RAPID COMMUNICATION

Insoluble Low-Density Lipoprotein-Proteoglycan Complexes Enhance Cholesteryl Ester Accumulation in Macrophages

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The interaction of arterial proteoglycans (PGs) and low-density lipoproteins (LDLs) has been postulated to be an important factor in extracellular cholesterol accumulation in the arterial wall. In the present study, insoluble complexes of LDL and PG (LDL-PG) were prepared and their effects on cholesteryl ester accumulation in mouse peritoneal macrophages was tested. The cholesteryl ester content of macrophages incubated with LDL-PG for 3 days was greater than 20 times that observed in cells incubated with LDL alone. The uptake of ^{125}I-LDL by macrophages was markedly stimulated if LDL was incorporated into a complex with PG. However, in contrast to either LDL or acetylated LDL (ALDL), the extent of subsequent degradation of LDL-PG by the cells was reduced. The uptake and degradation of LDL-PG complexes stimulated macrophage incorporation of ^{14}C-oleic acid into cholesteryl oleate 4- to 5-fold over LDL alone; however, esterification was significantly less than that observed with ALDL, even though more LDL-PG was degraded. Ultrastructurally, macrophages incubated with LDL-PG complexes contained lipid droplets as well as numerous phagocytic vacuoles often containing material similar in appearance to insoluble complexes. These results demonstrate that components of the extracellular matrix, such as PG, can modify the catabolism of LDL by scavenger cells. This phenomenon may be potentially important with respect to foam-cell genesis from macrophages in the arterial wall. (Am J Pathol 1985, 120: 6–11)

ARTERIAL proteoglycans (PGs) are large multivalent extracellular proteins which are capable of interacting with a variety of ligands and are believed to play important roles in maintaining structural and functional integrity of the vascular wall.[1] They have also been postulated to be involved in extracellular lipid deposition and atherogenesis.[1-3] Notably, PGs bind low-density lipoproteins (LDLs) and very low density lipoproteins *in vitro*[4,5]; and lipoprotein-PG complexes, studied as lipoprotein-glycosaminoglycan (GAG) complexes, have been extracted from vascular tissue.[6,7] Even though much lipoprotein in the arterial wall is believed to be complexed extracellularly to PG, the interaction of lipoprotein-PG complexes with vascular cells has not been studied. In addition, lipoprotein-PG complexes may have profound effects on the subsequent cholesterol metabolism by vascular cells such as macrophages. The interaction between lipoproteins and macrophages assumes much importance because many foam cells in atherosclerotic lesions are believed to be of macrophage origin.[8-10]

Studies described here were designed to determine whether or not lipoprotein–PG complexes would elicit lipid accumulation in macrophages. Results indicate that insoluble complexes of purified aortic PGs and plasma LDLs were readily ingested by resident peritoneal macrophages, which led to the intracellular accumulation of cholesteryl ester.

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Materials and Methods

Isolation and Preparation of Human Low-Density Lipoproteins

LDLs ($1.019 \leqslant d \leqslant 1.063$) were isolated from fresh human plasma (New York Blood Center, New York, NY) by ultracentrifugation.[11] Isolated LDL was dialyzed against 150 mM NaCl/0.3 mM disodium-EDTA, pH 7.4, at 4 C for 24 hours prior to use. Acetylated LDL (ALDL) was prepared as described by Goldstein et al[12] and dialyzed as above. Both LDL and ALDL were iodinated by the method reported by Bilheimer et al.[13] After dialysis of the iodinated preparations trichloroacetic acid-precipitable radioactivity was found to be $\geqslant 99.9\%$. The purity of various LDL preparations was determined as described previously.[14]

Isolation and Characterization of Aortic Proteoglycans

Intima-inner media of bovine thoracic aorta was coarsely ground in a food mill and immediately placed in chilled 4.0 M guanidine HCl/50 mM acetate, pH 5.8. The extraction media contained the following protease inhibitors in addition to those described previously[15]: 1.0 mM phenylmethylsulfonyl fluoride, 1.0 mM sodium iodoacetate, 3.0 mM 1,10-phenanthrolene, 10.0 mM N-ethylmaleimide, and 0.5 mg/ml soybean trypsin inhibitor. Extraction was carried out with constant stirring at 4 C and at a volume/tissue ratio of 5.0 ml/g wet weight.

