

Differential uptake of proteoglycan-selected subfractions of low density lipoprotein by human macrophages

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Abstract Macrophages and arterial chondroitin sulfate proteoglycans (CSPG) are probably associated with extracellular and intracellular lipoprotein deposition during atherogenesis. We found that human arterial CSPG can be used to select subclasses from low density lipoprotein (LDL) with different structural properties and capacities to interact with human monocyte-derived macrophages (HMDM). Four subclasses, LDL(PG)1 to LDL(PG)4, in order of decreasing CSPG-complexing capacity, were prepared and characterized in terms of their ability to interact with HMDM. The LDL subclasses with highest avidity for CSPG, LDL(PG)1 and LDL(PG)2, were bound, internalized, and degraded more efficiently than those of lower avidity for CSPG. From LDL(PG)1 to LDL(PG)4, the gradual decrease in uptake by HMDM and decreasing avidity for CSPG were associated with a gradual decrease in isoelectric point (from 5.93 to 5.68) and an augmented ratio of surface polar lipid to core nonpolar components (from 0.35 to 0.54). Competition experiments indicated that the proteoglycan-selected subfractions shared the binding sites and uptake mechanisms of native LDL. ■ The results suggest the existence of a structurally related gradation in the avidity of LDL subpopulations for cells and matrix components. The presence within LDL subpopulations of a differential capacity to interact with intimal extracellular and cellular elements could be associated with a similar heterogeneity in their atherogenic potential. — Hurt-Camejo, E., G. Camejo, B. Rosengren, F. Lopez, O. Wiklund, and G. Bondjers. Differential uptake of proteoglycan-selected subfractions of low density lipoprotein by human macrophages. *J. Lipid Res.* 1990. 31: 1387–1398.

Supplementary key words macrophage uptake • surface to core lipid ratio • LDL subclass size and charge

Accumulation of lipoproteins during atherosclerotic lesion development appears to be a sequential process by which apoB-containing lipoproteins are retained in the extracellular compartment, from where they are taken up by macrophages and smooth muscle cells (1). The intimal extracellular matrix contains several sulfated proteo-

glycans which appear responsible for the formation of complexes with apoB-containing lipoproteins. This may result in an increase of the lipoproteins' time of residence in the intima and, as suggested by in vitro experiments, stimulate their uptake by macrophages (2–6). The interaction of serum lipoproteins and proteoglycans can lead to soluble and insoluble LDL-PG complexes that are formed initially through negatively charged sulfate groups in the proteoglycans and lysine, arginine-rich segments in apoB-100 (7–9).

By adjusting the ionic strength, divalent ion concentration, and ratio of proteoglycan to lipoprotein, it is possible to form reversible associations of human chondroitin-6-SO₄ proteoglycan (CSPG) and LDL. These complexes can be solubilized by addition of physiological solutions. There is no evidence of LDL aggregates or LDL-CSPG associations remaining after solubilization and filtration of the solutions (6, 10). We have found that individual LDL preparations show dissimilar capacities to interact with CSPG and that this is related to small differences in isoelectric point and composition (11). Furthermore, within the same LDL preparation, when a large ratio of LDL to CSPG is used, the fraction that is insolubilized shows small differences in charge and composition from the lipoprotein that remains in solution (12). This indicates that when LDL is in excess, the arterial CSPG selects the LDL subclasses with higher affinity. Case-controlled studies suggest that frequently the presence in

Abbreviations: HMDM, human monocyte-derived macrophages; LDL, low density lipoprotein (d 1.019–1.063 g/ml); CSPG, arterial chondroitin sulfate-rich proteoglycan; apoB-100, apolipoprotein B-100; Tris, tri(hydroxymethyl)-aminomethane; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid); EDTA, ethylenediamine tetraacetic acid-Na₂; PMSF, phenyl methylsulfonyl fluoride; PBS, phosphate-buffered saline; TC, tyramine-cellobiose.

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serum of LDL with high in vitro affinity for arterial CSPG is associated with atherosclerosis and coronary heart disease (13–15). Furthermore, in rabbits LDL with increased positive charge, which augments several-fold its reactivity for arterial CSPG, is deposited to a higher extent in vivo in arterial intima (12). However, it is not clear whether the distinctive reactivity of LDL from different individuals for CSPG is a consequence of dissimilar amounts of subclasses with varied affinity and structure, or whether it is a property common to the whole fraction. These possibilities appeared worth exploring because of recent findings that suggest that structural heterogeneities in LDL could be strongly associated with coronary heart disease (16). It is possible that the potential atherogenicity of LDL subclasses is related to their differential reactivity for intimal proteoglycans and subsequent differential uptake by macrophages. Accordingly, to test this hypothesis, in the present study we investigated the possible relation between differential LDL affinity for proteoglycans and subsequent uptake and degradation by human macrophages. The uptake and degradation by HMDM of the LDL subclasses was found to be related to their affinity for CSPG and to differences in size, charge, and structure within them. This suggests that macrophages can distinguish between the proteoglycan-selected LDL subclasses, although all of them seem to follow the same pathways for binding and internalization used by native LDL.

MATERIALS AND METHODS

Materials

Sodium [^{125}I]iodide (14.4 mCi/ μg iodine), was purchased from Amersham International (Amersham, England). Iodogen was purchased from Pierce (Rockford, IL). Plastic culture dishes and tubes were purchased from Nunclon (Delta, Denmark). Ficoll-Hypaque and PD-10 Sephadex G-25 columns were purchased from Pharmacia (Uppsala, Sweden). Millex-GV, nonprotein binding filters for lipoprotein and medium filtering, were purchased from Millipore S. A. (Molsheim, France). All cell culture supplies were obtained from Flow Laboratories (Irvine, Scotland). Other salts, buffer substances, and solvents used in this work were of analytical grade and purchased from Merck (Darmstadt, West Germany).

Lipoproteins and proteoglycans

Differential and centrifugation in KBr solutions was used for the preparation of LDL within the density range 1.019–1.063 g/ml, as described (10). The lipoproteins were maintained in KBr with 1 mg/ml EDTA and 0.2 mM PMSF at 2–4°C. Just before use, they were equilibrated with the appropriate solution using PD-10 gel exclusion columns (Pharmacia). In the experiments to be described

lipoproteins from three different donors were used. Equilibrium density gradient centrifugation was used to obtain LDL subfractions with average densities 1.030, 1.040, and 1.051 g/ml (17).

