Interaction of LDL with human arterial proteoglycans stimulates its uptake by human monocyte-derived macrophages

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Abstract The aim of this work was to investigate the possible mechanisms for uptake by human monocyte-derived macrophages (HMDM) of low density lipoprotein (LDL) pretreated with human arterial chondroitin-6-SO_4-rich proteoglycan (LDL-PG). HMDM were incubated with ^{125}I-labeled tyramine cellobiose-labeled LDL-PG, native LDL, and acetylated LDL (Ac-LDL). The results showed that two to four times more LDL-PG than LDL was bound and internalized by the HMDM. Competition experiments showed that LDL-PG competed with native LDL for the apoB,E (LDL) receptor, but not for the Ac-LDL scavenger receptor. Both the LDL and LDL-PG uptake were reduced after preincubation of the macrophages with unlabeled native LDL, though to a lesser extent with LDL-PG. The specific binding of ^{125}I-labeled LDL and ^{125}I-labeled LDL-PG at 4°C was both saturable and concentration-dependent. The dissociation constant (K_d) for binding was 8.6 × 10^{-9} M for LDL and 9.4 × 10^{-9} M for LDL-PG, but the maximum binding (B_{max}) was 1.5-times higher for LDL-PG. Cholesterol derived from LDL-PG was less effective than native LDL in suppressing HMG-CoA reductase activity. The results indicate that the uptake of LDL-PG is mediated not only by the LDL-receptor, but also by another unspecific pathway, which may not be subjected to regulation. These results provide further support for the hypothesis that LDL modifications induced by arterial PG may contribute to the formation of foam cells. — **Hurt, E., G. Bondjers, and G. Camejo.** Interaction of LDL with human arterial proteoglycans stimulates its uptake by human monocyte-derived macrophages. *J. Lipid Res.* 1990. **31:** 443–454.

Supplementary key words LDL-proteoglycan • human macrophages • receptor pathway

Macrophages and macrophages-foam cells have been reported as cellular components of the aortic intimal lesion in experimental animal models and in humans (1–5). The transformation of blood monocyte-derived macrophages into foam cells involves an intracellular accumulation of lipids. How macrophages accumulate lipids to become arterial foam cells is still a point for conjecture.

The hypothesis that the interaction of apoB lipoproteins with extracellular matrix component could stimulate their uptake by macrophages, and thus cause the transformation of macrophages into foam cells, has received substantial experimental support (6–8). In particular, there is much evidence to suggest that the proteoglycans (PG) of the arterial tissue are involved in the deposition of apoB lipoproteins and arteries (9, 10). The interaction between apoB lipoproteins and arterial PG appears to be initiated by electrostatic interactions that lead to the formation of irreversible and reversible association between apoB lipoprotein and the arterial PG. This interaction induces structural modifications of the lipoproteins due to a rearrangement of the lipid and protein moieties (11).

The irreversible type of association produces large lipoprotein aggregates or insoluble complexes, and their contribution to the formation of foam cells has been explored. Insoluble aggregates of LDL with glycosaminoglycans, dextran sulfate, or proteoglycans have been shown to induce cholesterol and cholesteryl ester accumulation in mouse peritoneal macrophages (7, 8, 12) and in subendothelial cells from human aortic intima (13).

The ligand for arterial chondroitin sulfate proteoglycan has been reported to be located within the putative ligand for the LDL receptor (14, 15). It is therefore possible that the irreversible binding of LDL to proteoglycans might affect that interaction between LDL and the human monocyte-derived macrophage (HMDM) apoB,E(LDL) receptor.

Abbreviations: LDL, low density lipoprotein; Ac-LDL, acetylated LDL; PG, arterial chondroitin-rich proteoglycan; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase; apoB, apolipoprotein B; Tris, tri(hydroxymethyl)-aminomethane; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; EDTA, ethylenediamine tetraacetic acid-Na_2; PMSF, phenylmethylsulfonyl fluoride; PBS, phosphate-buffered saline; CS, chondroitin sulfate; LPDS, lipoprotein-deficient human serum; HMDM, human monocyte-derived macrophages; PBS-CMF, calcium- and magnesium-free PBS; ^{125}I-TC, ^{125}I-labeled tyramine cellobiose.