The PG-containing extract was partially purified by dissociative isopyknic centrifugation after adjusting the loading density to 1.41 g/ml by the addition of CsCl.[15] Centrifugation was carried out at 100,000*g* for 50 hours at 12 C. The bottom three-fourths of the gradient, containing approximately 75% of the extracted uronate, was exhaustively dialyzed against 7.0 M urea/50 mM Tris-HCl, pH 7.2, and concentrated by ultrafiltration (Diaflo PM-10; Amicon Corp., Danvers, Mass). The concentrated fraction was applied to a column (1.6 × 28 cm) of DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ). The unbound material was eluted with 7.0 M urea/50 mM Tris-HCl, pH 7.2, followed by a linear NaCl gradient (0 to 0.75 M) in the same buffer. The gradient slope was monitored by conductivity measurements.

Proteoglycans used in the present study eluted from the DEAE column between 0.36 and 0.45 M NaCl and were recovered by precipitation with the addition of three volumes of 95% ethanol containing 1.3% potassium acetate. The GAG composition of the PG preparation was determined by cellulose acetate electrophoresis[16] and found to contain 12% hyaluronic acid, 45% dermatan sulfate, and 43% chondroitin sulfate. Chromatography of the PG preparation on a column (1.6 × 30 cm) of Sepharose CL-2B (Pharmacia) by elution with Dulbecco's phosphate-buffered saline (DPBS) revealed both excluded (41%) and included (59%) populations. These fractions generally represent PG aggregates and monomers, respectively.

Isolation of Mouse Peritoneal Macrophages

Resident peritoneal macrophages were obtained from female NCS:Rku mice (Rockefeller University, New York, NY) essentially as described by Edelson and Cohn.[17] Briefly, peritoneal cells were harvested by lavage with DPBS. Ater centrifugation (400*g* for 10 minutes) the cells were resuspended in Dulbecco's modified Eagle's medium (DME) supplemented with 20% heat-inactivated fetal calf serum (FCS), penicillin (100 IU/ml), and streptomycin (100 μg/ml). Isolated cells were dispensed into plastic Petri dishes and incubated in a humidified 5% CO_2 incubator at 37 C. After 2 hours the dishes were washed twice with DPBS and the adherent cells incubated with supplemented DME overnight. All experiments were initiated the following day.

Preparation of LDL-PG Complexes

Low-density lipoprotein (355 μg LDL-protein) was diluted with 2.2 ml DPBS, and 50 μg PG (measured as GAG) in 0.4 ml distilled water. The addition of PG to the LDL-containing solution elicited an immediate turbidity that dissipated upon mixing. However, turbidity returned upon the addition of $CaCl_2$ to a final concentration of 9 mM. The preparations were left overnight in the cold (4 C). The resulting precipitates were centrifuged (1000*g* for 30 minutes), and the pellets were resuspended in DME before the addition to cell monolayers. The amount of PG (50 μg) used was sufficient to complex and precipitate all of the LDL when total cholesterol was measured.

Incorporation of ^{14}C-Oleic Acid Into Cholesteryl Esters

The incorporation of ^{14}C-oleic acid into cholesteryl esters by macrophages was determined by the method of Goldstein et al[18] as described previously.[14] Esterification was expressed as nanomoles ^{14}C-oleic acid incorporated into cholesteryl esters per milligram cell protein.

