Enhanced synthesis and accumulation of proteoglycans in cholesterol-enriched arterial smooth muscle cells

Parakat VIJAYAGOPAL

Departments of Medicine and Anatomy, Louisiana State University Medical Center, New Orleans, LA, U.S.A.

To determine the effects of lipid accumulation on proteoglycan synthesis, we studied proteoglycan biosynthesis in rabbit aortic smooth muscle cells in culture. Cholesterol-enrichment was accomplished by incubating confluent smooth muscle cells with cationized low-density lipoprotein. Control and cholesterol-enriched cells were incubated with [^{35} S]sulphate, [^{3} H]glucosamine, or [^{3} H]serine. Metabolically labelled proteoglycans in the cell layer and medium were quantified. During a 20 h incubation period, proteoglycan synthesis in cholesterol-enriched cells increased by 40–50% above that in control cells. A similar increase in precursor incorporation into proteoglycans was also noted following a short 15 min pulse. The cholesterol-enriched cells also showed a 45–50% increase over control rates in the intralysosomal accumulation of a large chondroitin sulphate proteoglycan and a small dermatan sulphate proteoglycan. The enhanced synthesis of proteoglycans in cholesterol-enriched

cultures was inhibited by cycloheximide and actinomycin D, which are inhibitors of protein synthesis and transcription respectively. Proteoglycan turnover was investigated by pulse-chase analysis. Following a 2-h pulse, intracellular proteoglycans in cholesterol-enriched cells disappeared, having a half-life of 26.5 h compared with 2.8 h for those in the control cells. The amount of trypsin-releasable proteoglycan was significantly reduced in cholesterol-enriched cells. In addition, the degradation of proteoglycans was severely retarded in cholesterol-enriched cultures. The activities of three acid hydrolases, *N*-acetyl- β -hexosaminidase, β -glucuronidase and cathepsin C, were significantly reduced in cholesterol-enriched cells compared with activities in control cells. The results indicate that proteoglycan metabolism is altered in cholesterol-enriched smooth muscle cells.

INTRODUCTION

Proteoglycans are large molecules containing one or more glycosaminoglycan chains covalently linked to a protein core. They are widely distributed in all connective tissues including the blood vessel wall. In the vessel wall, proteoglycans provide structural support, regulate tissue permeability and influence viscoelasticity and haemostasis [1–3]. In addition, based on studies conducted *in vitro*, proteoglycans might also play a role in facilitating lipid deposition in atherosclerosis [4–9].

Vascular smooth muscle cells in culture synthesize proteoglycans. Even though the major proteoglycans synthesized by these cells have been characterized [10,11], the pattern of proteoglycan synthesis (both composition and quantity) by smooth muscle cells fluctuates with the physiological state of the cells. Thus, factors such as the age of the cells, state of confluency and culture conditions can modulate proteoglycan synthesis by smooth muscle cells in culture [1].

Several other factors are capable of modulating proteoglycan metabolism by vascular cells in the absence of cell proliferation. For example, Chen et al. [12] and Schonherr et al. [13] recently reported that transforming growth factor- β stimulates proteoglycan synthesis by arterial smooth muscle cells without stimulating cell proliferation. I and others have reported that endothelial cell-conditioned medium and heparin also stimulate the synthesis of proteoglycans by smooth muscle cells [14–17].

Accumulation of lipids, notably cholesteryl ester, in macrophages and smooth muscle cells is an important initial event in atherosclerosis. Proteoglycans also accumulate in the lesions in early and late atherosclerosis. However, it is not known whether these two events are unrelated or whether cholesterol deposition

induces phenotypic changes in smooth muscle cells resulting in increased proteoglycan production. Therefore, in the present study, we have examined the effect of cholesterol enrichment of rabbit aorta smooth muscle cells on the synthesis of proteoglycans by the cells. The results indicate that compared with control cells, proteoglycan metabolism is altered in cholesterol-enriched smooth muscle cells.

MATERIALS AND METHODS

Materials

Na₂³⁵SO₄ (43 Ci/mg of sulphate) and [^{3} H]glucosamine (25 Ci/mmol) were purchased from ICN Radiochemicals (Irving, CA, U.S.A.). L-[G- ^{3} H]Serine (20 Ci/mmol) was obtained from American Radiochemicals (St. Louis, MO, U.S.A.). Dulbecco's modified Eagle's medium (DMEM) containing 4500 mg/l glucose and fetal bovine serum were from Gibco Laboratories (Grand Island, NY, U.S.A.). Tissue-culture plasticware was obtained from Corning (Corning, NY, U.S.A.). Trypsin, chondroitin ABC lyase (from *Proteus vulgaris*), chondroitin AC II lyase (from *Atherobacter aureus*), guanidinium chloride, Sephadex G-50, DEAE-Sephacel, Sepharose CL-4B, octyl-Sepharose and the different substrates for the assay of lysosomal enzymes were obtained from Sigma Chemical (St. Louis, MO, U.S.A.).

Low-density lipoprotein (LDL) and cationized LDL

LDLs ($d = 1.019$ – 1.063 g/ml) were isolated from pooled human serum by sequential ultracentrifugation [18]. Antioxidants, EDTA and butylated hydroxytoluene, were added to serum

immediately after collection. Cationized LDL was prepared by covalent attachment of *N,N*-dimethyl-1,3-propanediamine to LDL using 1-ethyl-3-(3-diethylaminopropyl)carbodiimide/HCl as catalyst [19]. Cationized LDL migrated toward the cathode in agarose-gel electrophoresis at pH 8.6. Lipoproteins were dialysed against 0.15 M NaCl/0.01 % EDTA, pH 7.4, and sterilized by membrane filtration (0.22 μ m pore diam.).

Smooth muscle cell culture

Primary cultures of rabbit thoracic aorta smooth muscle cells were established by the explant technique [20]. Briefly, aortas were obtained from New Zealand White rabbits. Explants of the vessel media were plated in 60-mm-diam. culture dishes in DMEM containing 20 % (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μ g/ml streptomycin and 2.5 ml/l amphotericin B. Cells began to grow out from the explants in 6–7 days and became confluent in 2–3 weeks. The explants were then removed and subcultures established in 35-mm-diam. culture dishes. Smooth muscle cells between passages four and eight were used in the studies. Cholesterol enrichment of the cells was achieved by incubating confluent smooth muscle cells in DMEM containing 10 % (v/v) fetal bovine serum and 100 μ g/ml cationized LDL for 7 days [21]. The medium was replaced with fresh medium containing cationized LDL every 3 days.

