Marked alteration of proteoglycan metabolism in cholesterol-enriched human arterial smooth muscle cells

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To elucidate the correlation between vascular cholesterol metabolism and proteoglycan (PrGl) biosynthesis, we investigated PrGl synthesis in human aortic smooth muscle cells (SMCs) after cholesterol enrichment with cationized low-density lipoproteins (LDL). Compared with normal SMCs, total PrGl synthesis by cholesterol-enriched cells decreased 2.4-fold (11 874 ± 530 d.p.m. per 10^5 cells compared with 4890 ± 385 d.p.m. per 10^5 cells). This was the net result of a 6.9-fold reduction in medium PrGl (11 000 ± 490 d.p.m. per 10^5 cells compared with 1580 ± 246 d.p.m. per 10^5 cells) and a 3.8-fold increase in cellular PrGl over controls (874 ± 27 d.p.m. per 10^5 cells compared with 3310 ± 193 d.p.m. per 10^5 cells). Prior incubation of SMCs with native LDL had no effect on PrGl synthesis by these cells. The decrease in PrGl synthesis in cholesterol-enriched cells correlated with a 90% and 20% reduction in the steady-state level of mRNA for biglycan and decorin respectively, and a virtual elimination of the steady-state level of mRNA for versican over controls. Despite the down-regulation of PrGl synthesis, cholesterol-loaded cells produced a 2-fold increase in a PrGl subfraction with high affinity for LDL. Compared with the corresponding PrGl subfraction from normal cells, that from the cholesterol-enriched cells exhibited increased charge density and a higher molecular mass and contained relatively larger proportions of chondroitin 6-sulphate and dermatan sulphate. These results show that PrGl metabolism is dramatically altered in cholesterol-enriched human SMCs.

INTRODUCTION

Proteoglycans (PrGls) are important structural components of the blood vessel wall. Several studies have shown that these macromolecules play a major role in influencing such arterial properties as viscoelasticity, permeability, lipid metabolism, haemostasis and thrombosis [1–3]. Moreover, PrGls also may play a significant role in atherogenesis [4–9]. Therefore alterations in PrGl content and type are likely to influence normal functions of arterial tissue.

Smooth muscle cells (SMCs) synthesize a significant proportion of artery wall PrGls and chiefly produce a large chondroitin sulphate PrGl (versican) and two small dermatan sulphate PrGls (biglycan and decorin) [10,11]. In addition, vascular SMCs also express cell-surface heparan sulphates [12]. Although Schonherr et al. [13,14] reported recently that platelet-derived growth factor and transforming growth factor had different effects on the biosynthesis of versican, biglycan and decorin by SMCs in culture, generally little is known about regulation of the biosynthesis of different classes of artery wall PrGl.

Foam cells, the hallmarks of atherosclerotic lesions, originate from vascular SMCs and resident macrophages [15–18]. Because PrGls also accumulate in atherosclerotic lesions, we recently investigated whether PrGl synthesis was altered in cholesterol-enriched SMCs [19]. Our results indicated that PrGl synthesis in cholesterol-enriched rabbit SMCs increased by 40–50% above that in control cells. Although these results are interesting, they are not directly relevant to human atherosclerosis. Therefore in the present study we sought to determine whether our initial observations in rabbit cells could be extended to cholesterol-enriched human aortic SMCs. Using Northern blot analysis we also examined the effect of cholesterol loading on the regulation of the biosynthesis of different PrGl subclasses, such as versican, biglycan and decorin, by these cells. In addition, because interaction between plasma low-density lipoproteins (LDL) and artery wall PrGls is implicated in atherogenesis, we determined whether cholesterol-enriched SMCs synthesized PrGls that exhibited enhanced binding affinity for LDL. Our results demonstrate that, in contrast with our earlier report of stimulation of PrGl synthesis in cholesterol-enriched rabbit SMCs, PrGl synthesis is suppressed in cholesterol-enriched human SMCs owing to a decrease in the steady-state levels of mRNA for versican, biglycan and decorin. Despite the decrease in synthesis, the PrGls produced by cholesterol-enriched cells exhibit increased binding to LDL.