Proteoglycans were extracted from intima-media minces prepared from human aortas obtained at necropsy within 12 h of accidental death and extracted for 24 h with 15 volumes of 6 M urea, 1 M NaCl in the presence of 10 mM EDTA, 0.2 mM PMSF, 5 mM benzamidine-HCl, and 10 mM ϵ -aminocaproic acid as protease inhibitors. After centrifugation at 100,000 g for 60 min, the supernatant was dialyzed for 48 h against 4 volumes of 6 M urea with the enzyme inhibitors to reduce the NaCl concentration to 0.25 M. Fifty-ml portions of the dialyzed material were loaded in a 10 \times 1.5 cm DEAE-Sephacell column (Pharmacia) equilibrated with 6 M urea, 0.25 M NaCl, 10 mM CaCl_2 , 0.05 M acetate buffer, pH 6.2, and containing protease inhibitors. The column was washed with 100 ml of the initial buffer to eliminate most of the contaminant protein and afterwards a 0.25–1.0 M NaCl 100 ml linear gradient was passed through at 20 ml/h. Two 280-nm absorbing peaks were obtained with maxima around 0.4 and 0.6 M NaCl. Most of the LDL complexing activity was found in the second peak after dialysis. This peak was collected, dialyzed against the initial buffer, and rechromatographed in a DEAE-Sephacell column.

On cellulose acetate electrophoresis in 0.2 M CaCl_2 , a papain-treated sample of the second peak moved as a single band in a position similar to that of chondroitin-6- SO_4 . High performance liquid chromatography (18) of the unsaturated disaccharides produced by chondroitinase ABC (EC 4.2.2.4) and AC (EC 4.2.2.5) indicated that the CSPG preparations used here contained 50–55 % chondroitin-6- SO_4 , 20–25 % chondroitin-4- SO_4 , and 12–15 % of the dermatan isomer. With both enzymes, approximately 10 % of the unsaturated disaccharides was nonsulfated. The protein content was 20–25 % of glycosaminoglycan content.

Chondroitin-4- SO_4 (whale cartilage), chondroitin-6- SO_4 (shark cartilage), and dermatan sulfate (pig skin) were Special grade and purchased from Seikagaku Kogyo Co. Ltd (Tokyo, Japan). The chondroitinases ABC and AC and the standard disaccharides were also obtained from this supplier. Heparin (170 $\mu\text{g}/\text{mg}$ from porcine intestine), papain, and TPCK-trypsin were purchased from Sigma Chemical Co. (St. Louis, MO).

Isolation and culture of macrophages

Human monocyte-derived macrophages were obtained from buffy coats by the Ficoll-Hypaque procedure (19). In the present experiments, cells from eight donors were used. Mononuclear cells were washed five times with phosphate-buffered saline, pH 7.2, without calcium or magnesium, which contained 10 mM EDTA to remove

platelets. Mononuclear cells were resuspended to 5×10^6 cells in serum-free RPMI 1640 medium supplemented with: 24 mM NaHCO_3 , 10 mM HEPES, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 1 mM sodium pyruvate, 4 mM glutamine, and nonessential amino acids. One ml or 0.5 ml of cell suspension was seeded in 30-mm or 16-mm plastic wells, respectively, and monocytes were allowed to adhere overnight. Nonadhered cells were eliminated the next day with six washes of PBS, and adhered monocytes (10^6) were cultured with 1 ml of RPMI 1640 medium supplemented with: 10% (v/v) fetal calf serum, 10% (v/v) human serum, 24 mM NaHCO_3 , 10 mM HEPES, 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 1 mM sodium pyruvate, 4 mM glutamine, and nonessential amino acids (hereafter referred to as medium A). The experiments were performed with HMDM cultured for 4 and 7 days after plating. Twenty four hours before addition of labeled lipoproteins, the cells were washed three times with 1 ml of PBS, pH 7.2, containing 2 mg/ml BSA (PBS-BSA) and three times with 1 ml of PBS, pH 7.2. The cells were then incubated in 1 ml of culture medium containing 5 mg protein/ml apolipoprotein B-free lipoprotein-deficient human serum (LPDS) in order to induce LDL receptor expression (medium B, see below). All the incubations were performed at 37°C in a 5% CO_2 humid atmosphere.

Lipoprotein labeling

For the studies of cellular uptake, iodination was performed with ^{125}I -labeled tyramine-cellobiose (TC) (20). For the studies of cellular degradation, conventional labeling of the lipoproteins with [^{125}I]iodide was performed with the iodine monochloride method (21). For both iodination procedures the specific activity was between 50 and 180 cpm/ng of protein. More than 99% of the radioactivity could be precipitated with 15% TCA (w/v). Less than 5% of the total was lipid-extractable.

Preparation of LDL(PG) subfractions

Arterial CSPG solutions (1 mg/ml of glycosaminoglycan calculated from the hexuronate content $\times 3.3$) were prepared from lyophilized samples, using buffer A which contained 5 mM HEPES, 4 mM CaCl_2 , 2 mM MgCl_2 , 0.2 mM PMSF, and 20 mM NaCl and adjusted to pH 7.20. ^{125}I -labeled tyramine cellobiose-labeled LDL or unlabeled LDL preparations were equilibrated in buffer A and adjusted to 2 mg protein/ml. To obtain four sequential subfractions of LDL, 10 to 20 μl of CSPG was added to 1 ml of lipoprotein solution. The insoluble LDL-CSPG complex that formed immediately was collected by centrifugation for 10 min at 10000 g in conical plastic tubes. The supernatant containing the uncomplexed LDL was collected, another 10 to 20 μl of CSPG was added to it, and the second batch of LDL-CSPG complex was col-

lected in the same way as the first, and the supernatant was again saved. The procedure was repeated twice; therefore, four subfractions of LDL were obtained. By adjusting the volumes of CSPG between 10 and 20 μl (10 to 20 μg) the four LDL subfractions added up to 80–90% of the original LDL. The amount of LDL in each fraction was 10–20% for fraction 1, 20–40% for fractions 2 and 3, and 10–20% for fraction 4. Due to the large excess of LDL, each amount of CSPG added was quantitatively collected in each pellet, and no detectable proteoglycan remained in each sequential supernatant when measured with the procedure of Lammi and Tammi (22), which can detect 0.05 μg . The collected LDL subfractions, hereafter designated LDL(PG)1 to LDL(PG)4, were washed with 1 ml of buffer A–20 mM NaCl. A mixture of the different LDL(PG) subfractions was prepared by adding together LDL(PG)1 through LDL(PG)4, plus the low affinity supernatant LDL, in their proper proportions. The subfractions to be used for cell uptake were redissolved in cell culture media and filtered through 0.22- μm filters. Those to be used for structural studies were redissolved in buffer A made 150 mM in NaCl and also filtered. Under these conditions, the LDL that precipitates is easily redissolved in less than an hour without the need for mechanical stirring. Since 1 μg of CSPG-glycosaminoglycan precipitates with 25 to 40 μg of LDL-protein, the amount of proteoglycan present in the lipoprotein solutions added to the cells can be at most 4 μg per dish when adding 100 μg of apoB protein.