Uptake and Degradation of ^{125}I-LDL

The uptake and degradation of ^{125}I-LDL—as LDL, ALDL, or LDL-PG—by macrophages was also measured as described previously.[14,19] Cell content of ^{125}I-

LDL was assayed in 0.2 M NaOH digests of trypsinized cell monolayers. Degraded ^{125}I-LDL was measured as non-iodine, trichloroacetic acid-soluble material present in the postculture media. All data were corrected for nonspecific adsorption to the dishes by the inclusion of control dishes containing no cells. Data were expressed as micrograms LDL per milligram cell protein.

Analytic Procedures

Lipids

The total cholesterol content of LDL-PG complexes was determined by the method of Rudel and Morris[20] after saponification and hexane extraction of the pelleted LDL-PG precipitates. *In situ* lipid extraction of macrophage cultures was carried out with hexane/isopropanol (3:2, vol/vol) according to the method of Hara and Radin.[21] Extracted lipids were separated into individual classes by thin-layer chromatography and quantified by scanning fluorometry as previously described.[22]

Uronic Acid

Uronic acid was quantified by the method of Blumenkrantz and Asboe-Hansen.[23] D-(+)-glucuronic acid was used as a standard.

Protein

Protein was determined by the method of Lowry et al[24] with bovine serum albumin as standard. Protein content of macrophage cultures was assayed on 0.2 M NaOH cell extracts.

Light and Electron Microscopy

After trypsinization (20 minutes) and three rinses with DPBS, macrophage monolayers were fixed with either 10% neutral-buffered formalin or 1.5% glutaraldehyde in 0.1 M cacodylate, pH 7.4, with 0.5% $CaCl_2$. Formalin-fixed cells were examined for the presence of lipid droplets after staining with 0.5% oil red O in 70% triethylphosphate for 15 minutes.[25] Glutaraldehyde-fixed macrophages were postfixed and processed for electron microscopy by the procedure of Augermuller and Fahimi.[26] Briefly, fixed cells were rinsed with 0.2 M imidazole HCl, pH 7.5, and postfixed for 30 minutes with 2% OsO_4 in 0.2 M imidazole HCl, pH 7.5.

Results

Incubation of resident peritoneal macrophages with LDL-PG complexes for 3 days resulted in the accumulation of neutral lipid as evidenced by the presence of numerous oil red O-positive intracellular inclusions. This morphologic observation was corroborated by results of lipid analysis. In two experiments, macrophages incubated with LDL-PG complexes for 3 days were found to contain 115 ± 25 (Mean ± SD; n = 5) and 191 ± 61 (n = 3) μg cholesteryl ester/mg cell protein. This amount of cholesteryl was similar to that found for ALDL (92 ± 34 [n = 4] μg cholesteryl ester/mg) but much greater than that of LDL alone (<5.0 μg/mg). Nonspecific adsorption of the complexes to the cells or culture dishes did not contribute to the observed lipid content because macrophage monolayers were trypsinized (20 minutes at 37 C) prior to *in situ* lipid extraction. For comparison, bovine aortic smooth-muscle cells were also incubated for 3 days with either LDL-PG complexes or LDL alone. No significant lipid accumulation was observed in either group of cells when stained with oil red O.

The mechanism of cholesteryl ester accumulation in macrophages incubated with LDL-PG was investigated in two ways. First, the uptake and degradation of LDL-PG complexes were examined after 5- and 24-hour incubation periods using ^{125}I-labeled LDL as a tracer. The

Table 1—Uptake and Degradation of ^{125}I-LDL, ^{125}I-ALDL, and ^{125}I-LDL-PG by Mouse Peritoneal Macrophages

	Time	Total uptake	Degraded	Undegraded
	(hr)	(μg LDL/mg cell protein)		
LDL	5	0.99 ± 0.32	0.92 ± 0.29[a] (93%)	0.07 ± 0.04[c] (7%)
ALDL	5	6.18 ± 1.08	5.25 ± 1.28[a] (85%)	0.93 ± 0.27[d] (15%)
LDL-PG	5	21.16 ± 4.31	8.17 ± 1.53[a] (39%)	12.99 ± 3.13[cd] (61%)
LDL	24	7.63 ± 1.20	7.42 ± 1.18[b] (97%)	0.21 ± 0.04[e] (3%)
ALDL	24	23.23 ± 2.40	22.41 ± 2.46[b] (96%)	0.82 ± 0.08[f] (4%)
LDL-PG	24	45.22 ± 2.05	32.46 ± 3.25[b] (72%)	12.76 ± 1.78[ef] (28%)