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The reversible type of associations between apoB lipoproteins and arterial PG lead to the formation of structurally modified lipoproteins that are soluble, monomerically disperse, and not altered in their surface charge. Their capacity to associate with apoB,E(LDL) receptor should not be affected because proteoglycans do not remain bound to the lipoproteins.

In a previous study, we showed that this nonaggregated human LDL, pretreated with a human arterial chondroitin-6-SO_4-rich proteoglycan (LDL-PG), induces an increase in the uptake of LDL, stimulation of lipid synthesis, and transformation of HMDM into foam cells (16). The present study was conducted to investigate the possible mechanisms for LDL-PG uptake by HMDM. The results indicate that the uptake of LDL-PG is mediated both by the apoB,E(LDL) receptor and another unspecific pathway which is not subject to down-regulation. Possible differences in the cell processing of LDL-PG, compared to native LDL, are discussed. Based on previous work and on the experiments reported here, we propose that small structural changes induced in LDL by its reversible interaction with human chondroitin-6-sulfate-rich arterial PG may contribute to the lipid accumulation in arterial macrophages.

MATERIALS AND METHODS

Materials

Sodium [^{125}I]iodide (14.4 mCi/μg iodine), 3-hydroxy-3-methyl [3-^{14}C]glutaryl coenzyme A (52 mCi/mmol), and RS-[2-^{14}C]mevalonic acid lactone (56.7 mCi/mmol) were 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, Sephacryl S-400 superfine and PD-10 Sephadex G-25 columns were purchased from Pharmacia (Uppsala, Sweden). Millex-GV, non-protein binding filters for lipoproteins 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).

Macrophage culture condition

Human monocyte-dervied macrophages (HMDM) were isolated by Ficoll-Hypaque discontinuous gradient from buffy coats, which were obtained from the Blood Department (17). The mononuclear cell preparations were washed five times with phosphate-buffered saline, pH 7.2, without calcium and magnesium (PBS-CMF) but containing 10 mM EDTA to remove platelets. The cell preparation procedure was performed at room temperature. Cell suspension was 95% viable, as determined by trypan blue exclusion. Mononuclear cells were resuspended to 10^7 cells/ml in 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 μg/ml streptomycin, 1 mM sodium pyruvate, 4 mM glutamine, and nonessential amino acids (hereafter referred to as medium A). One ml of cell suspension was seeded in 30-mm plastic wells and monocytes were allowed to adhere overnight. The next day, nonadhered cells were eliminated with six washes of PBS and adhered monocytes (10^6) were cultured with 1 ml of medium A (1st day). All the experiments were performed with HMDM cultured for 3 days. One day before assay, 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. They were then incubated in 1 ml of culture medium containing 5 mg protein/ml apoprotein B-free lipoprotein-deficient human serum (LPDS), in order to induce LDL receptor expression (medium B, see below). All the incubations except for the specific binding assay were performed at 37°C in a 5% CO_2 humid atmosphere.

Human aorta proteoglycan preparation

The chondroitin-6-sulfate-rich fraction (PG) of human intima-media aortic proteoglycan was prepared and characterized as previously described (14, 18). The proteoglycan preparations were lyophilized and stored at −20°C before use.

Lipoprotein and lipoprotein-deficient serum

Human LDL (d 1.030–1.055 g/ml) were prepared by sequential ultracentrifugation (4°C) of plasma from healthy, fasted male volunteers (19). Immediately after the collection of blood, 1 mg/ml of EDTA and 20 mM PMSF were added. After recentrifugation, the pure LDL preparations, as judged by agarose electrophoresis, were filtered through 0.22 μm Millex-GV filters, stored at 4°C, and used within 48 h of preparation. Acetylation of LDL was performed with acetic anhydride (20).