EXPERIMENTAL

Materials

Eagle's minimum essential medium (MEM), fetal bovine serum, antibiotics, non-essential amino acids, molecular grade agarose, and the cDNA probe for human versican were purchased from Gibco BRL. CNBr-activated Sepharose CL-4B came from Pharmacia LKB Biotechnology Inc. $Na_2{}^{35}SO_4$ (43 Ci per mg of sulphate) was bought from ICN. [^{32}P]dCTP and random priming labelling kit were purchased from Amersham. Nitrocellulose filters were obtained from Schleicher and Schuell. The cDNA probes for human biglycan (P16) and decorin (P2) were kindly provided by Dr. L. Fisher, National Institutes of Dental Research, Bethesda, MD, U.S.A. Guanidine hydrochloride (grade I), *N*-ethylmaleimide, PMSF, 6-aminohexanoic acid, benzamidine hydrochloride, DEAE-Sephacel, Sephadex G-50, Sepharose CL-4B, Sepharose CL-2B, chondroitin ABC lyase, chondroitin AC II lyase and anti-α-SMC actin antibody were supplied by Sigma.

Abbreviations used: LDL, low-density lipoprotein; MEM, minimal essential medium; PrGl, proteoglycan; SMC, smooth muscle cell.

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Lipoproteins

LDL (ρ 1.019–1.063 g/ml) was isolated from human serum by sequential ultracentrifugation [20]. EDTA (0.05 %), butylated hydroxytoluene (10 μM) and PMSF (2 mM) were added to the serum before centrifugation. Cationized LDL was prepared by covalent attachment of *N*,*N*-dimethyl-1,3-propanediamine to LDL by using 1-ethyl-3-(3-diethylaminopropyl)carbodi-imide/HCl as catalyst [21]. Cationized LDL was dialysed against 0.15 M NaCl/0.01 % EDTA, pH 7.4, and sterilized by membrane filtration (0.22 μm pore diameter) before its addition to cell cultures. Cationized LDL migrated toward the cathode in agarose gel electrophoresis at pH 8.6.

Cell culture

SMCs were isolated from segments of human aorta free of atherosclerotic lesions by digestion with collagenase and elastase [22]. Aortic tissue was obtained from heart donors from the cardiac transplantation program at Ochsner Foundation Hospital, New Orleans. After removal of the endothelial cells, the aortic segments were digested with a mixture of collagenase (0.2 %) and elastase (0.02 %) for 1 h at 37 °C. The SMCs were harvested by scraping the luminal surface of the aorta with a sterile cotton swab. Cells were recovered by swirling the swab in a sterile centrifuge tube containing culture medium [Eagle's MEM supplemented with 1 % (v/v) non-essential amino acids, 10 % (v/v) fetal bovine serum, 100 i.u./ml penicillin, 100 μg/ml streptomycin, and 1 μg/ml amphotericin B]. The cells were pelleted by centrifugation (250 ***g*** at 25 °C for 10 min), resuspended in culture medium and plated in gelatin-coated culture wells [1 % (w/v) gelatin]. When confluent, SMCs were subcultured by trypsinization. The cells were identified as SMCs by their morphology and positive staining with an anti-α SMC actin antibody and negative staining for Factor VIII-related antigen (specific for endothelial cells). Cells between passages three and five were used in the study. Cholesteryl ester accumulation in SMCs was induced by incubating cells in MEM containing 2 % (v/v) fetal bovine serum and 20 μg of protein per ml of cationized LDL for 48 h [23]. Cells incubated in MEM + 2 % fetal bovine serum served as control. In some cases SMCs were also incubated in the above medium containing 20 μg/ml LDL.