Mouse peritoneal cells (2–3 × 10^6) were dispensed into 35 × 10-mm plastic dishes, and monolayers of adherent cells were prepared as described. At time 0, each dish received 1 ml supplemented DME and 50 μg of LDL-protein equivalent of ^{125}I-LDL, ^{125}I-ALDL, or ^{125}I-LDL-PG complexes. After a 5- or 24-hour incubation, the postculture medium was collected and assayed for trichloroacetic acid-soluble, non-iodine radioactivity. Cells were trypsinized (0.5% trypsin for 20 minutes at 37 C), washed three times with DPBS, then solubilized with 0.2 M NaOH. The total protein content and ^{125}I radioactivity were determined on the solubilized extracts. All data were corrected for nonspecific adsorption to the dishes by inclusion of cell-free control dishes for each treatment. The data represent the mean ± standard deviation for four individual dishes. Data within the degraded and undegraded groups were compared separately for each time period with single-factor analysis of variance.[40] When indicated by a significant F statistic, subsequent pairwise comparisons were performed with the use of multiple-range testing.[40] Values with similar superscripts are statistically different from each other ($P < 0.01$).

Table 2—Incorporation of ^{14}C-Oleic Acid Into Cholesteryl Esters by Mouse Peritoneal Macrophages Incubated With LDL, ALDL, or LDL-PG

	^{14}C-Oleic acid incorporated into cholesteryl esters (nmol oleate/mg cell protein)	
	Experiment 1	Experiment 2
LDL	0.20 ± 0.05[a]	0.25 ± 0.09[b]
ALDL	12.20 ± 0.76[a]	4.39 ± 0.81[b]
LDL-PG	1.04 ± 0.08[a]	0.97 ± 0.21[b]

Mouse peritoneal cells (2–3 × 10^6) were dispensed into 35 × 10-mm plastic dishes, and monolayers of adherent cells prepared as described. At time 0, each dish received 1 ml supplemented DME, 100 nmol ^{14}C-oleate/20 nmol albumin, and 50 μg LDL-protein equivalent of LDL, ALDL, or LDL-PG complexes. After 5 hours of incubation, the cellular content of cholesteryl ^{14}C-oleate was determined. The data represents the mean ± standard deviation for four individual dishes. Data for each experiment were compared separately with single-factor analysis of variance.[40] When indicated by a significant F statistic, subsequent pairwise comparisons were performed with the use of multiple-range testing.[40] Values with similar superscripts are statistically different from each other ($P < 0.01$).

total amount of LDL-PG endocytosed by macrophages after 5 hours was much greater than that of native LDL or ALDL (Table 1). However, only 39% of endocytosed LDL-PG was degraded during this time period, as compared with 93% and 85% for LDL and ALDL, respectively. Similar to the values obtained at 5 hours, incubation of LDL and ALDL for 24 hours resulted in nearly total degradation (97% and 96%, respectively) even though the total amount taken up by the macrophages increased during this time. The amount of endocytosed LDL-PG doubled between 5 and 24 hours; and although the proportion that was degraded (72%) increased, it was still significantly reduced when compared with LDL and ALDL.

In another experiment cholesterol esterification was monitored by measuring ^{14}C-oleic acid incorporation into cholesteryl esters in the presence of LDL, ALDL, or the complexes (Table 2). Incubation of macrophages with native LDL resulted in a relatively low level of esterification, whereas ALDL elicited a characteristic stimulation of cholesteryl ester synthesis. Incubation with LDL-PG complexes also resulted in an increased incorporation of ^{14}C-oleic acid, although the magnitude (fourfold) was well below that observed with ALDL. This differential effect of LDL, ALDL, and LDL-PG appears to have been specific for cholesteryl ester synthesis, because results indicated no differences in ^{14}C-oleate incorporation into triacylglycerides and phospholipids among the three lipoprotein preparations.

