chains, may be comprised of free GAG chains.
TABLE II

<table>
<thead>
<tr>
<th>Smooth muscle cell type</th>
<th>Incubation time (h)</th>
<th>Total protein* dpm/10^6 cells</th>
<th>Decrease in WC vs. SR %</th>
<th>PG core protein dpm/10^6 cells</th>
<th>Decrease in WC vs. SR %</th>
<th>PG core protein (total protein) %</th>
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<tr>
<td>WC</td>
<td>12</td>
<td>4.5 x 10^6</td>
<td>32</td>
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<td>4.6</td>
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<tr>
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<td>4.6 x 10^6</td>
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</tr>
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<td>2.5 x 10^6</td>
<td>1.6 x 10^7</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>4.8 x 10^6</td>
<td>3.2 x 10^7</td>
<td>6.7</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Nondialyzable (Mr > 3500) radioactivity.

** CPC-precipitable radioactivity.

FIG. 2. Pulse-chase labeling kinetics of PG from WC and SR cells. Confluent 35-mm cultures were labeled with 50 μCi/ml [35S]sulfate for 24 h. The labeling medium was removed, and the cells were incubated for the indicated times in unlabeled medium containing 1 mM Na2SO4, media; 0 and A, pericellular and intracellular fractions, respectively. PG were isolated as trichloroacetic acid precipitates at each time point. Values represent the mean ± S.E. of triplicate cultures. A, SR cells; B, WC cells.

populations increased over time, the relative increase of peak 1 PG in the WC cultures was lower than that of SR peak 1, resulting in a peak 1:peak 2 ratio in WC cells at 60 h of 0.54 (using 35S) and of 0.50 (using 3H) compared to 0.67 (35S) and 0.78 (3H) in SR PG. Therefore, although both populations were reduced in WC compared to SR cultures, a relatively lower proportion of PG comprising peak 1 was present over time.

Following separation of the two major PG on Sepharose CL-4B, fractions were pooled as indicated in Fig. 4; dialyzed against 9.02 mM NaCl, 0.03 M Na2SO4; and precipitated with CPC. To identify the type of PG present, an aliquot of each peak from the cultures labeled for 60 h was digested with papain, and the resulting GAG chains were separated on cellulose acetate (Fig. 6). Autoradiograms indicated that the major sulfated component in peak 1 from Sepharose CL-4B from both cell types co-migrated with the CS standard, whereas a minor component migrated as heparan sulfate. In peak 2, a broad band of sulfated GAG migrating with the DS standard was the predominant species. In addition, small amounts of chondroitin sulfate and heparan sulfate migrating material were detected in this fraction. Differences in the migration position for DS suggest compositional differences or differences in sulfation patterns.

GAG Size and Disaccharide Composition—Aliquots of peaks 1 (CS-PG) and 2 (DS-PG) from Sepharose CL-4B were digested with papain, and the released GAG were isolated on Sepharose CL-6B. For either PG type, WC and SR cultures had similar GAG chain sizes (Fig. 7). Based upon published calibration curves (22), DS chains had an approximate molec-
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Fig. 4. Elution profiles of $^{35}$S-labeled proteoglycans secreted into culture media by 12 h (A (SR) and B (WC)), 36 h (C (SR) and D (WC)), and 60 h (E (SR) and F (WC)) (see Fig. 1). The media from three 60-mm cultures for each time point were exhaustively dialyzed, adjusted to 4 M GdnHCl, and chromatographed on a 60 x 0.9-cm column of Sepharose CL-4B in 4 M GdnHCl, 0.05 M sodium acetate, pH 5.8. The horizontal bars indicate the pools of peaks 1 and 2 used for further analyses.

ular size of 55 kDa, whereas CS chains were >55 kDa.

The GAG chains obtained from Sepharose CL-6B were digested with chondroitinase ABC, and the unsaturated disaccharides were identified (Fig. 8). The digestion products of CS-PG from SR cells contained ΔDi-0S (15%), ΔDi-6S (17%), and a predominance of ΔDi-4S (68%) (Fig. 8A). CS-PG from the WC cells, however, contained low levels of ΔDi-4S (22%) and a predominance of ΔDi-6S (70%) (Fig. 8C). Very little ΔDi-0S (8%) was detected. The products of DS-PG showed similar changes; those from WC cultures had a major ΔDi-6S component (52%) (Fig. 8D), and those from SR cultures had a majority of ΔDi-4S isomers (78%) (Fig. 8B). No disulfated disaccharides were detected in any preparation. These data illustrate major changes in the CS isomers comprising CS-PG of WC compared to SR cells as well as significant shifts in the composition of DS of DS-PG.