Radiolabelling of proteoglycans

The medium was removed from control and cholesterol-enriched confluent smooth muscle cell cultures. The cells were rinsed twice with PBS. Fresh DMEM containing 20 μ Ci/ml [^{35} S]sulphate and 10 μ Ci/ml [^{3} H]glucosamine was added to each dish and the cells were incubated for different times at 37 °C. In some experiments the proteoglycans were labelled with [^{35} S]sulphate (20 μ Ci/ml) and [^{3} H]serine (20 μ Ci/ml).

Isolation of proteoglycans

At the end of the labelling period the culture medium was harvested from each 35-mm-diam. dish and chilled on ice. The cell layer was quickly rinsed with 1 ml of ice-cold DMEM and the rinsing solution was combined with the medium fraction. Representative cultures were then either trypsinized and counted for cell number or dissolved in 0.4 M NaOH and used for protein assay. Solid guanidinium chloride was added to the medium fraction to a final concentration of 4 M together with protease inhibitors (0.1 M ϵ -aminohexanoic acid, 10 mM *N*-ethylmaleimide, 1 mM phenylmethanesulphonyl fluoride, 20 mM EDTA and 50 mM benzamidinium hydrochloride).

The cell layer in each dish was extracted with 2 ml of 4 M guanidinium chloride/0.05 M sodium acetate, pH 5.8, containing 0.1 % Triton X-100 and protease inhibitors (as listed above) for 24 h at 4 °C. This procedure removed almost all (> 98 %) of the proteoglycans from the cell layer and there was no difference in extractability between control and cholesterol-enriched cultures.

Unincorporated radioactive precursors were removed from the medium and cell-layer extracts by chromatography on a Sephadex G-50 column (1 cm $\times$ 30 cm). The column was eluted with 8 M urea/0.15 M NaCl/0.05 M sodium acetate, pH 6.0, containing 0.5 % (w/v) Triton X-100 and protease inhibitors. The excluded material containing labelled proteoglycans was then applied to a column of DEAE-Sephacel (bed volume of 3 ml) equilibrated in the same buffer used in the Sephadex G-50 chromatography. The column was washed with 30 ml of the same buffer at a flow rate of 5 ml/h to remove labelled glyco-

proteins. Labelled proteoglycans were then eluted with a continuous NaCl gradient (from 0.15 to 1.0 M) in the same buffer, using a total volume of 60 ml. Fractions (1 ml) were collected at a flow rate of about 3 ml/h and counted for ^{35} S and ^{3} H radioactivity. Conductivity of each fraction was also monitored. Two radiolabelled proteoglycan peaks eluted at 0.47 M NaCl and 0.58 M NaCl respectively for both medium and cell layer of control and cholesterol-enriched cultures. The peaks were either separately pooled or in some cases combined and dialysed against distilled water at 4 °C. Carrier chondroitin sulphate (500 μ g) was added to the dialysates and proteoglycans were precipitated with 1 % (w/v) 1-hexadecylpyridinium chloride (HPC) for 24 h at 25 °C. The proteoglycans were then re-isolated from their HPC complexes [22] and dissolved in distilled water. An aliquot was counted for radioactivity and the remainder was lyophilized.

Analysis of proteoglycans

The purified proteoglycans were treated with alkaline borohydride to remove the protein core [23] and analysed for their component glycosaminoglycans. The individual glycosaminoglycans were determined by treatment with nitrous acid [24], chondroitin ACII lyase and chondroitin ABC lyase [25]. The digests were eluted from a column of Sepharose CL-6B (0.6 cm $\times$ 90 cm) with 0.5 M sodium acetate, pH 7.0. Fractions (1 ml) were analysed for radioactivity. The relative proportions of heparan sulphate, dermatan sulphate and chondroitin sulphates were calculated from the proportion of radiolabelled material resistant to, or degraded by, each treatment.

Proteoglycans that accumulated in the cell layer of control and cholesterol-enriched smooth muscle cell cultures were analysed by a combination of gel-filtration chromatography and by hydrophobic chromatography. Briefly, cholesterol-enriched smooth muscle cells grown in 25 cm² culture flasks were incubated with 20 μ Ci/ml [^{35} S]sulphate for 24 h. Labelled proteoglycans were extracted from the cell layer and purified by Sephadex G-50 chromatography followed by DEAE-Sephacel chromatography as described earlier. The purified proteoglycans were dialysed against 4 M guanidinium chloride/0.05 M sodium acetate, pH 6, containing protease inhibitors and applied to a Sepharose CL-4B column (0.9 cm $\times$ 100 cm). The column was eluted with the above dialysis medium containing 0.5 % Triton X-100 at a flow rate of 10 ml/h. Fractions (1 ml) were analysed for ^{35} S radioactivity and peak fractions were separately pooled. Two major proteoglycan peaks were eluted, one near the V_0 and the other in the inclusive volume of the column. Proteoglycans in the second peak were further analysed by octyl-Sepharose chromatography [26]. A column (3 ml) of octyl-Sepharose was equilibrated with 3 M guanidinium chloride/0.15 M sodium acetate, pH 6.3, at room temperature. The proteoglycan fraction from Sepharose CL-4B chromatography was dialysed against the 3 M guanidinium chloride buffer and applied to the octyl-Sepharose column. After 2 h, the column was eluted with 3 column vol. of 3 M guanidinium chloride buffer and then with a linear gradient of 0–1 % CHAPS prepared in 3 M guanidinium chloride buffer in a total volume of 50 ml, at a flow rate of 3 ml/h. Fractions (1 ml) were analysed for radioactivity.