Lipoprotein binding and uptake

HMDM were cultured for 4 or 7 days and preincubated 24 h with lipoprotein-deficient culture medium. The cells were washed three times with PBS before adding medium B (lipoprotein-deficient serum) containing ^{125}I -labeled tyramine-cellobiose-labeled LDL(PG) subfractions. After incubation for 14 h at 37°C , the cells were washed, first with 1 ml PBS containing 2 mg BSA/ml and then with 1 ml PBS alone. The medium and the two washes were pooled and the radioactivity corresponding to the unbound lipoproteins was measured. The cells were treated with 5 mg/ml trypsin in 1 ml of Hank's medium without calcium and magnesium for 10 min at 37°C . The medium with trypsin was transferred to a tube and the trypsinized cells were washed with 1 ml of Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum and with 1 ml PBS. The trypsin medium and the two washes were mixed and radioactivity corresponding to the cell surface-bound lipoprotein was counted. Adhered cells were dissolved by addition of 0.5 ml of 0.2 N NaOH ($2 \times$) and transferred to a tube. The radioactivity corresponding to the amount of lipoprotein internalized by the cells was counted. Aliquots from the cell extraction were used to measure protein.

Competition experiments were performed similarly as above but by adding a constant amount of ^{125}I -labeled TC-LDL(PG)1, ^{125}I -labeled TC-LDL(PG)4, or ^{125}I -labeled TC-LDL (10 $\mu\text{g}/\text{ml}$ cell medium) and increasing amounts of unlabeled native LDL.

Lysosomal degradation of ^{125}I -labeled LDL and ^{125}I -labeled LDL-PG lipoprotein subfractions was determined as follows. HMDM were cultured 7 days and preincubated 24 h with lipoprotein-deficient culture medium (medium B). The cells were washed three times with PBS before adding medium B containing ^{125}I -labeled LDL, ^{125}I -labeled LDL(PG)1 to LDL(PG)4 subfractions or a pool of ^{125}I -labeled LDL(PG) subfractions at the concentrations indicated in the figure. After 4 h of incubation at 37°C , the extent of degradation was determined by measuring the amount of ^{125}I -labeled material soluble in trichloroacetic acid present in the media (^{125}I -labeled monotyrosine) (23). Adhered cells were washed one time with PBS containing 2 mg BSA/ml and two times with PBS alone. The cells were dissolved by adding 0.4 ml of 0.2 N NaOH two times and then were transferred to a tube. The radioactivity corresponding to the amount of lipoprotein associated with the cells was counted. Aliquots from cell extraction were used to measure protein.

In all cell assays, parallel dishes without cells were always included as control for unspecific adsorption or degradation of the label. Radioactivity in these dishes was subtracted from that of the dishes with cells.

Analytical procedures

Protein was determined by the Bradford procedure using bovine gamma globulin as standard (Bio-Rad Protein Assay, Bio-Rad Chemical Division, Richmond, CA). Cholesterol was analyzed by a colorimetric procedure (24). ^{125}I radioactivity was measured with an LKB 1282

Compugamma Universal Gamma Counter (LKB, Bromma, Sweden). Lipid composition of the lipoprotein fractions was performed on Folch extracts (25) by means of thin-layer chromatography on silica bound to capillary quartz rods (Chromatorods SIII) with the use of three sequential solvents: hexane-ethyl ether 90:10 (v/v), hexane-ethyl ether-acetic acid 85:10:5 (v/v), and chloroform-methanol-water 65:25:4. After each solvent, the separated lipids were quantitatively evaluated by passing the rods through a flame ionization detector in an Iatronscan TH-10 analyzer (Iatron Laboratories, Inc. Tokyo, Japan). The response of the system was calibrated with a standard mixture containing equal amounts of cholesteryl oleate, free cholesterol, palmitate, phosphatidylserine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine obtained from Supelco (Supelco Park, Bellefonte, PA). Analytical isoelectric focusing of LDL subclasses was carried out on 0.5-mm thick agarose gels containing 10% (w/v) sorbitol and 5% (v/v) of a mixture of Pharmalyte 4-6.5 and 5-8 (26). The gels were run in a Phastsystem and isoelectric point standards were included in each run. After washing and drying, the gels were stained with Coomassie brilliant blue. The chemicals and instrument were purchased from Pharmacia. Nondenaturing electrophoresis was run using 2/16 PAA agarose-acrylamide gels obtained from Pharmacia, according to Krauss and Burke (27), with the difference that LDL and its subfractions were prestained with 5% (v/v) of 0.65% (w/v) Sudan black in ethylene glycol. The results from isoelectric focusing and electrophoresis were evaluated with an LKB Ultrascan LX laser densitometer (LKB). Proteoglycan (glycosaminoglycan) content was measured with a nanogram-sensitive Safranin O method (22). The statistical significance of the data was evaluated with the Statgraphics program (Statistical Graphics Corporation, Rockville, MD).

TABLE 1. Percentage composition of the parent LDL and LDL subclasses prepared by sequential precipitation with arterial CSPG

LDL	% Protein ^a	% Lipid ^a	% of the Lipids					
			CE	TG	Chol	PC	Sph	PS + PE ^b
LDL _{tot} 1	25.8	72.1	64.2 \pm 0.7	5.3 \pm 0.5	10.1 \pm 0.3	12.7 \pm 0.9	4.3 \pm 0.1	3.0 \pm 0.2
LDL(PG)1	27.9	72.1	69.9 \pm 0.8	3.9 \pm 0.8	8.2 \pm 0.2	10.8 \pm 0.6	4.5 \pm 0.4	3.5 \pm 0.7
LDL(PG)2	26.2	73.7	68.9 \pm 1.0	3.9 \pm 0.2	7.6 \pm 0.3	11.9 \pm 0.8	4.1 \pm 0.8	3.5 \pm 0.7
LDL(PG)3	26.1	73.9	65.5 \pm 0.5	4.8 \pm 0.4	10.9 \pm 0.9	11.2 \pm 0.5	3.9 \pm 0.3	3.7 \pm 0.6
LDL(PG)4	24.9	75.1	62.4 \pm 0.7	4.6 \pm 0.1	10.0 \pm 0.2	12.8 \pm 0.3	5.3 \pm 0.3	4.8 \pm 0.3
LDL _{tot} 2	24.6	75.4	62.8 \pm 2.0	4.1 \pm 0.1	10.2 \pm 0.5	13.7 \pm 0.7	4.9 \pm 0.8	3.8 \pm 0.9
LDL(PG)1	28.6	71.4	70.1 \pm 2.0	4.9 \pm 0.3	8.9 \pm 1.8	10.2 \pm 1.2	3.7 \pm 0.1	2.2 \pm 0.2
LDL(PG)2	26.2	73.8	67.8 \pm 0.7	4.8 \pm 0.1	9.7 \pm 0.1	10.9 \pm 1.5	5.0 \pm 2.4	3.6 \pm 1.0
LDL(PG)3	24.5	75.5	63.7 \pm 3.0	3.9 \pm 1.4	11.4 \pm 2.0	12.4 \pm 1.5	5.0 \pm 2.4	3.6 \pm 1.0
LDL(PG)4	23.8	76.2	59.5 \pm 2.0	3.8 \pm 0.2	9.9 \pm 2.4	15.1 \pm 1.2	6.4 \pm 0.6	5.4 \pm 0.7

CE, cholesteryl ester; TG, triglyceride; Chol, cholesterol; PC, phosphatidylcholine; Sph, sphingomyelin; PS, phosphatidylserine; PE, phosphatidylethanolamine.