Metabolic labelling

Control, LDL-treated and cholesterol-enriched SMCs were incubated in MEM (without serum and lipoproteins) containing 30 μCi/ml [^{35}S]sulphate for 20 h. In some cases the cells were incubated with 75 μCi/ml [^{35}S]sulphate for 30 min.

Isolation of PrGls

^{35}S-labelled PrGls were isolated from the culture medium and cell layer as described before [19]. Briefly, at the end of incubation, the culture medium was removed and the cell layer rinsed with ice-cold MEM. The wash was combined with the media. After the addition of solid guanidine hydrochloride (final concn. 4 M) and protease inhibitors (100 mM aminohexanoic acid, 50 mM benzamidine hydrochloride, 20 mM EDTA, 10 mM *N*-ethylmaleimide and 1 mM PMSF), the PrGls were precipitated (see below). Representative cultures were used for cell count and protein assay.

The cell layer was extracted with 4 M guanidine hydrochloride containing 0.05 M sodium acetate, pH 5.8, with 0.1 % Triton X-100 and protease inhibitors for 24 h at 4 °C. There was no difference between the control and cholesterol-enriched cells in the extractability of PrGl, which was approx. 98 %.

Unincorporated radioactivity was removed from the media and cell extracts by Sephadex G-50 chromatography. ^{35}S-labelled PrGls in the excluded fraction of the column were precipitated with cetylpyridinium chloride after addition of 200 μg of carrier chondroitin sulphate [19]. The PrGls were then re-isolated from their cetylpyridinium chloride complexes [24], dissolved in distilled water, and the aliquots counted in a liquid scintillation spectrometer.

RNA extraction and Northern analysis

Total cellular RNA was isolated from confluent control and cholesterol-enriched SMCs by acid phenol extraction [25] and quantified by measuring absorbance at 260 nm. Total RNA (20 μg) for each cell treatment was separated by denaturing agarose gel electrophoresis and transferred to a nitrocellulose membrane [26]. Membranes were probed with ^{32}P-labelled cDNAs specific for versican, decorin, biglycan and β-actin [27]. After hybridization and stringent washing, the filters were subjected to autoradiography. Ribosomal RNA band intensity and hybridization to β-actin cDNA served as controls for RNA loading. Specific bands were subsequently quantified in a Molecular Dynamics PhosphorImager by using the manufacturer's image analysis software. Band intensities were normalized to β-actin to correct for loading differences.

Affinity chromatography

LDL was coupled with cyanogen bromide-activated Sepharose CL-4B in accordance with the manufacturer's instructions. In preliminary studies we also included heparin (50 μg per ml of gel) in some of the coupling reactions, because Camejo et al. [28] observed that heparin protected the lysine and arginine residues of LDL, which are essential for PrGl binding, from being blocked, by reacting with the activated gel.

PrGls from Sephadex G-50 chromatography were dialysed against interaction buffer (10 mM Tris, 30 mM $CaCl_2$, 10 μM butylated hydroxytoluene and 0.1 % BSA, pH 7.0; ionic strength 0.1). Aliquots containing equal amounts of radioactivity (25000 d.p.m.) were applied to a 3 ml column of LDL–Sepharose that had previously been equilibrated with the interaction buffer. The PrGl samples were allowed to stand in the gel for 5 min at room temperature, after which the column was washed with 10 ml of interaction buffer. The column was then eluted stepwise with interaction buffer containing increasing molarities of NaCl and no BSA. The column eluates were assayed for ^{35}S radioactivity. Earlier we used affinity chromatography to determine the binding affinities of PrGls for LDL [29].