Pulse-chase studies

The turnover of proteoglycans in control and cholesterol-enriched smooth muscle cells was investigated by pulse-chase analysis. Confluent smooth muscle cells in 35-mm-diam. culture dishes were cholesterol loaded as described above. Control and

cholesterol-enriched cells were then incubated in DMEM containing 50 $\mu\text{Ci/ml}$ [^{35}S]sulphate for 2 h at 37 °C. The medium was removed and the cell layers were washed six times with DMEM containing 0.5 mM Na_2SO_4 . The cultures were then chased in isotope-free DMEM for up to 8 h. After each chase period proteoglycans were extracted from the cell layer and medium as described above. The cell-layer extract was dialysed exhaustively against 0.15 M NaCl/0.05 M Na_2SO_4 , pH 7.4, containing protease inhibitors. Proteoglycans were precipitated from the dialysates as described before and counted for radioactivity. The extracts from the medium were chromatographed on a column of Sephadex G-50 in the dialysis buffer used for cell extracts. The material eluting at the V_t of the column representing free [^{35}S]sulphate generated by intracellular degradation of labelled proteoglycans was quantified. The macromolecular proteoglycans excluded from the Sephadex column were precipitated as described above and quantified.

In some cases, at the end of the 2-h pulse, the cells were washed with serum-free medium and treated with trypsin (50 $\mu\text{g/ml}$) for 10 min at 37 °C. The trypsin solution was removed and mixed with an equal volume of DMEM containing 20% (v/v) fetal bovine serum. Trypsin-releasable proteoglycans were then quantified. The cell layer was washed with soybean trypsin inhibitor (500 $\mu\text{g/ml}$) and the trypsin-resistant proteoglycans were extracted and quantified.

Preparation of radiolabelled proteoglycans

Normal smooth muscle cells grown to confluency in 75 cm^2 flasks were incubated with 20 $\mu\text{Ci/ml}$ of [^{35}S]sulphate in DMEM containing 5% (v/v) fetal bovine serum for 48 h at 37 °C. The culture medium was removed and dialysed exhaustively against 0.15 M NaCl/0.05 M Na_2SO_4 containing protease inhibitors at 4 °C. The dialysate was concentrated by ultrafiltration and radiolabelled proteoglycans were isolated by DEAE-Sephacel chromatography. The proteoglycan peaks were pooled and proteoglycans were precipitated with HPC as described earlier.

Endocytosis and degradation of radiolabelled proteoglycans

Confluent smooth muscle cell cultures in 35-mm-diam. culture dishes were cholesterol loaded. Control and cholesterol-enriched cells were incubated in 1 ml of DMEM containing ^{35}S -labelled proteoglycan isolated as described above (2.4×10^5 d.p.m./ml) for various periods (30 min to 24 h). Endocytosis and degradation of labelled proteoglycans were assayed as described by Kresse et al. [27]. Briefly, at each time period, the medium was removed from duplicate dishes and the cell layer was washed exhaustively with calcium- and magnesium-free Hanks' solution. The cells were detached with trypsin and sedimented. The cell pellet was washed with 0.15 M NaCl and assayed for radioactivity.

The culture medium was mixed with 3 vol. of ethanol. The solution was left for 3 h at room temperature and the precipitated proteoglycans were sedimented by centrifugation. Radioactivity in the ethanol supernatant was measured and taken as a measure of degraded proteoglycans.

Endocytosis represents the sum of intracellular radioactivity and ethanol-insoluble radioactivity of the culture medium. Both endocytosis and degradation data were corrected for blank values obtained from parallel incubations conducted in dishes without cells.

Protein synthesis

Protein synthesis in smooth muscle cells was measured by determining the incorporation of [^3H]leucine into secreted and

cellular proteins [28]. Briefly, control and cholesterol-enriched smooth muscle cells were incubated in DMEM containing 10 $\mu\text{Ci/ml}$ of [^3H]leucine. The medium was removed and dialysed against 0.15 M NaCl/0.001 M leucine and proteins were precipitated by the addition of an equal volume of cold trichloroacetic acid (15%). The cell layer was washed and dissolved in 0.05% Triton X-100 followed by sonication. The cellular proteins were precipitated with trichloroacetic acid. The medium-derived and cellular proteins were washed three times with trichloroacetic acid and dissolved in 1 M NaOH. Aliquots were counted for ^3H radioactivity.

Subcellular fractionation of cholesterol-enriched cells

Cholesterol-enriched confluent smooth muscle cells were incubated with ^{35}S -labelled proteoglycan isolated from the medium of normal smooth muscle cell cultures. After 12 h, the cells were washed and harvested and a post-nuclear supernatant was prepared [29]. The post-nuclear supernatant was then sub-fractionated on a Percoll gradient ($d = 1.065$ g/ml) [29], prepared without the sucrose cushion [30]. The gradient was centrifuged for 40 min at 20000 rev./min in a Beckman VTi50 rotor. Fractions (2 ml) were collected and analysed for radioactivity and their densities were measured.

Assays of lysosomal enzymes

Assays of the lysosomal enzymes *N*-acetyl- β -hexosaminidase (EC 3.2.1.30), β -glucuronidase (EC 3.2.1.31) and acid phosphatase (EC 3.1.3.2) were performed on cell homogenates as described by Seymour and Peters [31], and cathepsin C (EC 3.4.14.1) was assayed by the procedure of Peters et al. [32] as modified by Haley et al. [33]. All assays contained 0.1% Triton X-100. Control and cholesterol-enriched smooth muscle cells in 60-mm-diam. culture dishes were washed and scraped off the dishes with a rubber policeman in ice-cold medium (pH 7.2) containing 0.25 M sucrose, 1 mM EDTA and 22 mM ethanol. The cell suspension was homogenized and stored at -20 °C before use in enzyme assays. For all enzymes 1 unit of activity represents the hydrolysis of 1 μmol of substrate/min at 37 °C.

Analytical methods

Protein concentration was determined by a modified Lowry procedure [34]. Cellular cholesterol content was determined fluorometrically [35].

RESULTS

Effect of cholesterol enrichment of smooth muscle cells on cellular cholesterol and protein contents and cell proliferation and viability

Smooth muscle cells were grown to confluency in 35-mm-diam. culture dishes and then incubated with 100 $\mu\text{g/ml}$ cationized LDL for 7 days. Table 1 compares cell number, total cellular protein content, and free and esterified cholesterol levels in control and cholesterol-enriched cells. There was no difference in the cell number between control and cholesterol-enriched cells. Total cell protein and esterified cholesterol were increased significantly in cells incubated with cationized LDL as compared with control cells ($P < 0.001$). The increase in levels of esterified cholesterol was 10-fold. Free cholesterol concentrations increased slightly. Cells exposed to cationized LDL for 7 days showed the same viability as control cells ($> 95\%$).