Macrophages incubated with LDL-PG were also examined by transmission electron microscopy. As shown in Figure 1, macrophage cytoplasm contained numerous, often large, phagocytic vacuoles as well as nonmembrane-bound lipid droplets. Phagocytic vacuoles frequently contained amorphous flocculent material, presumably representing ingested LDL-PG complexes, that was similar in appearance to material observed extracellularly.

Discussion

The interaction of polyanionic GAG chains of PG macromolecules with LDL has been implicated as a mechanism for lipoprotein sequestration in the arterial wall.[1–3] In addition, this binding may have important effects on subsequent cellular processing. For example, macrophages possess few LDL receptors and do not

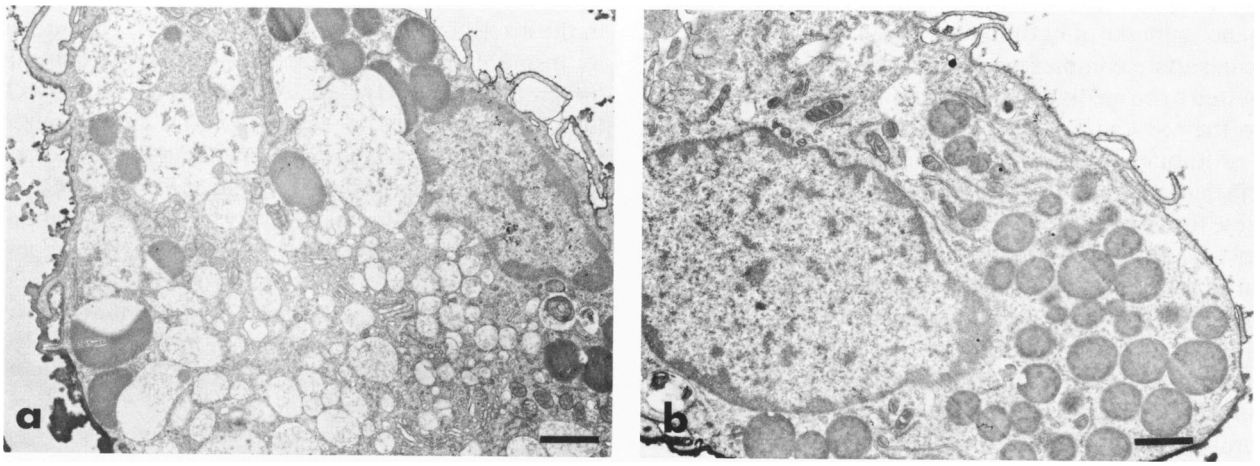

Figure 1—Representative appearance of cultured mouse peritoneal macrophages incubated with LDL-PG complexes (50 μg LDL-protein equivalent/ml) for 3 days. Cells were trypsinized (0.5% trypsin for 20 minutes at 37 C) prior to fixation. Both phagocytic vacuoles and cytoplasmic lipid droplets can be seen. Bar = 1 μ. (**a**, ×7100; **b**, ×6900)

accumulate lipid when incubated with high concentrations of lipoprotein.[12,27] In contrast, LDL that has been chemically modified (eg, acetylation) is taken up very efficiently by macrophages, which results in intracellular accumulation of cholesteryl ester.[28] However, it remains to be determined how much LDL in the arterial wall is chemically modified in a manner that enhances its uptake by macrophages and stimulates cholesterol accumulation *in vivo*. Alternatively, we have recently demonstrated that LDL insolubilized by other extracellular matrix components (heparin, fibronectin, and denatured collagen) was rapidly endocytosed by macrophages, which resulted in cholesteryl ester accumulation.[14] These results demonstrated clearly that interaction of LDL with extracellular matrix components can alter lipoprotein endocytosis and catabolism. However, since such complexes have not yet been demonstrated to exist in the arterial wall, the significance of this observation with respect to atherogenesis remains uncertain. In the present study we have presented evidence that resident peritoneal macrophages readily endocytose insoluble LDL-PG complexes and subsequently accumulate esterified cholesterol. Morphologic and biochemical observations corroborate that this net accumulation results from avid uptake of LDL-PG complexes, accumulation of LDL cholesterol, and increased cholesterol esterification.