PrGl characterization

The PrGl fractions in the culture media from normal and cholesterol-enriched cultures that exhibited the strongest binding to LDL were characterized with respect to their charge density, glycosaminoglycan composition and molecular size. The charge density of PrGls was determined by DEAE-Sephacel ion-exchange chromatography. The PrGl fractions were dialysed against urea buffer (8 M urea, 0.1 M Tris, 0.15 M NaCl, 0.3 % Triton X-100, pH 7.0, containing protease inhibitors) and applied to a DEAE-Sephacel column (bed volume 3 ml). The column was eluted with a continuous gradient of 0.15–1.0 M NaCl in the

urea buffer at a flow rate of 10 ml/h. Fractions were analysed for radioactivity. The glycosaminoglycan composition of the PrGls was determined by a combination of enzymic and chemical methods. Chondroitinase ABC and chondroitin ACII lyase digestion were used to determine the presence of chondroitin sulphates and dermatan sulphate [30], and nitrous acid degradation served to detect heparan sulphate [31]. The relative proportions of chondroitin 4- and 6-sulphates were determined by disaccharide analysis [32].

The hydrodynamic size of the PrGls was determined by gel exclusion chromatography on a column (0.9 cm × 100 cm) of Sepharose CL-2B. The column was eluted with 4 M guanidine hydrochloride, 0.05 M sodium acetate, pH 7.0, containing protease inhibitors, at a flow rate of 10 ml/h. Fractions were analysed for radioactivity. Freeze-dried *Escherichia coli* was used to determine the void volume of the column and [^{35}S]sulphate the total volume.

Analytical methods

Free and esterified cholesterol were determined enzymically [33] and protein concentration was assayed by a modified Lowry procedure [34].

RESULTS

Effect of cholesterol enrichment on cell viability, proliferation and cellular cholesterol content

At the end of the two-day incubation, over 95 % of the control cells as well as the cells exposed to cationized LDL or LDL remained viable, as determined by Trypan Blue exclusion. As shown in Table 1, incubation of human aortic SMCs in the presence of 20 μg/ml cationized LDL for 2 days increased cell number significantly ($P < 0.0001$). In contrast, similar incubation of SMCs with LDL did not increase cell number. Table 1 also shows that exposure of SMCs to cationized LDL for 2 days increased the intracellular free cholesterol by 1.3-fold and esterified cholesterol by 82-fold over the control. Incubation of cells with cationized LDL for 24 h also increased cellular cholesteryl ester content to a lesser extent (20-fold over control; results not shown). Earlier studies reported similar increases in cellular cholesterol content after incubation of human aortic SMCs with cationized LDL [23]. Exposure of SMCs to LDL did not increase cellular cholesterol content. Phase-contrast microscopy revealed no difference in cell morphology between control cells and cells exposed to LDL or cationized LDL.

Table 1 Effect of exposure of SMCs to cationized LDL on cell number and cellular cholesterol content

SMCs were incubated in the presence and absence of 20 μg/ml cationized LDL, and cell number and cell cholesterol contents were determined. Values are means ± S.E.M. for four experiments conducted in triplicate. Statistical significances: **, $P < 0.0001$; *, $P < 0.05$ compared with control cultures.

		Cholesterol (μg per 10^6 cells)	
Exposure	10^{-5} × Cell number	Free	Esterified
None	6.45 ± 0.13	3.6 ± 0.2	0.31 ± 0.05
LDL	6.56 ± 0.16	3.4 ± 0.4	0.36 ± 0.12
Cationized LDL	7.86 ± 0.14**	4.7 ± 0.4*	25.4 ± 2.8**

Table 2 Effect of cholesterol enrichment of SMCs on PrGl synthesis

SMCs were incubated with cationized LDL for either 24 or 48 h. Subsequently, control and cholesterol-enriched SMCs were incubated in MEM containing 30 Ci/ml [^{35}S]sulphate for 20 h. PrGls in media and cell layer were extracted and quantified. Results are the means ± S.E.M. for three experiments, each performed in triplicate. Statistical significance: **, $P < 0.0001$ compared with controls. Chol, cholesterol.