Table 1 Effect of cholesterol enrichment of smooth muscle cells on cell number, total cellular protein and cholesterol content

Smooth muscle cells were incubated in DMEM containing 10% (v/v) fetal bovine serum in the presence and absence of cationized LDL (100 µg/ml). After 7 days at 37 °C, cell number and cellular protein and cholesterol contents were determined. Values are the mean ± S.E.M. of triplicate cultures. Statistical significance: *P < 0.001.

	10 ⁻⁵ × Cell number	Total protein (µg)	Cholesterol (µg/mg of protein)	
			Free	Esterified
Control	5.8 ± 0.3	70.9 ± 3.2	15.3 ± 2.1	1.2 ± 0.3
Cholesterol-enriched	5.6 ± 0.4	104.8 ± 6.5*	18.8 ± 3.5	11.4 ± 2.5*

Table 2 Effect of cholesterol enrichment of smooth muscle cells on the incorporation of [³⁵S]sulphate and [³H]glucosamine into proteoglycans

Control and cholesterol-enriched smooth muscle cells were incubated with [³⁵S]sulphate and [³H]glucosamine for 20 h and radiolabelled proteoglycans were isolated and quantified. Numbers are d.p.m. × 10⁻⁴/10⁵ cells ± S.D. of triplicate cultures. Significant difference from control (*, *P* < 0.001; †, *P* < 0.005; ‡, *P* < 0.01).

	Control		Cholesterol-enriched	
	[³⁵ S]Sulphate	[³ H]Glucosamine	[³⁵ S]Sulphate	[³ H]Glucosamine
Cell layer	0.97 ± 0.03	1.54 ± 0.80	4.5 ± 0.50*	6.83 ± 0.12*
Medium	2.6 ± 0.40	3.63 ± 0.15	0.71 ± 0.05†	1.28 ± 0.12‡
Cell layer + medium	3.6 ± 0.42	5.17 ± 0.15	5.2 ± 0.53‡	8.11 ± 0.14*

Table 3 Incorporation of [³⁵S]sulphate and [³H]serine into proteoglycans after short-term pulse

Cultures were incubated in DMEM containing 20 µCi/ml each of [³⁵S]sulphate and [³H]serine for 15 min. Proteoglycans were isolated and quantified. Results represent the mean ± S.D. of triplicate assays. Significantly different from control (*P < 0.001).

Cells	Incorporation of label (d.p.m./10 ⁵ cells)	
	[³⁵ S]Sulphate	[³ H]Serine
Control	1796 ± 86	2632 ± 110
Cholesterol-enriched	2557 ± 54*	3664 ± 122*

Effect of cholesterol enrichment on the incorporation of labelled precursors into proteoglycans

Control and cholesterol-enriched smooth muscle cells were incubated with [³⁵S]sulphate and [³H]glucosamine for 20 h and the radiolabelled proteoglycans were isolated and quantified. In control cultures the cell-layer proteoglycans contained approx. 30% of ³⁵S and ³H radioactivities, while the medium-derived proteoglycans contained 70%. In contrast, in cholesterol-enriched cultures this distribution was reversed, with the cell layer containing 84–87% of the radioactivities and the medium 13–16%.

Table 2 shows the incorporation of [³⁵S]sulphate and [³H]glucosamine into proteoglycans of the cell layer and the

medium in control and cholesterol-enriched smooth muscle cells. Compared with control cultures, in cholesterol-enriched cultures there was a 4.6-fold increase for ³⁵S-labelled proteoglycans in the cell layer, a 3.7-fold decrease of levels in the medium with an overall increase of 1.4-fold. The corresponding values for ³H-labelled proteoglycans were a 4.3-fold increase in the cell layer, a 3-fold decrease in medium, with an overall increase of 1.5-fold. Thus, cholesterol enrichment of smooth muscle cells produced a 40–50% increase in the incorporation of labelled precursors into proteoglycans and a major shift in the distribution of the proteoglycans between the cell layer and the medium.

We also measured the incorporation of [³H]serine into proteoglycan core protein. Following incubation with [³H]serine for 20 h, there was a 53% increase over control rates in the incorporation of radioactivity into total (cell layer and medium) proteoglycans in cholesterol-enriched cultures (d.p.m. × 10⁻⁵/10⁵ cells ± S.D.: control, 1.5 ± 0.2; cholesterol-enriched, 2.3 ± 0.25). Cycloheximide (10 ng/ml) and actinomycin D (50 ng/ml) abolished the increase in [³H]serine and [³⁵S]sulphate incorporation into proteoglycans in cholesterol-enriched cells (results not shown).

To determine whether the increase in the level of precursor incorporation into proteoglycans observed during the long-term labelling was due to increased proteoglycan synthesis, cells were exposed to labelled precursors for 15 min and proteoglycan synthesis was quantified (Table 3). In cholesterol-enriched cells, [³⁵S]sulphate and [³H]serine incorporation into newly synthesized proteoglycans were increased respectively by 42% and 39% over levels in control cells. After the 15-min pulse, the majority of the labelled proteoglycans was intracellular (92–95% for control cells and 97–98% for cholesterol-enriched cells), indicating that very few of the newly synthesized proteoglycans were secreted. These studies thus showed that proteoglycan synthesis was elevated significantly in cholesterol-enriched cells.

Analysis of proteoglycans

On a column of DEAE-Sephacel the radiolabelled proteoglycans in the cell-layer and medium compartments of control and cholesterol-enriched cell cultures resolved into two peaks, one eluting at 0.47 M NaCl and the other at 0.58 M NaCl. This indicated that there was no over-sulphation of proteoglycans in cholesterol-enriched cultures. Peak 1 for the cell layer contained 10–15% of the total ³⁵S and ³H radioactivities incorporated into proteoglycans and peak 2 contained the remainder. The corresponding values for medium proteoglycans were: peak 1, 6–10%; and peak 2, 90–94%. There was no difference between control and cholesterol-enriched cells in the relative proportion of radioactivity in the two peaks. The proteoglycans were analysed for their glycosaminoglycan composition. Almost all of the ³⁵S radioactivity in peak 1 from the cell layer and medium of control and cholesterol-enriched cells was susceptible to degradation with nitrous acid, indicating that the proteoglycan contained predominantly heparan sulphate chains. Peak 2 proteoglycans were resistant to nitrous acid treatment; but about 86–90% of the ³⁵S radioactivity was susceptible to chondroitin AC lyase digestion. The remainder was susceptible to chondroitin ABC lyase. Therefore, proteoglycans in peak 2 from control and cholesterol-enriched cells contained predominantly chondroitin sulphate chains and a small proportion (10–14%) of dermatan sulphate chains.