Lipoproteins insolubilized by PG at physiologic pH and ionic strength were readily internalized by macrophages. On the basis of ultrastructural observations, the enhanced uptake of insolubilized LDL represents phagocytosis of particulate material. In contrast, smooth-muscle cells, generally considered nonphagocytic, apparently did not ingest the insoluble complexes to any significant degree. The possible role of specific or receptor-mediated adsorption to the macrophage cell surface in this process is not clear. For example, the enhanced uptake of both soluble and insoluble LDL–dextran sulfate complexes by macrophages has been suggested to be mediated by the binding of dextran sulfate to the cell's surface.[29,30]

Although pinosomes are probably too small for efficient uptake of insoluble complexes, it is conceivable that stimulation of pinocytosis could augment total LDL uptake if some of the LDL-PG complexes were in a smaller, more soluble form in the incubation medium. In this regard, it has been demonstrated that anionic molecules can act as nonspecific inducers of pinocytosis in mouse macrophages.[31] Some of the most striking increases in pinocytic vesicle formation were brought about by heparin, chondroitin sulfate, dextran sulfate, and hyaluronic acid. Thus, the presence of dermatan sulfate–chondroitin sulfate PG, as well as hyaluronic acid, in the LDL-PG complexes could have further enhanced or stimulated the interiorization process.

The cholesteryl ester cycle in macrophages has now been extensively studied and described.[28] Cholesteryl ester-rich particles, including cholesteryl ester/albumin coarcervates,[32] ALDL,[12] soluble LDL–dextran sulfate complexes,[29] or atherosclerotic aortic extracts,[33] are effectively internalized by macrophages with a subsequent stimulation of cholesterol esterification. This mechanism is dependent on the hydrolysis of ingested LDL cholesteryl ester to free cholesterol and its reesterification. In the present study, although insoluble LDL-PG complexes were avidly taken up by macrophages, they were slowly degraded when measured with ^{125}I-LDL as a tracer. This apparent reduced rate of breakdown may have resulted from overloading the cells' capacity to degrade the complexes. Although not specifically examined in this study, our previous results indicated that removal of uningested or excess complexes did not further enhance degradation over the short term.[14] However, in view of the large amount of ^{125}I-LDL-PG that was degraded, even after 5 hours of incubation, it was surprising to observe only a modest increase in ^{14}C-oleic acid incorporation when compared to LDL alone. One possible explanation for this phenomenon is the failure of cholesteryl ester hydrolysis to parallel protein ^{125}I-LDL hydrolysis in the lysosomes. In this regard, the polyanionic GAGs have been shown to inhibit some lysosomal enzymes.[34,35] A second possibility is the existence of some extracellular hydrolysis of the complexes or LDL. Any cholesterol freed under these conditions would not be available for intracellular reesterification.

Intact lipoprotein-PG complexes that are saline-extractable (soluble) from the arterial wall represent a minor component of the total insudated lipoprotein.[3] Thus, the majority of lipoprotein remains tightly bound in the arterial wall,[36] and this pool may be considered as insoluble. Furthermore, it is probable that this lipoprotein fraction is in close association with extracellular matrix proteins, including PG. LDLs insolubilized by arterial PG represent a physiologic model of lipoprotein modification, without the necessity of chemical derivitization. In this study we have clearly demonstrated that LDL-PG complexes are avidly phagocytosed by macrophages and lead to intracellular cholesteryl ester accumulation. This mechanism may be potentially important with respect to the genesis of foam cells from macrophages in the arterial wall.

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