Conventional labeling of the lipoproteins with [^{125}I]iodide was performed with the iodine monochloride method (21). For the studies of cellular uptake, iodination was performed with ^{125}I-labeled tyramine-cellobiose (^{125}I-TC) (22). For both iodination procedures the specific activity was between 50 and 100 cpm/ng of protein. For ^{125}I-labeled LDL, 98 ± 1.1% of the radioactivity could be precipitated with 15% TCA (w/v), while the equivalent figure for ^{125}I-TC-LDL was 99 ± 0.2%. Less than 5% of the total was lipid-extractable.

The formation of the LDL-arterial proteoglycan complexes has been described previously (16). The difference between this procedure and those in other studies (6–8) is that under our conditions, with the use of 5–10 mM $CaCl_2$ and a high ratio of LDL to PG, reversible associa-

tions were formed. Briefly, LDL-proteoglycan reaction was carried out after equilibration of the labeled LDL or unlabeled LDL and the human arterial proteoglycan (PG) with a sterile low ionic strength solution containing 5 mM Tris-HCl, pH 7.2, 2 mM $MgCl_2$, and 10 mM $CaCl_2$. LDL (1.0–2.0 mg cholesterol/ml). The LDL was added to proteoglycan solutions containing 10–20 μg/ml of glycosaminoglycans (calculated from their hexuronate content) and incubated on ice for 30 min. The aggregated LDL was collected by centrifugation at 10,000 *g* for 15 min at 4°C. The pellet, containing between 20 and 60% of the added LDL, was washed once with the reaction solution, redissolved in medium B, and filtered through a 0.22-μm Millex-GV filter. Under these conditions the LDL that precipitates is easily redissolved either in culture media or in buffer solution containing 140 mM NaCl. LDL redissolved without the need for mechanical stirring. Since 1 μg of PG-glycosaminoglycan precipitates with 40–50 μg of LDL-cholesterol, the amount of PG present in the lipoprotein solutions added to the cells can be at most 2–4 μg of PG-glycosaminoglycan present.

To test whether the labeled PG-treated LDL, after solubilization with cell culture media, contained lipoprotein aggregates due to self-association or by tight complexing with PG, the following control experiment was performed. Aliquots (500 μl) of medium containing 100 μg/ml of ^{125}I-TC-LDL-PG (80 cpm/ng) before and after being incubated for 18 h with HMDM and ^{125}I-TC-LDL (100 cpm/ng) that had not been exposed to cells were loaded separately in the same Sephacryl-400 SF 50 × 1.0 cm column, equilibrated with 5 mM HEPES, pH 7.2, 150 mM NaCl, 4 mM $CaCl_2$, and 2 mM $MgCl_2$, and eluted at 10 ml/h. Radioactivity was measured in the fractions collected from the chromatographic runs.

Lipoprotein-deficient human serum (LPDS, d > 1.215 g/ml) was prepared by ultracentrifugation of the infranatant obtained after sequential lipoprotein isolation. LPDS, judged to be devoid of apoprotein B by immunoelectrophoresis, was inactivated at 56°C for 30 min and stored at −20°C. Lipoprotein-depleted medium for cell incubation was prepared by equilibrating apoB-free LPDS in RPMI 1640-supplemented medium with a PD-10 Sephadex G-25 column, filtering it through a 0.22 μm Millex-GV filter, and diluting it to 5 mg of protein/ml with RPMI 1640 medium supplemented with: 24 mM $NaHCO_3$, 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin, 1 mM sodium pyruvate, 4 mM glutamine, and nonessential amino acids (hereafter referred to as medium B). Labeled and unlabeled LDL and Ac-LDL were equilibrated in medium B with PD-10 Sephadex G-25 columns and filtered through 0.22-μm Millex-GV filters before being added to the cells.