The cell-associated chondroitin sulphate–dermatan sulphate proteoglycans isolated by DEAE-Sephacel chromatography were further analysed by a combination of Sepharose CL-4B chromatography and octyl-Sepharose chromatography.

Figure 1 Octyl-Sepharose chromatography of dermatan sulphate proteoglycans

Dermatan sulphate proteoglycans from control (a) and cholesterol-enriched (b) cells obtained by Sepharose CL-4B chromatography were subjected to octyl-Sepharose chromatography. The column was eluted with a linear gradient of 0–1% CHAPS in 3 M guanidinium chloride/0.15 M sodium acetate, pH 6.3 at room temperature. Fractions were analysed for radioactivity.

Sepharose CL-4B chromatography resolved the proteoglycans from control and cholesterol-enriched smooth muscle cells into two fractions, a large proteoglycan ($K_{av} = 0.17$) and a small proteoglycan ($K_{av} = 0.65$). The relative proportions of ^{35}S radioactivity in the two fractions from control and cholesterol-enriched cells were similar (fraction 1: control, 23%; cholesterol-enriched, 21%; fraction 2: control, 77%; cholesterol-enriched, 79%); however, there was a 45–50% increase of radioactivity in both fractions from cholesterol-enriched cells compared with levels in control cells. Proteoglycan fraction 1 from control and cholesterol-enriched cells was digested completely by chondroitin ABC lyase while about 6% resisted digestion by chondroitin ACII lyase, indicating that the proteoglycans contained predominantly chondroitin 4- and 6-sulphate chains. Similar analysis of proteoglycan fraction 2 indicated that this proteoglycan, from both control and cholesterol-enriched cells, contained 75–77% chondroitin sulphate chains and 23–25% of dermatan sulphate chains.

Octyl-Sepharose chromatography in 3 M guanidinium chloride was used to fractionate the smaller dermatan sulphate proteoglycans (DS-PG). Earlier Choi et al. [26] showed that although both DS-PG I and DS-PG II bound quantitatively to octyl-Sepharose in 3 M guanidinium chloride, DS-PG II bound less strongly and was eluted at lower concentrations of CHAPS, while DS-PG I was eluted at higher concentrations of CHAPS. Figure 1 shows the elution profile of the DS-PG from control and cholesterol-enriched cells. The elution profile was similar for both cultures. A small portion of the DS-PG did not bind to the octyl-Sepharose and was eluted in the column wash. However,

Figure 2 Pulse-chase analysis of ^{35}S -labelled proteoglycans

Confluent cultures of control and cholesterol-enriched smooth muscle cells were radiolabelled with 50 $\mu\text{Ci}/\text{ml}$ [^{35}S]sulphate for 2 h, washed and chased. After each chase period proteoglycans in the cell layer and medium were extracted, precipitated with HPC and analysed for ^{35}S radioactivity. Disappearance of labelled proteoglycans from the cell layer in control (●) and cholesterol-enriched cells (○) is shown in panel (a). ^{35}S -labelled proteoglycans [from control (○) and cholesterol-enriched cells (●)] in the medium and free [^{35}S]sulphate [from control (■) and cholesterol-enriched cells (□)] generated by intracellular degradation of proteoglycans are shown in panel (b). Each value represents the average of results from triplicate cultures.

the majority of the DS-PG exhibited strong binding to octyl-Sepharose and was eluted at approx. 0.72% CHAPS, indicating that the proteoglycan was predominantly DS-PG I [26].

Pulse-chase studies

The effect of cholesterol enrichment on proteoglycan turnover in smooth muscle cells was investigated by pulse-chase analysis. Control and cholesterol-enriched cells were incubated with [^{35}S]sulphate for 2 h, washed thoroughly and chased in isotope-free medium for 1, 2, 4, 6 and 8 h. After each chase period proteoglycans in the cell layer and chase medium were quantified along with free [^{35}S]sulphate in the chase medium. Since heparan sulphate constituted only 6–15% of the medium-derived and

cell-layer proteoglycans, no attempt was made to analyse this proteoglycan separately. The results of the pulse-chase studies are presented in Figure 2. The ³⁵S-labelled proteoglycans from the cell layer of cholesterol-enriched cultures disappeared more slowly than those from control cultures (Figure 2a). The half-lives of cell-layer proteoglycans for control and cholesterol-enriched cultures were 2.8 ± 0.2 h and 26.5 ± 1.8 h (mean $\pm$ S.E.M. of five experiments) respectively. As a result, at the end of the 8 h chase, about 86 % of the total ³⁵S-labelled proteoglycans present at time 0 in cholesterol-enriched cultures still remained in the cell layer. The corresponding value for control cultures was only 18 %. For cholesterol-enriched cells about 4 % of the cell-layer proteoglycans appeared in the medium as intact proteoglycans and 10 % as free [³⁵S]sulphate (Figure

2b). This was significantly less than the corresponding values of 14 % and 68 % respectively for control cells. The rate of degradation of labelled proteoglycans to free [³⁵S]sulphate was significantly lower for cholesterol-enriched cultures than for control cultures (Figure 2b). Similarly, the rate of secretion of labelled proteoglycans into the medium for cholesterol-enriched cultures was also lower than that for control cultures (Figure 2b). At the end of an 8 h chase, 35 % of the cell-associated proteoglycans in control cultures was releasable by trypsin. In contrast, only 12 % of the cell-layer proteoglycans was trypsin-releasable in cholesterol-enriched cultures. The remainder was trypsin-inaccessible (results not shown).