PG Monomeric Structure—Although insufficient purified PG was available for core protein analysis, we used a crude (CPC-precipitated media) preparation to compare core proteins of the CS-PG and DS-PG monomers from the WC and SR media (Fig. 9). DS-PG extracted from pigeon arterial tissue has a core protein with approximate molecular mass of 50 kDa (26), whereas the core protein of CS-PG is polydisperse from 200 to 300 kDa. For PG of smooth muscle cells, $^3$H-labeled proteins of 200–300 kDa and a major $^3$H-labeled protein of ~50 kDa appeared following polyacrylamide gel

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electrophoresis of chondroitinase ABC-digested PG. PG core sizes were similar for both cell types.

Overall sulfation of the CS-PG and DS-PG monomers was compared by using PG labeled with \[^{35}\text{S}\]sulfate and \[^{3}\text{H}\]serine. For the estimate, we have assumed that the serine content per core protein and serine specific activity are similar within a PG. For any given PG, we have demonstrated similarity in core protein sizes between WC and SR cells (Fig. 9). Ratios of \[^{35}\text{S}\]sulfate to \[^{3}\text{H}\]serine calculated for PG monomers showed that per a standard size of monomer, DS-PG is relatively more sulfated compared to CS-PG (Table III). This suggests that conditions which favor DS-PG accumulation impart more electronegativity to a matrix.

Since we demonstrated a larger chain size for CS comprising CS-PG compared to DS of the DS-PG monomers (Fig. 7), the higher \[^{35}\text{S}\]sulfate/\[^{3}\text{H}\]serine ratio in DS suggested either a greater number of chains per given unit of core protein or an increased sulfation of the chain disaccharides. We investigated a possible increased sulfation of CS or DS using two radioactive labels for the GAG chain. For this study, WC and SR smooth muscle cells were incubated for 48 h with \[^{35}\text{S}\]sulfate and \[^{3}\text{H}\]glucosamine. In order to separate CS- and DS-containing PG from heparan sulfate PG and hyaluronic acid, radiolabeled media PG were separated by a continuous sodium chloride gradient on DEAE-Sephacel. Small amounts of hyaluronic acid and heparan sulfate PG were separated from the major CS-DS peak (Fig. 10). The pooled fractions of the CS-DS peak had \[^{35}\text{S}:^{3}\text{H}\] ratios of 0.20 in WC-derived PG and 0.16 in SR-derived PG. On Sepharose CL-4B, 68-74\% \(^{35}\text{S}\) and 67-74\% \(^{3}\text{H}\) of the WC CS-DS peak was isolated as DS compared to 56-64\% \(^{35}\text{S}\) and 52-60\% \(^{3}\text{H}\) of the SR CS-DS peak. The radioactivity of these PG is shown in Table IV and indicates a higher \[^{35}\text{S}:^{3}\text{H}\] ratio for both PG types from WC cells. A greater percent of DS-PG in the CS-PG/DS-PG mixture in the WC compared to SR cells would impart a greater overall electronegativity to the entire mixed population of PG produced by the WC cells.

With regard to the position of sulfation, the results of disaccharide analysis showed that both CS-PG and DS-PG from WC cells differed from the respective PG in SR smooth muscle cells. For the two individual PG types, the sulfation of individual GAG chains was examined in an additional experiment. In this study, GAG were prepared following papain treatment of PG radiolabeled with \[^{35}\text{S}\]sulfate and \[^{3}\text{H}\]glucosamine. For CS-PG, \[^{35}\text{S}:^{3}\text{H}\] ratios were 0.055 for WC cells and 0.045 for SR cells. This finding of greater sulfation of CS in WC compared to SR cells agrees with the results of the analysis of disaccharides (see Fig. 8) since a greater proportion of unsulfated chondroitin was observed in GAG from SR cells. For DS, similar \[^{35}\text{S}:^{3}\text{H}\] ratios were seen for WC (0.056) and SR (0.056) cells, suggesting a similar degree of sulfation of the repeating disaccharide units. The fact that the DS-PG monomer of WC cells had higher ratios than that of SR cells (Table IV) suggests basic structural differences in the monomer, for example, that there are other radiolabeled oligosaccharides containing glucosamine convolutely linked to the core protein (27). These may be present in lesser amounts in DS-PG of WC cells.