	^{35}S radioactivity (d.p.m. per 10^5 cells)			
	24 h		48 h	
	Control	Chol.-enriched	Control	Chol.-enriched
Cell layer	1074 ± 23	1720 ± 46**	874 ± 27	3310 ± 193**
Medium	9891 ± 146	7651 ± 123**	11000 ± 490	1580 ± 246**
Cell + medium	10965 ± 131	9371 ± 113**	11874 ± 530	4890 ± 385**

Effect of cholesterol enrichment of SMCs on proteoglycan synthesis

To determine whether cholesteryl ester enrichment altered PrGl synthesis, we compared the incorporation of [^{35}S]sulphate into PrGls by normal and cholesteryl ester-enriched cells. The results are shown in Table 2. Exposure of SMCs to cationized LDL for 24 or 48 h significantly altered PrGl synthesis by the cells. After 24 h of incubation with cationized LDL, cholesterol-enriched cells had a 1.3-fold decrease in media PrGls, a 1.6-fold increase in cellular PrGls and a 1.17-fold net decrease in overall PrGl synthesis (media + cell PrGl) compared with control cells; 48 h of exposure to cationized LDL resulted in greater alterations in PrGl synthesis, as evidenced by a 6.9-fold decrease in media PrGls, a 3.8-fold increase in cellular PrGls and a 2.4-fold net decrease in overall PrGl synthesis over control cells. In all subsequent experiments, therefore, PrGl synthesis was investigated after exposure of SMCs to cationized LDL for 48 h. In similar experiments, prior incubation of SMCs in the presence of 20 μg/ml LDL for 24 or 48 h had no effect on PrGl synthesis by these cells (results not shown). Cholesterol accumulation resulted in altered SMC PrGl distribution in the medium and in the cell layer. In control cultures, 93 % of total PrGl was found in the medium, whereas only 32 % of PrGl from cholesterol-enriched cells was in the medium. This finding suggests decreased PrGl secretion in cholesterol-enriched cells.

The above results indicate that PrGl synthesis might decrease in cholesterol-enriched cells. To confirm this, cells were pulsed with 75 μCi/ml [^{35}S]sulphate for 15 min and PrGl synthesis was assayed. Because secretion and degradation of newly synthesized PrGls are minimal, all newly synthesized PrGls will be intracellular and their quantification should be a true reflection of PrGl synthesis. Similar to the results of the long-term labelling experiment, synthesis of newly synthesized PrGls by cholesterol-loaded cells was decreased significantly after the 15 min pulse (in d.p.m. per 10^5 cells: control, 3125 ± 150; cholesterol-loaded, 1420 ± 95; $P < 0.001$). These results thus confirmed that PrGl synthesis was depressed in cholesterol-enriched cells compared with normal cells.

Northern blot analysis

To examine whether the decreased PrGl synthesis in cholesterol-loaded SMCs was due to a decrease in the mRNAs for PrGls, we determined the mRNA expression for versican, biglycan and decorin by Northern blot analysis. Results indicated that normal human aortic SMCs express mRNA transcripts that hybridize to

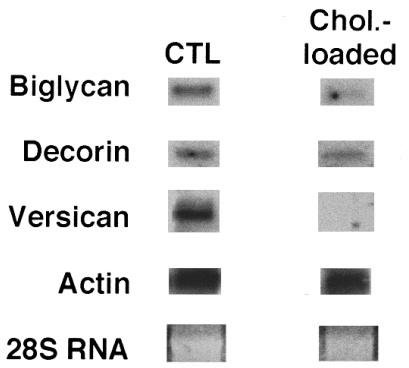

Figure 1 Effect of cholesterol enrichment of SMCs on steady-state mRNA for three PrGl core proteins

Total RNA (20 μg) from control and cholesterol-enriched SMCs were probed with radiolabelled cDNAs for biglycan, decorin, versican and β-actin. Specific bands were detected and quantified with a Molecular Dynamics PhosphorImager. The 28 S rRNA bands are also shown, to demonstrate equal loading. This is a representative experiment, which was repeated four times with similar results.