Endocytosis and degradation of ³⁵S-labelled proteoglycans

The pulse-chase studies indicated that degradation of proteoglycans was severely retarded in cholesterol-enriched smooth muscle cells. This was further confirmed by studying the endocytosis and degradation of intact ³⁵S-labelled proteoglycans isolated from smooth muscle cell cultures. Figure 3 shows the time course of endocytosis and degradation of ³⁵S-labelled proteoglycans by control and cholesterol-enriched smooth muscle cells. Previously Kresse et al. [27] have shown that the ethanol-soluble radioactivity in the medium represented free [³⁵S]sulphate generated by cellular degradation of labelled proteoglycans. Endocytosis (Figure 3; upper panel) of labelled proteoglycans by control and cholesterol-enriched cells was virtually identical. The degradation of ³⁵S-labelled proteoglycans (Figure 3; lower panel) by control cells proceeded linearly after an initial lag period of 1.5 h, so that by 24 h, almost 90 % of the endocytosed proteoglycans were degraded. In contrast, the lag period was significantly prolonged for cholesterol-enriched cells (6 h). Additionally, degradation proceeded at a much slower rate, resulting in only 34 % degradation at the end of 24 h. There was virtually no degradation of proteoglycans in cell-free culture medium.

Effect of cholesterol-enrichment on protein synthesis

The above studies showed that both proteoglycan synthesis and secretion were altered in cholesterol-enriched smooth muscle cells. To determine whether this was specific for proteoglycans, the effect of cholesterol enrichment on general protein synthesis was investigated. Table 4 shows the incorporation of [³H]leucine

Figure 3 Endocytosis and degradation of [³⁵S]proteoglycans as a function of time

Control and cholesterol-enriched smooth muscle cells were incubated with [³⁵S]proteoglycans (2.4×10^5 d.p.m./ml) synthesized by confluent smooth muscle cells. At the indicated time, endocytosis (upper panel) and degradation (lower panel) of proteoglycans were determined as described under the Materials and methods section. Each data point is the average of triplicate assays.

Table 4 Protein synthesis in control and cholesterol-enriched smooth muscle cells

Cells were incubated in DMEM containing 10 μ Ci/ml [³H]leucine for either 30 min or 24 h. Protein synthesis was measured as trichloroacetic-acid-precipitable radioactivity. Each value is given as the mean $\pm$ S.D. of triplicate cultures. Significantly different from control (*, $P < 0.0005$; †, $P < 0.0001$).

	Trichloroacetic-acid-precipitable radioactivity			
	30 min incubation (d.p.m./10 ⁵ cells)		24 h incubation (10 ⁻³ $\times$ d.p.m./10 ⁵ cells)	
	Medium	Cell	Medium	Cell
Control	308 $\pm$ 26	5901 $\pm$ 93	51 $\pm$ 2.1	180 $\pm$ 3.7
Cholesterol-enriched	274 $\pm$ 14	6782 $\pm$ 243*	68 $\pm$ 2.5†	213 $\pm$ 6.8*

Figure 4 Subcellular fractionation of cholesterol-enriched cells

Cholesterol-enriched smooth muscle cells were incubated with ^{35}S -labelled proteoglycans. After 12 h, the cells were washed, homogenized and post-nuclear supernatant was prepared. The post-nuclear supernatant was then fractionated on a Percoll gradient. Fractions were analysed for ^{35}S radioactivity and density.

Table 5 Enzymic activities in homogenates of control and cholesterol-enriched smooth muscle cells

Values are given as the mean $\pm$ S.D. of quadruplicate assay. Significantly different from control (*, $P = 0.0007$; †, $P < 0.0001$).

Enzyme	Specific activity (units/ 10^5 cells)	
	Control	Cholesterol-enriched
Acid phosphatase	94.1 ± 5.1	85.3 ± 5.8
N-Acetyl- β -hexosaminidase	12.4 ± 1.6	$6.7 \pm 0.8^*$
β -Glucuronidase	17.1 ± 2.2	$3.4 \pm 0.4^\dagger$
Cathepsin C	19.2 ± 2.1	$5.0 \pm 0.4^\dagger$

into proteins after a 30 min and 24 h pulse. In cholesterol-enriched cells, there was a 15% increase over control levels in cell-associated proteins after the 30-min pulse. The increase remained about the same (18%) following the 24-h pulse. However, after the 24-h labelling, there was a 33% increase over control levels in secreted proteins.

Subcellular fractionation of cholesterol-enriched cells

The retarded degradation of proteoglycans observed in cholesterol-enriched smooth muscle cells resulted in their excessive intracellular accumulation. Subcellular fractionation of cholesterol-enriched cells was conducted after 12 h of endocytosis, in order to determine in which cellular compartment the proteoglycans accumulated. Figure 4 shows that most of the radioactivity was confined to the bottom fraction of the Percoll gradient, corresponding to dense lysosomes. Fractions corresponding to endosomes contained only small amounts of radio-

activity. Similarly, the nuclear pellet also contained very little radioactivity (results not shown).

Lysosomal enzymes

To determine whether cholesterol enrichment of smooth muscle cells affected lysosomal enzymes, we assayed the activities of four marker enzymes in control and cholesterol-enriched cells (Table 5). Compared with control cells, the specific activities of N-acetyl- β -hexosaminidase, β -glucuronidase and cathepsin C were significantly inhibited in cholesterol-enriched cells.

DISCUSSION

Lipids and proteoglycans accumulate in vascular smooth muscle cells in atherosclerosis. However, it is not known whether lipid accumulation influences cellular proteoglycan metabolism. We used cholesterol-enriched smooth muscle cells to study the effect of lipid loading on proteoglycan synthesis by these cells. Exposure of rabbit aorta smooth muscle cells to cationized LDL for 7 days produced a 10-fold increase in cellular cholesteryl ester content as compared with levels in control cells. Others have reported similar increases in cholesteryl ester content as well as a significant increase in cholesteryl ester synthesis, over control levels in smooth muscle cells exposed to cationized LDL [21,36].