**DISCUSSION**

The purpose of this study was to compare PG metabolism in arterial smooth muscle cells of atherosclerosis-susceptible WC and -resistant SR pigeons. Cultures prepared from the aortas of newly hatched birds were used in order to examine
with chondroitinase ABC. The core proteins were reduced with chondroitin ABC lyase-treated SR PG. The migration position

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there is a difference in monomer composition favoring 6-sulfation in SR cells; and

WC and SR smooth muscle cells were compared at confluence. In all experiments, total PG production was estimated for both cell lines; and although this contributed significantly to the lower PG content in WC cultures, a 37% reduction in synthesis was also a factor. These findings are in marked contrast to a study reported by Wight (33), where a greater incorporation of $^3$S into GAG of both the media and cell pellets was reported for aortic explants of WC compared to SR cells. In spite of minor differences in experimental design and the use of different passages of cells, the opposite finding between the two studies remains unexplained. Our findings do confirm our previous study (6) on PG synthesis by aortic tissue segments. In that study, at four different ages studied, both $[^3]$S sulfate incorporation and hexuronic acid determinations indicated a lower synthesis and artery content of PG in WC compared to SR tissues. Sites of lesion predilection in the distal thoracic aorta in WC cells prior to actual development of atherosclerosis had the lowest PG synthesis. When atherosclerosis developed, there were increases in PG synthesis and content related to the extent of atherosclerosis. This agrees with reports (21, 35–37) demonstrating a positive correlation of GAG concentration and atherosclerosis. We suggest, from our observations, that reduced PG synthesis in WC smooth muscle cells precedes and may be a responsible factor for atherosclerosis progression.

Our data indicate that CS-PG and DS-PG are the predominant PG secreted into the medium by both cell types. Additionally, lesser amounts of heparan sulfate PG and hyaluronic acid are produced. Although both CS-PG and DS-PG production is reduced in WC cultures, CS degradation in WC cultures resulted in a lower CS-PG:DS-PG ratio in these cultures. The difference in CS-PG processing by the WC cells may result from differences in PG structure and synthesis. Whereas our data indicate similarities both in core protein and in GAG chain sizes for any given PG type, a difference in sulfation position of both the CS and DS chains from the two cell types was noted. Disaccharide analysis indicated that at 48 h, $\Delta$Di-6S was the major disaccharide in CS in WC cells, whereas $\Delta$Di-4S was predominant in CS of SR cells. Low levels of $\Delta$Di-4S in CS-PG of the WC cells suggest that there may be less of some GAG chains enriched in 4-sulfated N-acetylgalactosamine, loss of GAG chains exclusively sulfated in at C-4, or loss of a specific type of CS-PG monomer containing exclusively chondroitin 4-sulfate chains. For both types of cells, when GAG chains from CS-PG were chromatographed on Sepharose 6B, a similar GAG size and no evidence of low molecular weight chains were observed. Loss of chains enriched in chondroitin 4-sulfate does not appear to be likely since this would imply that there is selective turnover of some GAG chains within a PG monomer, which is inconsistent with the present knowledge of PG degradation. Disaccharide composition of CS-PG at earlier times than 36 h of incubation has not been examined. We therefore cannot conclude that the lower level of CS-PG sulfated at C-4 in WC cells is attributed to a loss over time or whether CS-PG initially synthesized has a composition favoring 6-sulfation of N-acetylgalactosamine.

In DS-PG, the presence of significant levels of $\Delta$Di-6S following chondroitinase ABC treatment in WC cells was surprising since a majority of reports of DS-PG from a variety of sources show DS to be sulfated primarily in at C-4 of N-acetylgalactosamine. However, DS-PG of tissues such as skin contain primarily only one GAG chain covalently linked to the core protein (38), whereas the majority of artery DS-PG appear to have more than one GAG chain/core protein (39). It is therefore possible that one of these chains is sulfated at C-6 of N-acetylgalactosamine. There also remains the possibility that in WC cells CS-PG enriched in chondroitin 6-sulfate is partially degraded over time and becomes coisolated with the DS-PG preparation.

Since differences in transit time through intracellular com-

TABLE III


Counts are taken from Fig. 5. The data were obtained from sample pools at each time point shown in Fig. 1.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Incubation time</th>
<th>CS-PG</th>
<th>DS-PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>WC</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.019</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>0.020</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>60</td>
<td>0.021</td>
<td>0.029</td>
<td></td>
</tr>
<tr>
<td>SR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.019</td>
<td>0.028</td>
<td></td>
</tr>
<tr>
<td>36</td>
<td>0.019</td>
<td>0.027</td>
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<td>60</td>
<td>0.019</td>
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</tbody>
</table>

FIG. 9. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography of PG core proteins. PG from culture media at 48 h were precipitated with CPC and hydrolyzed with chondroitinase ABC. The core proteins were reduced with $\beta$-mercaptoethanol and electrophoresed on a 7.5% polyacrylamide gel. $[^3]$H serine incorporated into the core protein was detected by autoradiography. Lane 1, intact PG from WC cells; lane 2, intact PG from SR cells; lane 3, chondroitin ABC lyase-treated WC PG; lane 4, chondroitin ABC lyase-treated SR PG. The migration position of protein standards is indicated. Major bands are observed with molecular sizes of 50 and $>200$ kDa. Minor bands represent contaminating radiolabeled proteins trapped in the PG-CPC complex.
The media fractions from cultures labeled for 36 h were dialyzed against 0-1 M NaCl in the urea/Tris buffer. The elution volume was 200 ml, and 3-ml fractions were collected and analyzed for radioactivity. The peaks of radioactivity were identified by susceptibilities to specific GAG-degrading enzymes. A, SR cells; B, WC cells.