^aThe amount of protein was obtained by spectrometric evaluation and the lipid content was determined by direct dry weight determination of aliquots of the lipid extracts. The values are the average of triplicate determinations.

^bThe values are calculated from the integrated areas obtained in the Iatronscan and represent the average \pm SD of four individual runs.

TABLE 2. Volume and area parameters for parent LDL and LDL subclasses obtained by sequential complexing with arterial CSPG

Lipoprotein	Vtl ^a	Dtl ^b	Vnpl ^c	Dnpl ^d	Vpl ^e	Anpl ^f	Apl ^g	% A Occ PL ^h	% A Accbl Protein ⁱ
LDL	2.81	17.5	1.98	15.6	8.40	7.6	4.2	55.2	44.8
LDL(PG)1	2.27	16.3	1.77	15.0	5.04	7.1	2.5	35.6	64.4
LDL(PG)2	2.56	17.0	1.93	15.4	6.30	7.5	3.2	42.0	58.0
LDL(PG)3	2.77	17.4	1.97	15.5	8.07	7.6	4.0	53.1	46.9
LDL(PG)4	2.86	17.6	1.91	15.0	9.46	7.5	4.7	63.3	36.7

^aVtl, volume of total lipid mass per spherical particle ($\text{nm}^3 \times 10^{-3}$).

^bDtl, diameter of total lipid mass per spherical particle (nm).

^cVnpl, volume of spherical nonpolar lipid core (cholesteryl ester + TG) ($\text{nm}^3 \times 10^{-3}$).

^dDnpl, diameter of spherical nonpolar lipid core (cholesteryl ester + TG) (nm).

^eVpl, volume of polar lipid mass (free cholesterol + phospholipids) ($\text{nm}^3 \times 10^{-2}$).

^fAnpl, surface area of the nonpolar lipid core ($\text{nm}^2 \times 10^{-2}$).

^gApl, area of the polar lipid monolayer assumed to be 2 nm thick ($\text{nm}^2 \times 10^{-2}$).

^h% A Occ PL, percentage of the nonpolar lipid core surface area theoretically occupied by a condensed monolayer of polar lipids.

ⁱ% A Accbl Protein, percentage of the nonpolar surface area accessible to the apoB-100 moiety.

RESULTS

Characteristics of the CSPG-selected LDL subclasses

Table 1 presents the composition of the sequential, CSPG-selected subclasses obtained from LDL preparations from two subjects. From fractions 1 to 4 there was a significant decrease in the protein content with a proportional increment in the total lipid content. Within the lipids, the most noticeable difference from LDL(PG)1 to LDL(PG)4 was a decrease in the percentage of cholesteryl esters and an increase in phosphatidylcholine. With these data, and knowing that each particle will contain at most one apoB-100 molecule, it is possible to calculate the number of each class of molecule per particle, assuming an average molecular weight for cholesteryl esters of 650; for triglycerides, 900; and phospholipids, 850. Once this is obtained and with the use of average volumes for each class of molecule (28), the volumes per particle occupied by the nonpolar core, containing cholesteryl esters and triglycerides, and the volume, occupied by the lipids in the polar surface monolayer, can be estimated (28, 29). In Table 2, the calculated parameters for one lipoprotein preparation and its CSPG-selected subclasses are presented; similar results were obtained with the other analyzed lipoproteins. It can be observed that the total volume of lipids (Vtl) increases from LDL(PG)1 to LDL(PG)4, mainly because the nonpolar lipid volume (Vnpl) increases from 1.77×10^{-3} to $1.98 \times 10^{-3} \text{ nm}^3$. Since the increment of polar lipid volume (Vpl) along the subclasses is almost twofold (5.04 to $9.46 \times 10^{-2} \text{ nm}^3$), the area of the nonpolar spherical core (Anpl) that can be covered by the polar lipid monolayer becomes higher (Apl), from LDL(PG)1 to LDL(PG)4. If one assumes a thickness of 2 nm for the polar monolayer, the percentage of the core surface covered by polar lipids can be estimated and an idea of the surface area left for the polar regions of apoB-100 that are also in the particle surface

can be obtained. In LDL(PG)1, the area that is "accessible" to the protein is 64.4%, but only 36.7% in LDL(PG)4. This suggests that, in the fractions with higher reactivity for the proteoglycan, the protein is more extended in the surface, whereas it is more compact in the

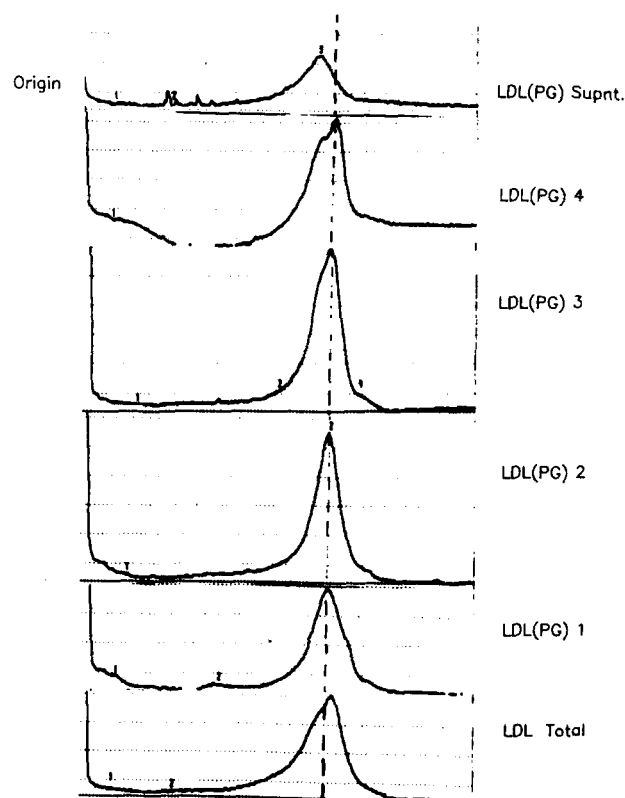


Fig. 1. Nondenaturing gradient gel electrophoresis of total LDL and LDL subclasses obtained by sequential complexing with the arterial chondroitin sulfate-rich proteoglycan (CSPG). The Sudan black pre-stained lipoproteins were run in a 2–16% agarose-polyacrylamide gel for 2400 volt-hours. The gel was scanned with a laser densitometer. The upper scan corresponds to the final supernatant remaining after collection of the final proteoglycan complexed LDL fraction: LDL(PG)4.