In cholesterol-enriched cells, there was a 40–50% increase over control cells in the total incorporation of radiolabelled precursors into proteoglycans during long-term incubation (20 h). This was not due to increased cell proliferation or over-sulphation of proteoglycans, but is the result of increased *de novo* synthesis. That the incorporation of [^{35}S]sulphate and [^3H]serine into proteoglycans in cholesterol-enriched cells was increased 42% and 39% respectively above that in control cells also following a 15-min pulse supports this conclusion. Recently Owens and Wagner reported increased proteoglycan synthesis in cholesterol-enriched macrophages isolated from pigeons [37]. Together, these studies suggest that cholesterol enrichment probably induces phenotypic modulation of cells and thus increases their proteoglycan synthesis.

The cholesterol-enriched cells exhibited major shifts in the distribution of proteoglycans into media and cell-layer compartments. More than 80% of the proteoglycans accumulated in the cell-layer compartment as compared with about 30% in the control cultures. Such a shift in the distribution of proteoglycans between the medium and cell-layer compartments exhibited by cholesterol-enriched smooth muscle cells was not evident in cholesterol-enriched macrophages [37]. Whether this is due to a difference in the cell type or some other factor is not known.

As well as producing increased proteoglycan synthesis, cholesterol enrichment of smooth muscle cells also stimulated general protein synthesis. However, unlike the increased intracellular accumulation of proteoglycans observed in these cells proteins did not accumulate, suggesting that they are under a different metabolic control.

The results of the present study demonstrate that in cholesterol-enriched cells proteoglycan metabolism is altered in two ways. First, there is increased production of proteoglycans. The mechanism responsible for this is not known. However, it involves *de novo* synthesis of proteoglycan core protein as shown by a 39% stimulation of [^3H]serine incorporation into proteoglycans following a 15-min pulse. This is supported by the observation that cycloheximide, an inhibitor of protein synthesis, inhibited the stimulation of proteoglycan synthesis in cholesterol-enriched cells. Therefore, there is a higher rate of core-protein synthesis in cholesterol-enriched cells than in control cells. In addition,

modulation of synthesis also possibly involves enhanced transcription of core protein, since actinomycin D inhibited synthesis.

Secondly, there is over accumulation of proteoglycans in cholesterol-enriched smooth muscle cells. We used pulse-chase studies to determine the cause of intracellular proteoglycan accumulation. In control cells, there was a relatively rapid disappearance of ^{35}S -labelled proteoglycans ($t_{1/2} = 2.8$ h) from the cell layer. After an 8-h chase, about 14% of the cell-layer proteoglycans appeared in the culture medium as intact proteoglycans. The remaining 86% was degraded and appeared as free [^{35}S]sulphate in the medium. In contrast, the intracellular ^{35}S -labelled proteoglycans in cholesterol-enriched cultures lost their radiolabel almost 10 times slower ($t_{1/2} = 26.5$ h) than control cells. Also, only 4% of the proteoglycans subsequently appeared intact in the medium and only 10% was degraded. Likewise, in spite of the similar uptake of labelled proteoglycans by control and cholesterol-enriched cells, the cholesterol-enriched cells degraded endocytosed proteoglycans at a much lower rate than the control cells (Figure 3). Taken together, these results indicate that proteoglycan degradation, as well as secretion, is severely retarded in cholesterol-enriched smooth muscle cells. This in turn leads to over-accumulation in the cells.

The present study shows that both control and cholesterol-enriched smooth muscle cells synthesize two different chondroitin sulphate-dermatan sulphate proteoglycans: a large chondroitin sulphate-rich proteoglycan and a small dermatan sulphate-rich proteoglycan (DS-PG I). Even though smooth muscle cells from other species, such as rat, synthesize a second dermatan sulphate-rich proteoglycan (DS-PG II) [38], it appears that this proteoglycan is not expressed by the rabbit cells. Since the proteoglycans were analysed after 24 h of labelling, there might be significant proteoglycan degradation. It is therefore possible that degradation might have limited the amount of DS-PG II available for detection by the method. However, this appears unlikely because, recently, Asundi et al. [38] also failed to detect transcripts homologous to bovine bone DS-PG II cDNA in rabbit aortic medial tissue. More definitive studies using specific antibodies are obviously needed to confirm our observation. Levels of both the chondroitin sulphate-rich proteoglycan and DS-PG I were elevated in cholesterol-enriched cells, compared with those in control cells. This indicates that there is no preferential accumulation of either proteoglycan species in the cholesterol-enriched cells. Therefore, the metabolism of the two proteoglycans does not appear to be regulated differently in the cholesterol-enriched smooth muscle cells.

The proteoglycans which accumulate in cholesterol-enriched cells are mostly confined to the dense lysosomes, with very little accumulation in the non-lysosomal compartment. This shows that the transport of endocytosed proteoglycans into the lysosomes proceeds normally in these cells. However, it appears that the transport of proteoglycans from the intracellular space to the cell surface is less efficient, or defective, in cholesterol-enriched cells. This is because, after a 2-h pulse the amount of trypsin-releasable proteoglycans was significantly reduced in these cells as compared with that in control cells. Such relocation of proteoglycans from the trypsin-accessible compartment to a trypsin-resistant compartment has been reported in rat ovarian granulosa cells treated with chloroquine [39]. Since trypsin-accessible proteoglycan mostly represents proteoglycans on the cell surface [40], a reduction in this could eventually lead to reduction in the amount of proteoglycan destined for secretion.

The mechanism responsible for retarded degradation of proteoglycans in cholesterol-enriched cells was investigated. The specific activities of three lysosomal marker hydrolases, *N*-acetyl-

β -hexosaminidase, β -glucuronidase and cathepsin C were reduced significantly in cholesterol-enriched smooth muscle cells compared with control cells. Reduced activities of *N*-acetyl- β -hexosaminidase and β -glucuronidase were reported earlier in pig aorta smooth muscle cells when they were cholesterol-enriched by incubating with LDL and chloroquine [41]. These two lysosomal hydrolases and cathepsins are involved in the degradation of proteoglycans [42]. A reduction in their activities will result in reduced degradation of the proteoglycans that have accumulated in the lysosomes of the cholesterol-enriched cells. However, what causes the reduced activities of the enzymes in these cells is not known. One possibility involves reduced synthesis of the enzymes in cholesterol-enriched cells. Alternatively, cholesterol enrichment might reduce the intralysosomal concentration of the enzymes by accelerating their rate of secretion from the cell to the culture medium.