Table 3 LDL affinity chromatography of PrGl in culture media and cell layer

LDL–Sepharose CL-4B columns (3 ml) were prepared and equilibrated with interaction buffer (10 mM Tris, 30 mM $CaCl_2$, 10 M butylated hydroxytoluene and 0.1% BSA, pH 7.0). PrGl samples were dialysed against the interaction buffer and aliquots containing equal amounts of radioactivity (25000 d.p.m.) were applied to the affinity column. After 5 min at room temperature, the column was eluted stepwise with 5 ml of interaction buffer containing increasing molarities of NaCl (no BSA). The fractions were counted for ^{35}S radioactivity. Results are the means of triplicate determinations.

	PrGl eluted (%)			
	Cell layer		Media	
[NaCl] (M)	Control	Chol.-enriched	Control	Chol.-enriched
0.15	32	13	29	17
0.25	20	12	21	8
0.50	33	40	30	35
1.00	15	35	20	40

human versican, biglycan and decorin (Figure 1). In contrast, cholesterol enrichment of SMCs resulted in a total or near-total elimination of the expression of the steady-state level of mRNA for versican, a 90% decrease in biglycan-specific mRNA, and a 20% decrease in decorin-specific mRNA. This observation was confirmed in four separate experiments.

LDL affinity chromatography of PrGl

We used affinity chromatography to determine the binding ability of PrGl to LDL. Preliminary studies indicated that approx. 85–93% of the total ^{35}S-labelled PrGls applied to the LDL–Sepharose column was recovered in the eluates and that the inclusion of heparin in the coupling reaction did not improve the binding of PrGl to the affinity column. In subsequent studies, therefore, LDL was coupled to Sepharose CL-4B in the absence of heparin. Table 3 shows representative affinity chromatographic profiles of PrGls from normal and cholesterol-enriched SMCs. There were differences in the binding of PrGls from the two cell

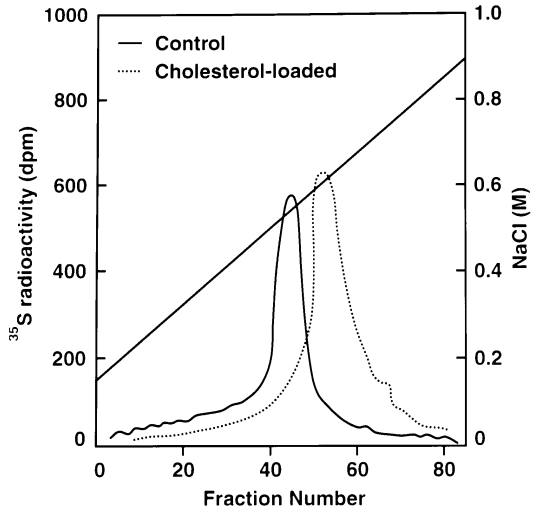

Figure 2 Representative DEAE-Sephacel chromatography of PrGl from three experiments

The PrGl fractions in the culture media of control and cholesterol-enriched cultures that exhibited the strongest binding to LDL were dialysed against 8 M urea, 0.1 M Tris, 0.15 M NaCl and 0.3% Triton X-100, pH 7.0, containing protease inhibitors. Aliquots were applied to a DEAE-Sephacel column and the column was eluted with a continuous NaCl gradient of 0.15–1.5 M in the above buffer. Fractions were counted for radioactivity.

cultures. These differences were not due to saturation of the LDL–Sepharose column because we added equal amounts of ^{35}S radioactivity to the column (25000 d.p.m. in a total volume of 1 ml). Compared with PrGls from normal cultures, lower proportions of PrGls from cholesterol-enriched cultures were eluted at low molarities of NaCl (0.15 and 0.25 M). In contrast, a higher percentage of PrGls from these cultures exhibited stronger binding to LDL (as evidenced by elution at 0.5 and 1.0 M NaCl) than did the PrGls from normal cultures. The proportion of PrGls that showed the strongest binding to LDL (elution at 1.0 M NaCl) was 2-fold higher in the cholesterol-loaded cultures than in the normal cell cultures (35 compared with 15 for cellular PrGls and 40 compared with 20 for media PrGls).