LDL subclasses with lower affinity. Although several assumptions are present in the above calculations, such as the polar phase/nonpolar phase lipid class distribution, the monolayer thickness, and the particle sphericity, they are applicable to all the CSPG-selected subclasses and are supported by experimental data (28, 29).

The total lipid volume of the particles increased from 2.27 to 2.82×10^{-3} nm. Because the protein content per particle is constant, the total volume of the particle should increase from LDL(PG)1 to LDL(PG)4. In terms of the particle diameter, this increase is modest: 0.7 to 1.2 nm. The results from a nondenaturing gel electrophoresis experiment confirmed the above supposition. **Fig. 1** shows the gradual decrease in mobility from LDL(PG)1 to LDL(PG)4. The calibration curve for this gel batch indicates that LDL(PG)1 and LDL(PG)4 differ by approximately 1 nm (10 Å) in diameter. This difference in hydrodynamic radius cannot be due to CSPG molecules associated to LDL. In this hypothetical situation, a much larger change would have been observed in the particle since the hydrodynamic radius of proteoglycan aggregates is larger than that of LDL (2–4). In the same gel, a concentrated sample of the supernatant remaining after the collection of LDL(PG)4 was included. This fraction, which amounts to less than 10% of the initial LDL and is not yet characterized, concentrates the largest LDL particles which appear to have low affinity for CSPG.

Surface charge of LDL is an important modulator of its association with CSPG and macrophages (6–11). Measurement of the apparent isoelectric point (pI) of the intact lipoprotein subclasses with the use of agarose isoelectric focusing is a convenient procedure to study this property. **Fig. 2** shows stained gels containing the pat-

terns of proteoglycan-selected LDL subclasses and the parent LDL from two preparations. It can be observed that from LDL(PG)1 to LDL(PG)4, pI values become more acidic. The average values \pm SD for the maxima of the densitometric evaluation were: 5.81 ± 0.04 , 5.93 ± 0.02 , 5.92 ± 0.01 , 5.90 ± 0.01 , 5.81 ± 0.02 , and 5.68 ± 0.02 for the parent LDL, LDL(PG)1 to LDL(PG)4, and the final supernatant, respectively. Although the differences are small, they are statistically significant between LDL(PG)1 and LDL(PG)4. Because the pI scale is logarithmic, the difference represents a 1.32 higher positive charge content in LDL(PG)1 than in LDL(PG)4.

LDL subclass binding and internalization by macrophages

Labeling of LDL with 125 I-labeled tyramine-cellobiose provides a convenient procedure for measurement of cell uptake because, upon degradation of the internalized LDL, the labeled disaccharide is retained in the cells as a cumulative marker of the process with nondetectable label leakage into the medium (20). This is especially useful with cells such as HMDM that can show low levels of LDL receptors (30). **Fig. 3** presents a comparison of internalization and surface-binding between LDL(PG)1 and LDL(PG)4 as function of lipoprotein concentration in the media. Both processes were higher for the LDL subclass that was more readily complexed by CSPG, in those cells that had been cultured for 4 days before the addition of the label.

In a second experiment, surface-binding and internalization were measured for the four proteoglycan-selected LDL subclasses. These results are summarized in **Fig. 4**. Although some of the values measured between neighbor-

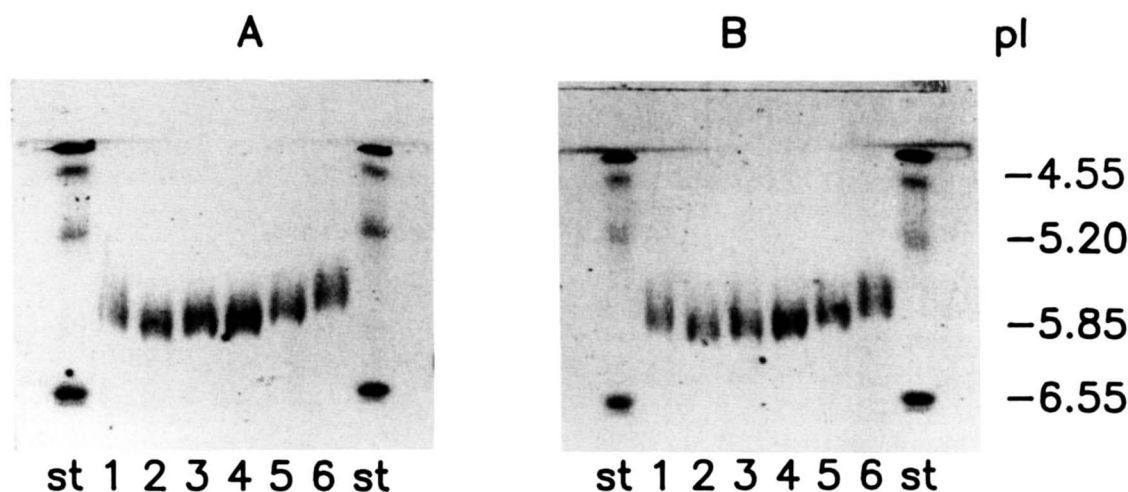


Fig. 2. Thin agarose isoelectric focusing of total LDL and LDL subclasses prepared by sequential precipitation with arterial chondroitin sulfate-rich proteoglycan (CSPG). The gels were stained with Coomassie brilliant blue. Gels A and B correspond to subfractions obtained from two different LDL preparations. The values on the right indicate the isoelectric points of standards (st). The band at pI 5.85 (carbonic anhydrase B) is barely visible in the photographic image. The number at the bottom identifies the samples: st, standard mixture; 1, total LDL; 2, LDL(PG)1; 3, LDL(PG)2; 4, LDL(PG)3; 5, LDL(PG)4; 6, supernatant remaining after collection of the last CSPG-complexed fraction; LDL(PG)4.

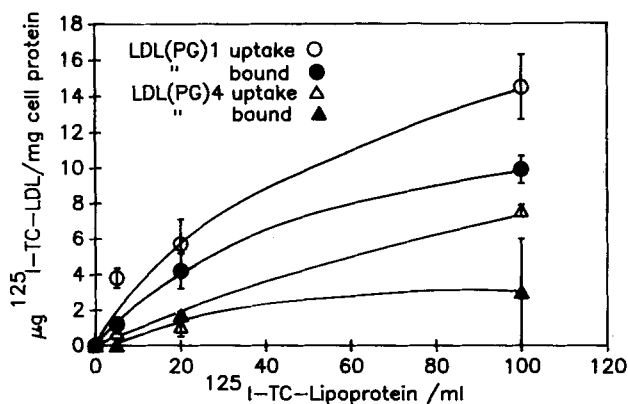


Fig. 3. Surface binding and uptake of proteoglycan-selected subclasses of ^{125}I -labeled tyramine-cellobiose-labeled LDL by human monocyte-derived macrophages. Only the fraction with highest affinity for the chondroitin-rich arterial proteoglycan, LDL(PG)1, and the one with the lowest, LDL(PG)4 were used in this experiment. HMDM were cultivated for 4 days. After 24 h incubation in lipoprotein-deficient medium, macrophages were incubated in 1 ml of lipoprotein-deficient medium containing the indicated concentrations of ^{125}I -labeled tyramine-cellobiose-labeled LDL(PG)1 and LDL(PG)4. After incubation for 18 h, the amount of labeled lipoprotein bound to the cell surface and internalized by the HMDM was determined as described in Materials and Methods. The values are averages of four determinations \pm SD.