In summary, these results show that cholesterol enrichment of rabbit aorta smooth muscle cells results in increased production and excessive intracellular accumulation of proteoglycans. Since proteoglycans bind to LDL, their accumulation in the cells can facilitate further intracellular retention of this lipoprotein.

This study was supported by grant HL-42993 from the National Institutes of Health.

REFERENCES

- Wight, T. N. (1988) *Arteriosclerosis* **9**, 1–20
- Camejo, G. (1982) *Adv. Lipid Res.* **19**, 1–53
- Berenson, G. S., Radhakrishnamurthy, B., Srinivasan, S. R., Vijayagopal, P., Dalferes, E. R. and Sharma, C. (1984) *Exp. Mol. Pathol.* **41**, 267–287
- Srinivasan, S. R., Vijayagopal, P., Dalferes, E. R., Abbate, B., Radhakrishnamurthy, B. and Berenson, G. S. (1986) *Atherosclerosis* **62**, 201–208
- Vijayagopal, P., Srinivasan, S. R., Jones, K. M., Radhakrishnamurthy, B. and Berenson, G. S. (1985) *Biochim. Biophys. Acta* **837**, 251–261
- Salisbury, B. G. J., Falcone, D. J. and Minick, C. R. (1985) *Am. J. Pathol.* **120**, 6–11
- Hurt, E. and Camejo, G. (1987) *Atherosclerosis* **67**, 115–126
- Srinivasan, S. R., Vijayagopal, P., Radhakrishnamurthy, B. and Berenson, G. S. (1991) *Biochim. Biophys. Acta* **1081**, 188–196
- Vijayagopal, P., Srinivasan, S. R., Radhakrishnamurthy, B. and Berenson, G. S. (1992) *Arterioscler. Thromb.* **12**, 237–249
- Wight, T. N. and Hascall, V. C. (1983) *J. Cell Biol.* **96**, 167–176
- Schmidt, A., Von Teutul, A. and Buddecke, E. (1984) *Hoppe-Seyler's Z. Physiol. Chem.* **365**, 445–456
- Chen, J.-K., Hoshi, H. and McKeehan, W. L. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 5287–5291
- Schonherr, E., Jarvelainen, H. T., Sandell, L. J. and Wight, T. N. (1991) *J. Biol. Chem.* **266**, 17640–17647
- Vijayagopal, P., Ciolino, H. P. and Berenson, G. S. (1992) *Biochim. Biophys. Acta* **1135**, 129–140
- Berrou, E., Breton, M., Duedon, E. and Picard, J. (1988) *J. Cell. Physiol.* **137**, 430–438
- Wight, T. N., Potter-Perigo, S. and Aulinskas, T. (1989) *Am. Rev. Respir. Dis.* **140**, 1132–1135
- Vijayagopal, P., Ciolino, H. P., Radhakrishnamurthy, B. and Berenson, G. S. (1992) *Atherosclerosis* **94**, 135–146
- Hatch, F. T. and Lees, R. S. (1968) *Adv. Lipid Res.* **6**, 2–63
- Basu, S. K., Goldstein, J. L., Anderson, R. G. W. and Brown, M. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 3178–3182
- Ross, R. (1971) *J. Cell Biol.* **50**, 172–186
- Pomerantz, K. B. and Hajjar, D. P. (1989) *J. Lipid Res.* **30**, 1219–1231
- Edwards, I. J., Wagner, W. D. and Owens, R. T. (1990) *Am. J. Pathol.* **136**, 609–621
- Carlson, D. M. (1968) *J. Biol. Chem.* **243**, 616–626
- Gowda, D. C., Bhavanandan, V. P. and Davidson, E. A. (1986) *J. Biol. Chem.* **261**, 4926–4934
- Saito, H., Yamagata, T. and Suzuki, S. (1968) *J. Biol. Chem.* **243**, 1536–1542
- Choi, H. U., Johnson, T. L., Pal, S., Tang, L. H., Rosenberg, L. and Neame, P. J. (1989) *J. Biol. Chem.* **264**, 2876–2884
- Kresse, H., Tekolf, W., Von Figura, K. and Buddecke, E. (1975) *Hoppe-Seyler's Z. Physiol. Chem.* **356**, 943–952
- Jougasaki, M., Kugiyama, K., Saito, Y., Nakao, K., Imura, H. and Yasue, H. (1992) *Circ. Res.* **71**, 614–619

-
- 29 Gieselmann, V., Pohlmann, R., Hasilik, A. and Von Figura, K. (1983) *J. Cell Biol.* **97**, 1–5
- 30 Hoppe, W., Rauch, U. and Kresse, H. (1988) *J. Biol. Chem.* **12**, 5926–5932
- 31 Seymour, C. A. and Peters, T. J. (1977) *Clin. Sci. Mol. Med.* **52**, 229–239
- 32 Peters, T. J., Muller, M. and DeDuve, C. (1972) *J. Exp. Med.* **136**, 1117–1139
- 33 Haley, N. J., Shio, H. and Fowler, S. (1977) *Lab. Invest.* **37**, 287–296
- 34 Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427
- 35 Gamble, W., Vaughan, M., Kruth, H. S. and Avigan, J. (1978) *J. Lipid Res.* **19**, 1068–1070
- 36 Goldstein, J. L., Anderson, R. G. W., Buja, L. M., Basu, S. K. and Brown, M. S. (1977) *J. Clin. Invest.* **59**, 1196–1202
- 37 Owens, R. T. and Wagner, W. D. (1991) *Arterioscler. Thromb.* **11**, 1752–1758
- 38 Asundi, V., Cowan, K., Matzura, D., Wagner, W. and Dreher, K. L. (1990) *Eur. J. Cell Biol.* **52**, 98–104
- 39 Yanagishita, M. and Hascall, V. C. (1984) *J. Biol. Chem.* **259**, 10270–10283
- 40 Schmidt, A. and Buddecke, E. (1988) *Exp. Cell Res.* **178**, 242–253
- 41 Leake, D. S. and Peters, T. J. (1982) *Atherosclerosis* **44**, 275–291
- 42 Poole, A. R. (1986) *Biochem. J.* **236**, 1–14
-

Received 13 October 1992/19 March 1993; accepted 2 April 1993