Characterization of PrGls

The PrGl subfractions in the culture media from normal and cholesterol-enriched cultures that exhibited the strongest binding to LDL were characterized with respect to their charge densities, glycosaminoglycan composition and molecular size. The DEAE-Sephacel ion-exchange chromatography of the PrGl fractions is shown in Figure 2. The high-affinity PrGl fraction from control cultures was eluted from the column as a single peak at a NaCl concentration of 0.58 M. Although the PrGl fraction from cholesterol-enriched cultures also gave a single peak, it was eluted at a higher concentration of NaCl (0.63 M).

Table 4 shows the glycosaminoglycan composition of the PrGls, which contained chondroitin 4- and 6-sulphates and dermatan sulphate. No heparan sulphate was detected in the PrGls. The PrGl fraction from cholesterol-enriched cultures contained a higher proportion of chondroitin 6-sulphate and dermatan sulphate than did the fraction from normal cultures.

The molecular masses of the PrGl fractions were determined by analytical Sepharose CL-2B chromatography. The elution profiles are shown in Figure 3. The PrGl fraction from control cultures eluted as a single peak with a K_{av} of 0.50. The elution

Table 4 Relative proportions of glycosaminoglycans in PrGl

The glycosaminoglycan composition of the PrGl fractions in the culture media from control and cholesterol-enriched cultures that exhibited the strongest binding to LDL was determined after digestion with various enzymes. Results are the averages of duplicate measurements in two separate experiments.

	Percentage of total radioactivity		
SMC culture	Chondroitin 4-sulphate	Chondroitin 6-sulphate	Dermatan sulphate
Control	36	40	24
Cholesterol-enriched	16	52	32

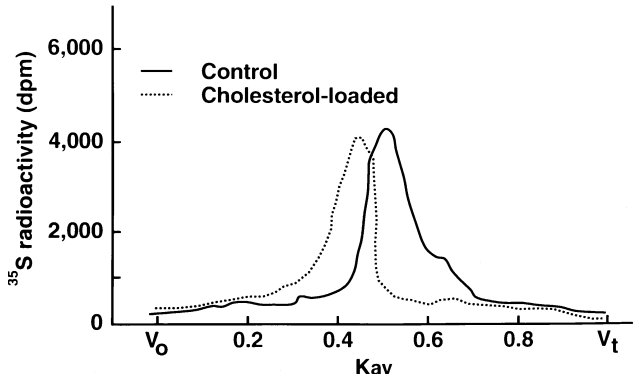

Figure 3 Analytical Sepharose CL-2B chromatography of PrGl

The high-affinity PrGl fractions in the media of control and cholesterol-enriched cultures were eluted on a Sepharose CL-2B column (0.9 cm × 100 cm) with 4 M guanidine/HCl, 0.05 M sodium acetate, pH 7.0, containing protease inhibitors. Fractions of 1 ml were collected at a flow rate of 10 ml/h and aliquots were counted for radioactivity. Similar results were obtained in two additional experiments.

position of the PrGl fraction from cholesterol-enriched cultures shifted to a K_{av} of 0.44, indicating an increase in the molecular mass.

DISCUSSION

The studies of Goldstein et al. [23] have established that human aortic SMCs become cholesteryl ester-enriched *in vitro* after incubation with cationized LDL. In agreement with this, our results show that incubation of human aortic SMCs with cationized LDL, but not native LDL, for 2 days increased the cellular cholesteryl ester 82-fold and free cholesterol 1.3-fold over controls. Our earlier studies and those of others have demonstrated that exposure to cationized LDL also causes cholesteryl ester accumulation in rabbit SMCs [19,35]. Therefore incubation with cationized LDL offers a convenient method for cholesterol enrichment of SMCs *in vitro*. Moreover this model can be used to study the consequences of cholesterol enrichment on SMC metabolism.