ing LDL subclasses were not statistically significant, as can be judged from the overlapping standard deviation values, there was a clear and significant tendency from LDL(PG)1 to LDL(PG)4 subfractions for a gradual decrease in net uptake and surface-binding (data not shown). In all cases measured, there was a negative correlation above -0.8 with $P < 0.05$ when the values for

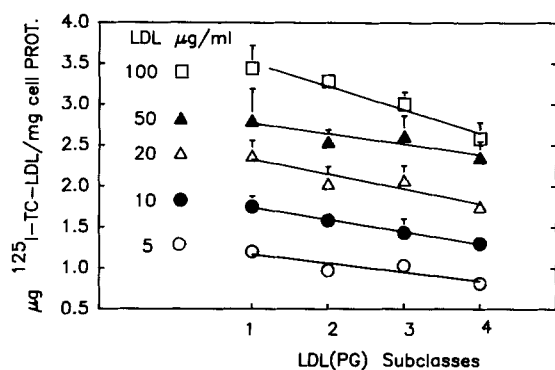


Fig. 4. Uptake by human monocyte-derived macrophages of proteoglycan-selected subclasses of ^{125}I -labeled tyramine-cellobiose-labeled LDL. In this experiment the four proteoglycan-selected LDL subclasses, LDL(PG)1 to LDL(PG)4, were studied. HMDM were cultured for 7 days. After 24 h preincubation in lipoprotein-deficient medium, macrophages were incubated in 1 ml of lipoprotein-deficient medium containing the indicated concentrations of each ^{125}I -labeled tyramine-cellobiose-labeled LDL(PG) subclass. After incubation for 18 h, the amount of labeled lipoprotein uptake by the macrophages was determined as described in Materials and Methods. The values represent the averages of four determinations and the bars indicate the standard deviation. The symbols indicate the concentrations of lipoprotein used in the media.

surface-binding and internalization were correlated with the number for the LDL subclasses.

It can be noted that the absolute values for uptake, expressed in μg ^{125}I -labeled TC-LDL/mg cell protein, are lower in the experiments in Fig. 4 than those in Fig. 3. These differences could have been caused by the longer culture period used for the cells in the latter experiments (30).

LDL(PG) subfraction degradation

The differential uptake by HMDM of proteoglycan-selected LDL subfractions could be a property of pre-existing LDL subclasses. Also, the differential uptake could be potentiated by modifications induced in the LDL particles during their interaction with the CSPG. To explore these two possibilities, we prepared a mixture of LDL(PG)1 through LDL(PG)4 subfractions plus the low affinity supernatant LDL in their proper proportions (LDL(PG)-pool) and measured its degradation by HMDM. If the differential uptake of LDL(PG)1 to LDL(PG)4 subfractions is a property of pre-existing LDL subclasses, then the LDL(PG)-pool should be internalized and degraded by HMDM to the same extent as the total native LDL. **Fig. 5** presents a comparison of HMDM degradation of ^{125}I -labeled LDL(PG)1 to LDL(PG)4 subfractions, total native LDL, and the mixture LDL(PG)-pool as a function of lipoprotein concentration in the media. The results show first that all four ^{125}I -labeled LDL(PG) subfractions were degraded to a higher extent than native ^{125}I -labeled LDL at protein concentrations from 5 to 50 μg of protein/ml of medium. Second, similar

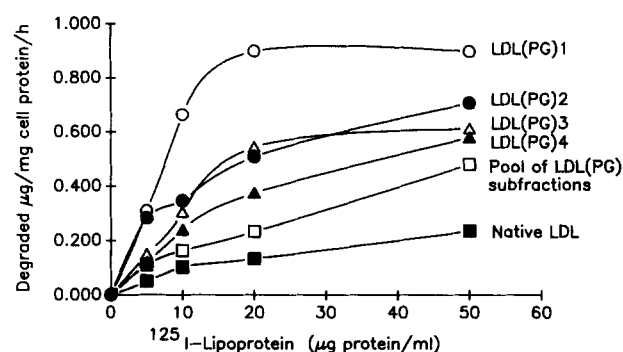


Fig. 5. Degradation by human monocyte-derived macrophages of proteoglycan-selected subclasses of LDL. In this experiment the four proteoglycan-selected LDL subclasses were studied: LDL(PG)1 (\circ), LDL(PG)2 (\bullet), LDL(PG)3 (\triangle), LDL(PG)4 (\blacktriangle); the pool of LDL(PG)1 to LDL(PG)4 subfractions plus the low affinity LDL supernatant in their proper proportions (\square), and total native LDL (\blacksquare). Human monocyte-derived macrophages were cultured 7 days. After 24 h preincubation in lipoprotein-deficient medium, macrophages were incubated in lipoprotein-deficient medium containing the indicated concentrations of each ^{125}I -labeled lipoprotein. After 4 h, the extent of lipoprotein degradation was determined by measuring the content of ^{125}I -labeled, acid-soluble monotyrosine. The values represent the average of duplicate determinations.

to the results obtained in the surface-binding and uptake experiments using ^{125}I -labeled TC, there was a significant tendency from LDL(PG)1 to LDL(PG)4 for a gradual decrease in cell internalization and degradation. These differences in cell degradation levels between the ^{125}I -labeled LDL(PG) subfractions were higher than the differences obtained in uptake levels using ^{125}I -labeled TC studies. One explanation may be that TC-labeling of protein could reduce the number of free arginine and lysine residues, therefore decreasing the LDL affinity for both the CSPG and the apoB (LDL) receptor (8, 20). Third, the mixture of LDL(PG)1 to LDL(PG)4 subfractions plus the low affinity LDL supernatant was degraded more than the total native LDL. This LDL(PG)-pool, as expected, was less degraded than the LDL(PG)1 to LDL(PG)4 subfractions, due to the presence of the low affinity LDL supernatant in the LDL(PG)-pool. From these results, we can conclude that LDL subclasses with differential reactivity for arterial CSPG pre-exist in preparations of total native LDL. Also, the results indicate that the interaction with CSPG potentiated the differential uptake and degradation between the proteoglycan-selected LDL subclasses.

The above results do not allow any conclusion as to whether subclasses exist within total LDL that are taken up differentially by HMDM. Uptake experiments carried out with ^{125}I -labeled TC-LDL density gradient subfractions show that denser and smaller particles are internalized more than lighter and larger subfractions. The uptake levels obtained after 16 h incubation with 10 $\mu\text{g}/\text{ml}$ of ^{125}I -labeled TC-LDL subfractions were: 0.190 ± 0.127 $\mu\text{g}/\text{mg}$ cell protein for LDL with average density 1.030 g/ml, and 0.400 ± 0.129 $\mu\text{g}/\text{mg}$ for LDL with average density 1.050 g/ml. These values were averages of duplicates from two LDL preparations.