![Figure 10. Elution profile of [35S]sulfate (●)- and [3H]glucosamine (○)-labeled PG on DEAE-Sephadex.](image)

**TABLE IV**

| Cell type | Intact PG, Dpm/10^6 cells | CS/DS | 35S | 3H | 35S|3H |
|-----------|--------------------------|-------|-----|----|----|--|---|
| CS-PG     |                         |       |     |    |    |    |   |
| WC-1      | 4.2                      | 21.3  | 0.20|    |    |    |   |
| WC-2      | 5.1                      | 27.1  | 0.19|    |    |    |   |
| SR-1      | 5.6                      | 39.1  | 0.14|    |    |    |   |
| SR-2      | 5.0                      | 33.1  | 0.15|    |    |    |   |
| DS-PG     |                         |       |     |    |    |    |   |
| WC-1      | 12.2                     | 59.8  | 0.21|    |    |    |   |
| WC-2      | 10.7                     | 54.0  | 0.20|    |    |    |   |
| SR-1      | 7.3                      | 42.9  | 0.17|    |    |    |   |
| SR-2      | 8.9                      | 50.8  | 0.18|    |    |    |   |

*1 and 2 refer to duplicate cultures.

*Values are Dpm x 10^-4.

Proteoglycans of Artery Smooth Muscle Cells

**Acknowledgments**—We would like to thank Drs. J. S. Parks, T. C. Register, and R. W. St. Clair for their critical reviews of the manuscript.

**REFERENCES**


Portions of the nucleus are believed to affect post-translational processing of PG, the slower appearance of secreted PG in WC cultures (Fig. 2) and the structural and compositional differences noted suggest a longer intracellular processing time in WC cells. Sulfation and chain elongation have been shown to be functions of the cis Golgi, whereas epimerization and 4-sulfation are functions of the trans Golgi (40). A longer dwelling time in WC PG was not different from that of SR PG, but greater 6-sulfation was observed. The enhanced 6-sulfation may effectively reduce the amount of substrate PG available for the C-5 uronosyl epimerase in the trans Golgi (41). In WC cells, the enhanced level of chondroitin 6-sulfate may therefore lead to the production of a DS-PG molecule with low levels of iduronic acid and 4-sulfation.

The significance of the increased level of chondroitin 6-sulfate to atherosclerosis susceptibility in WC pigeons is not fully understood at present, but several reports allude to its possible importance. Curwen and Smith (42) studied GAG extracted from WC and SR aortic tissue and found that the lesion-prone region of the thoracic aortas of WC pigeons became enriched with chondroitin 6-sulfate at 2 years of age when lesions developed. Similar segments in SR arteries which remained lesion-free retained a predominance of chondroitin 4-sulfate. An intriguing link to CS isomer predominance and lesion development is suggested by studies of the physical changes in core lipids of human LDL resulting from LDL-GAG interaction (43). Complexing LDL to chondroitin 4-sulfate did not affect physical changes in the LDL particle, whereas the interaction of LDL with chondroitin 6-sulfate resulted in an elevation in LDL lipid core transition temperature, thus allowing a mesomorphic liquid-crystalline state to exist at 37 °C. These events in the artery wall could lead to the accumulation of lipid in a physical state which impairs its metabolism and efflux. Cholesterol deposits that are liquid-crystalline are typically found in atherosclerotic lesions (44).

If PG metabolism by smooth muscle cells in culture is a reflection of the function of the cell in the artery wall, our observations on cells of the atherosclerosis-susceptible WC pigeons may have important pathological consequences. A reduction in PG production could lead to a matrix lacking structural integrity and allow increased permeability to plasma lipoproteins. A reduction or alteration of CS-PG, the PG responsible for the formation of high molecular weight aggregates with hyaluronic acid, could be of particular importance in diminishing the resistance of the tissue to compressible forces. Finally, in the artery wall the PG composition favors a more electronegative PG, the capacity for LDL retention will increase. Specific tissue locations are often dominated by one species of PG (34); and in the artery wall, this could result in pockets of more highly charged PG to provide an environment conducive to LDL binding and accumulation.

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