Our results show that cholesterol-enriched SMCs synthesized significantly less PrGls than did normal cells. This is not attributable to alterations in cell number or viability. In fact, cultures exposed to cationized LDL showed increased cell proliferation. Despite this, PrGl synthesis by these cells was depressed. The suppression of PrGl synthesis is a consequence of cellular cholesterol enrichment because incubation of SMCs with native LDL, which did not cause cellular cholesterol loading, did not suppress PrGl synthesis. The decrease in PrGl synthesis in cholesterol-enriched cells is attributable to inhibition of synthesis *de novo*. This is consistent with a 55% decrease in [^{35}S]sulphate incorporation into PrGls in cholesterol-enriched cells compared with normal cells after a 15 min pulse. These results, however, are inconsistent with our recent observations on cholesterol-enriched rabbit SMCs [19]. Although we used identical procedures for cholesterol enrichment of the cells in both studies, PrGl synthesis was significantly stimulated in cholesterol-loaded rabbit cells but not in the human cells. This discrepancy probably represents species differences in the response of SMCs to cholesterol accumulation.

Despite this species difference in total PrGl synthesis, cholesterol enrichment causes intracellular accumulation of newly synthesized PrGl in both cell types. Although the present study did not investigate the mechanism of over-accumulation of PrGls in cholesterol-enriched human cells, our earlier work determined that PrGl degradation and secretion are severely retarded in cholesterol-enriched rabbit SMCs [19]. Similar mechanisms could also be involved in the excessive accumulation of PrGls in the cell layer compartment of cholesterol-enriched human cells.

It seems that the decreased PrGl synthesis in cholesterol-enriched SMCs is due to inhibition of transcription. Cholesterol enrichment almost completely suppressed versican transcripts and inhibited biglycan and decorin transcripts by 90% and 20% respectively. Although the changes in mRNA levels reflect a general decrease in overall PrGl synthesis, as indicated by our results, the mRNAs of all three PrGl core proteins were not affected equally by cholesterol enrichment. These findings emphasize that cholesterol accretion in human aortic SMCs differentially regulates the expression of PrGl core protein mRNA. The decrease in PrGl mRNA could be due to either decreased transcription or increased mRNA degradation. However, this phenomenon is not likely to represent a global alteration in mRNA synthesis and processing because levels of β-actin mRNA were not different between the two groups.

It is evident from the current study that in cholesterol-enriched SMCs there was a 2-fold increase in a PrGl subfraction with high binding affinity for LDL. This implies that, despite the down-regulation of PrGl synthesis in these cells, at equivalent concentrations the PrGl synthesized by the cholesterol-loaded cells contains twice the subfraction that binds LDL with high affinity than the PrGl synthesized by normal SMCs. This PrGl subfraction is larger and has a higher charge density than does its counterpart from normal cells. In addition it also contains a higher proportion of chondroitin 6-sulphate and dermatan sulphate. Because all the above features increase the binding ability of PrGl to LDL [36,37], in all likelihood this PrGl fraction produced by the cholesterol-enriched SMCs will also bind LDL very avidly.

To our knowledge, this study shows for the first time that cholesterol accretion in human SMCs affects PrGl metabolism both at the transcriptional and post-translational levels. At present we can only speculate on the significance of these findings *in vivo*. The presence of SMCs enriched in cholesteryl ester is a major feature of atherosclerotic lesions. As PrGls are involved in maintaining the structural integrity of the artery wall and SMCs are the main source of arterial PrGl, a significant decrease in PrGl synthesis and secretion by cholesterol-loaded SMCs, as seen in our study, could seriously compromise artery wall integrity. At the same time, a PrGl subfraction synthesized by these cells could bind LDL with high affinity. This, in turn, could contribute to increased intracellular retention of this lipoprotein.

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