Lipoprotein uptake in the presence of proteoglycan

Polyanionic compounds at high concentrations have been shown to affect LDL binding to cells (31). Therefore, it could be possible that the differences observed in the association and uptake of LDL subclasses by the cells were related to the presence of dissimilar CSPG amounts in the LDL solutions added to the cells. However, the sequential procedure used to prepare the LDL subclasses leads to a quantitative precipitation of each aliquot of CSPG added, as established by measurements of glycosaminoglycans in the supernatant (22). Therefore, the amount of CSPG present in each of the resolubilized LDL subclasses is similar in the different aliquots added to the cells. As mentioned in the Methods section, the maximal amount of proteoglycan-glycosaminoglycan added to the cells with the lipoprotein subclasses could be 2–4 μg per dish. To test whether addition of the arterial CSPG affects the lipoprotein uptake and could explain the differences in uptake and binding between LDL sub-

classes, the following experiment was conducted. HMDM were incubated with 20 $\mu\text{g}/\text{ml}$ of ^{125}I -labeled TC-native LDL and increasing amounts of arterial CSPG were added (2 to 40 $\mu\text{g}/\text{ml}$ culture medium). The results presented in Fig. 6 indicate that the presence of CSPG in the medium in the concentrations that may be added with the LDL(PG) subfractions, if anything, reduced the lipoprotein uptake. Therefore, this effect cannot be responsible for the increased uptake observed for the proteoglycan-treated fractions, 1.85 ± 0.23 for LDL(PG)1 and 1.34 ± 0.2 for LDL(PG)4, when compared with native LDL, 0.96 ± 0.09 $\mu\text{g}/\text{mg}$ cell protein ($n = 4$). On the other hand, the differences in uptake between LDL(PG) subclasses are unlikely to be caused by dissimilar amounts of CSPG added with them because, as mentioned, they contain similar amounts of CSPG.

Competition experiments

Competition experiments in which native LDL was used to displace a constant amount of ^{125}I -labeled TC-LDL(PG)1, LDL(PG)4, and native LDL were used to explore the uptake pathways used by the CSPG-selected subclasses in macrophages. Fig. 7 presents the results for competition of uptake and binding. The addition of native LDL resulted in a dose-dependent inhibition of binding and uptake of the CSPG-selected LDL subclasses. We can conclude that both LDL subclasses are bound and internalized by mechanisms similar to those for the native total LDL. However, as also can be observed in Fig. 7, the concentration of unlabeled native LDL required for half-maximal inhibition was higher for LDL(PG)1 than for LDL(PG)4. LDL showed behavior intermediate between LDL(PG)1 and LDL(PG)4. These results may indicate that LDL subclasses with highest reactivity for the

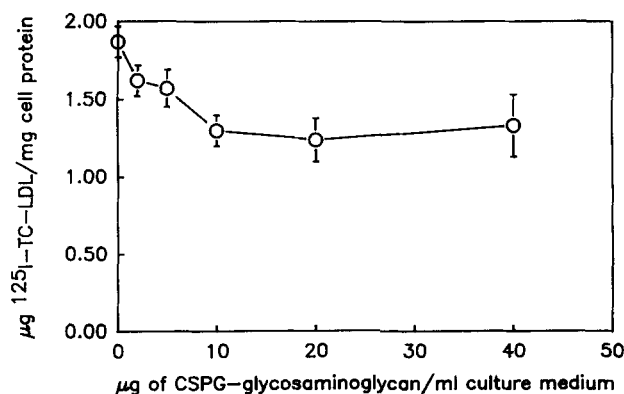


Fig. 6. Effect on uptake of total ^{125}I -labeled TC-LDL of arterial proteoglycans added to the media. HMDM were cultivated for 7 days. After 24 h incubation in lipoprotein-deficient medium, macrophages were incubated for 14 h in 1 ml of lipoprotein-deficient medium containing the indicated concentrations of arterial CSPG-glycosaminoglycan and 20 $\mu\text{g}/\text{ml}$ of total native ^{125}I -labeled TC-LDL protein. The uptake of labeled LDL was determined as described in methods. The values are averages of four determinations \pm SD.

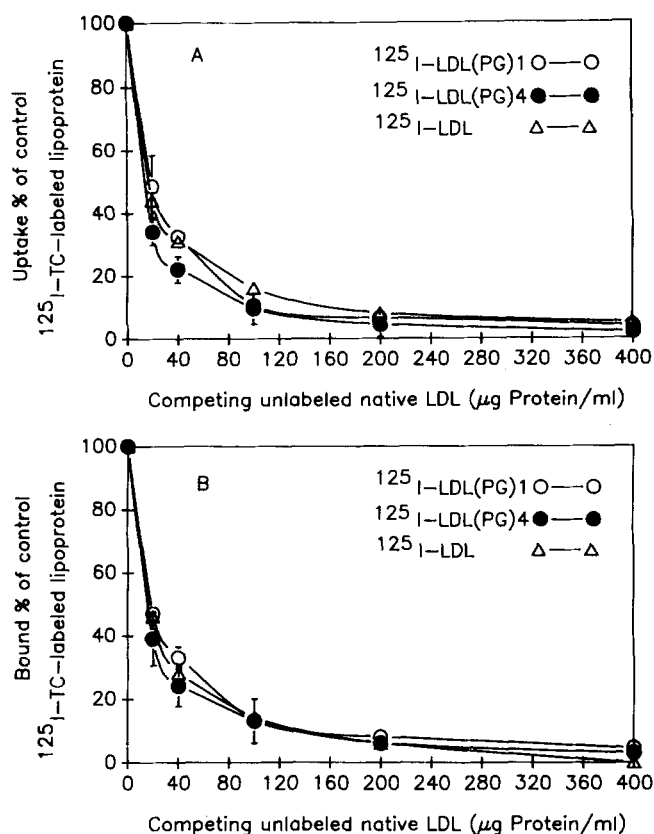


Fig. 7. Competition by unlabeled native total LDL of the uptake (panel A) and binding (panel B) of proteoglycan-selected LDL subclasses by human macrophages. HMDM were cultured for 7 days. After 24 h preincubation with lipoprotein-deficient medium, macrophages were incubated in 1 ml of lipoprotein-deficient medium containing 10 μ g of protein/ml of 125 I-labeled tyramine-cellobiose-labeled LDL(PG)1, LDL(PG)4, or native total LDL in the presence of the indicated concentration of unlabeled native total LDL. After 14 h incubation, the uptake of labeled lipoprotein (panel A) and lipoprotein bound to the cell surface (panel B) were determined as described in Material and Methods. Panel A: the 100% value for the uptake in the absence of competing lipoprotein was 1.85 ± 0.23 ($n = 4$) for LDL(PG)1, 1.34 ± 0.24 ($n = 4$) for LDL(PG)4, and 0.944 ($n = 2$) for native LDL μ g/mg cell protein. The values represent the average of four determinations \pm SD (bars).