Lipoprotein uptake, degradation, and binding studies

HMDM, preincubated 24 h in culture medium B, were washed three times with PBS before adding medium B containing ^{125}I-TC-LDL, ^{125}I-TC-LDL-PG, or ^{125}I-TC-Ac-LDL at the concentrations indicated in the figures. For the competition experiments, a 40-fold excess of unlabeled LDL, LDL-PG, or Ac-LDL was added to the medium. After incubation for 6 h at 37°C, the cells were washed first with 1 ml PBS-CMF containing 2 mg BSA/ml and then with 1 ml PBS-CMF alone. The medium and the two washes were pooled and the radioactivity corresponding to the unbound lipoproteins was measured. 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 pooled and radioactivity corresponding to the cell surface-bound lipoprotein was counted. Adhered cells were extracted three times with 0.5 ml of 0.2 N NaOH 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. Around 100–300 μg of protein per well was obtained. Parallel dishes without cells were always included as controls for unspecific adsorption of the label. Radioactivity in these dishes was subtracted from that of the dishes with cells in order to obtain the calculations. Unless otherwise stated, the procedure described above was the same for all the cell assays. All the experiments were performed in duplicate and repeated at least once.

For lipoprotein-receptor induction experiments, HMDM were preincubated with medium B alone or containing 100 μg protein/ml of LDL, LDL-PG, or Ac-LDL. After 24 h of incubation at 37°C, the medium was removed and the cells were washed three times with PBS-BSA and three times with PBS. The cells were then incubated with ^{125}I-TC-LDL, ^{125}I-TC-LDL-PG, or ^{125}I-TC-Ac-LDL (at the concentrations indicated in the figures) for 6 h at 37°C. The amount of each lipoprotein bound and internalized by the cells was measured as described above.

Lysosomal degradation of ^{125}I-labeled LDL or ^{125}I-labeled LDL-PG was determined as follows. The lipoprotein concentrations indicated in the figures were added to the HMDM cultures and incubated at 37°C for 12–16 h. The extent of degradation of ^{125}I-labeled LDL or ^{125}I-labeled LDL-PG was determined by measuring the amount ^{125}I-labeled trichloroacetic-soluble material (^{125}I-labeled monotyrosine) (23). The amount of intracellular lipoprotein was also determined after treatment with trypsin, as described above.

^{125}I-labeled LDL and ^{125}I-labeled LDL-PG cell-surface binding assay was performed at 4°C, essentially as described (20). In brief, HMDM preincubated in medium B for 24 h were washed three times with cold PBS-BSA

and three times with cold PBS. Prior to the experiment, the cells were incubated for 1 h at 4°C with 1 ml of cold medium B-HEPES (without NaH_2CO_3). The medium of the cells was replaced with cold medium B-HEPES containing ^{125}I-labeled LDL or ^{125}I-labeled LDL-PG at different concentrations, with or without unlabeled competing lipoprotein. After 6 h of incubation of 4°C, the cells were rapidly washed two times with 1 ml of cold PBS-BSA and once with 1 ml cold PBS-CMF. The cells were dissolved in 0.5 ml of 0.2 N NaOH and transferred to a tube. Each dish was washed twice with 0.5 ml of 0.1 N NaOH and each rinse was added to the tube. After counting, 200-μl aliquots in duplicate were used for protein determination. Nonspecific binding was defined as the amount of radiolabeled lipoprotein bound in the presence of 200 or 400 μg of protein/ml of unlabeled ligand. Specific binding was calculated by subtracting the nonspecific binding values from the total binding obtained in the absence of the corresponding unlabeled lipoprotein. The data from the specific binding curves were linearized according to the Scatchard method (24), using a radioligand binding analysis program by G. A. McPherson (Elsevier Science Publishers BV, P.O. Box 1527, 1000 BM Amsterdam, The Netherlands). This program performs linear regression analysis, calculates the equilibrium dissociation constant (K_d), the maximum amount of ligand bound at receptor saturation (B_{max}), and the Hill coefficient.