CSPG are bound and internalized more effectively than those with lower reactivity for the proteoglycan.

DISCUSSION

Several laboratories have presented evidence that there is a differential contribution of LDL subclasses to atherosclerosis (16, 32, 33). In cholesterol-fed nonhuman primates the presence of a cholesterol-rich large LDL subclass is associated with coronary atherosclerosis (34). However, in humans a low molecular weight LDL particle appears to be an important discriminator between normal subjects and patients with coronary heart disease (16, 32).

A distinct contribution of LDL subclasses to atherogenesis is probably related to their dissimilar fate during interaction with the cellular and noncellular arterial wall components. Size discrimination is a possibility, supported by results that indicate that the endothelium can distinguish between LDL(s) that differ by 10–20% in molecular weight. This means a 4–7% difference in hydrodynamic diameter (35).

Once in the intima, apoB-100 lipoproteins come in contact with the extracellular matrix, macrophages, and smooth muscle cells. The association of LDL, and probably Lp[a], with proteoglycans, collagen, and elastin, and its uptake by cells probably depends on the particles' residence time in the intima and on their affinity for intimal structures (1–7, 36, 37). The affinity of LDL for cells and proteoglycans appears to be structurally related (1–7). However, LDL (d 1.019–1.063 g/ml) is made of overlapping subpopulations that differ in composition and size and probably in other properties related to the arrangement and distribution of polar and nonpolar components of the particle (38, 39). Therefore, gradations of their affinities for cells and proteoglycans are to be expected, and each subject, due to metabolic heterogeneities within LDL and genetic factors, can contain a distinct distribution of LDL subclasses (17, 40). Part of the LDL contribution to atherosclerotic lesion progress seems to be associated with its entrapment by intimal proteoglycans (1–4), possible concomitant oxidative modifications, and intracellular accumulation in macrophages (41). Furthermore, these phenomena could be related since sulfated polysaccharide-induced changes in LDL appear to potentiate its uptake by macrophages (5, 6, 10, 42, 43). In previous work we have shown that this increased interaction of CSPG-treated LDL with HMDM is not caused by the presence of LDL aggregates in the cell culture media. Also, competition experiments suggest that the CSPG-treated LDL is taken up via the apoB,LDL receptor (6, 10).

Avila, Lopez, and Camejo (12) found that arterial CSPG could be used to selectively complex LDL subclasses. In the present work we have used an extension of this finding and isolated LDL subclasses within which the affinity for arterial CSPG is related to their uptake by macrophages. These results were expected because both associations depend on the extent of exposure on the surface of LDL of apoB-100 segments rich in lysine and arginine (8, 9, 44, 45). Analysis of the fractions suggests some of the structural parameters that could contribute to modulate affinity for proteoglycans and cells and how they change along the fractions. The more obvious of these parameters is the lower ratio of surface to core lipids in the LDL subfractions with higher affinity for CSPG and HMDM. This may control and area accessible to apoB-100, resulting in these fractions in a larger area of the particle which is protein covered. This probably has the

consequence of a more extensive exposure of peptide segments on the surface of the LDL subclass. Evidence obtained in monolayer studies of LDL lipids indicates that the surface of free cholesterol and phospholipids is a very condensed one (46). This suggests that any modification, along the subclasses, of the area covered by the required surface monolayer will have to depend on the protein flexibility, and not on modification of the spreading pressure of the lipids that appear to be very tightly associated. The LDL subclasses with lower affinity for the CSPG and the macrophage surface-receptors have a polar lipid surface monolayer that covers up to 20% more area, thus allowing for less extension of the apoB-100. We may speculate that in this situation, the cell receptor recognition sites and the proteoglycan binding regions, both rich in positive lysines and arginine, may be less accessible. The results from isoelectric focusing experiments, indicating that the fractions with less affinity for CSPG and macrophages are less positive, support this hypothesis. The immunoreactivity of lysine- and arginine-rich epitopes in apoB-100 has been shown to be different in VLDL and LDL. This change appears to be associated with the dissimilar triglyceride content (47). Nuclear magnetic resonance studies indicate that LDL segments that contain lysine residues exist in distinct microenvironments and that only about 50 lysines are involved in receptor binding (48). Segments of apoB-100 responsible for its association with sulfated proteoglycans, residues 3359–3377, overlap with segments that apparently recognize the apoB receptor (8, 9). Therefore, our findings that the extent of affinity for arterial proteoglycans of LDL subclasses, associated with particle structure, correlates with extent of binding and uptake by macrophages are not surprising. Related evidence had been obtained by Aviram et al. (48) who found that increasing the content of triglycerides in LDL (decreasing the lipid surface-to-core ratio) reduces its uptake by macrophages and diminishes the exposure of lysine groups. On the other hand, the reduction of triglyceride reverses such effects. These results support the hypothesis that differences in surface and core lipid content modulate the exposure of apoB-100 regions that control interactions with proteoglycans and cells.

The experiments with LDL subfractions obtained by density gradient centrifugation suggest that the differential binding and uptake by the HMDM could be caused by pre-existing LDL subclasses. However, the selective interaction with the arterial CSPG potentiates the differential uptake and degradation by human macrophages, thus supporting the hypothesis that motivated this work. We have previously found that treatment with CSPG alters the exposure of trypsin-hydrolyzable apoB-100 segments (6). It is then possible that LDL subclasses with high affinity for CSPG may be more extensively modified during its association with CSPG through arginine- and

lysine-containing segments of apoB-100, resulting in an increased interaction with HMDM. The binding and uptake by HMDM of LDL subclasses obtained by size exclusion or density gradient should be further studied, accompanied by a comparison of their composition with those of the CSPG-selected subclasses.

Our results raise the possibility that different subjects could contain subclasses within their apoB lipoproteins with dissimilar capacity to associate with arterial extracellular components and macrophages. Both phenomena appear to be closely related to atherogenesis (1–7, 31, 40), and they could be the connection between differences in LDL-proteoglycan affinity and the clinical manifestations of coronary heart disease (13–15). ■■

The authors thank Aira Lidell, Ulla-Britt Rignel, and Mutjaba Siddiqui for technical assistance. Tyramine-cellobiose was a generous gift from Dr. Ray C. Pittman, Department of Medicine, University of California, San Diego, La Jolla, CA. We thank Nadia Steinzor for her help during the preparation of the manuscript. This work was partially supported by grants from the Swedish Medical Research Council (proj.nr. 4531, 7142, 7515), The Swedish Association Against Heart and Chest Diseases, AB Hässle, the King V and Queen Victoria Foundations, and Proyecto SI-1987 from Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT).

Manuscript received 30 August 1989 and in revised form 1 May 1990.

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