HMG-COA reductase assay

The effect of the lipoproteins on the regulation of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA) on HMDM was studied in: *a*) cells preincubated for 24 h with medium B to allow HMG-CoA reductase activity to increase to unsuppressed levels, and *b*) cells cultured with medium A that contained cholesterol, where the enzyme levels are suppressed. LDL, LDL-PG, or Ac-LDL was added to the cells at the concentrations indicated in the corresponding figures. The cells were then incubated for 16 h. The medium was then removed and the cells were washed three times with both PBS-BSA and PBS. The cells were removed with a rubber policeman into 2.0 ml of PBS-CMF containing 10 mM EDTA, pH 8.0. The cells were then sedimented at 2000 rpm for 10 min, the supernatant was discarded, and the cell pellets were stored at – 80°C. Thawed cell pellets were incubated for 10 min at 37°C with 0.1 ml of 50 mM K_2HPO_4, pH 7.4, containing 5 mM dithiothreitol, 1 mM EDTA, and 0.25% Kyro EOB (a gift from Procter and Gamble, Cincinnati, OH). After centrifugation for 2 min at 10,000 rpm at room temperature, the clear supernatants (20 μl) were assayed in duplicate for protein concentration and for HMG-CoA reductase activity (25).

Analytical procedures

Protein was determined by a modification of the Lowry procedure, with bovine serum albumin as the standard (26). Cholesterol was analyzed by a colorimetric procedure (27). ^{125}I radioactivity was measured with an LKB 1282 Compugamma Universal Gamma Counter. ^{14}C radioactivity was measured with an LKB 1217 Rackbeta Wallac Liquid scintillation counter. The statistical significance of the data was evaluated by Student's *t* test, using the Statgraphics program (Statistical Graphics Corporation, Rockville, MD).

RESULTS

Size distribution of LDL-PG

Fig. 1 shows the pattern on gel exclusion chromatography of ^{125}I-TC-LDL and ^{125}I-TC-LDL-PG, the latter one obtained after incubation with HMDM for 18 h. There was coincidence between the fractionation pattern obtained with native LDL and the one with LDL-PG. Before incubation with HMDM ^{125}I-TC-LDL-PG was also run through the same column and the profile obtained was indistinguishable from those shown in Fig. 1. This indicated that LDL-PG before and after incubation with HMDM had a hydrodynamic size distribution similar to native LDL, and that no LDL polymers or LDL-PG aggregates were present. The recovery of radioactivity was approximately 100%, suggesting that no large aggregates had been filtered out by the column. No effort was made to measure hexuronic acid content in the fractions or to

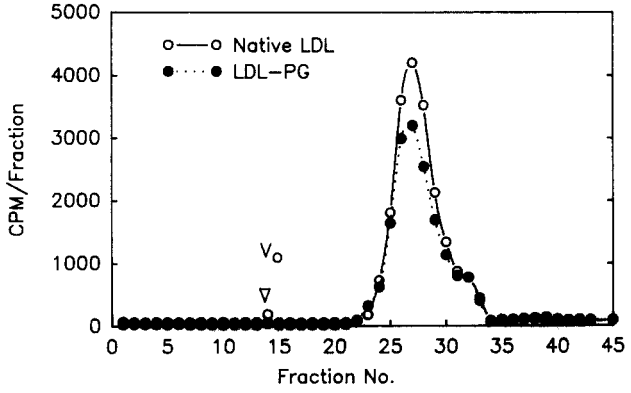

Fig. 1. Size distribution of native LDL and LDL-PG. Aliquots of 500 μl from cell culture medium containing 100 μg protein/ml of ^{125}I-TC-LDL-PG (80 cpm/ng) after being incubated for 18 h with HMDM and of ^{125}I-TC-native LDL (100 cpm/ng) that was not incubated with the cells were passed separately through a 50 × 1.0 cm Sephacryl 400 SF column equilibrated with 5 mM HEPES, pH 7.2, 150 mM NaCl, 4 mM $CaCl_2$, and 2 mM $MgCl_2$. The column was eluted at 10 ml/h. Radioactivity was evaluated in each 1-ml fraction.

evaluate the elution volume of proteoglycans, because the amount present in 0.5–1 ml of LDL-PG-containing medium (less than 2 μg) would be diluted beyond possible detection after gel exclusion chromatography.

Similar results were obtained when unlabeled native LDL and ^{125}I-TC-LDL-PG were mixed and passed through the chromatography column together. There was complete coincidence between the cholesterol determination from native unlabeled LDL and radioactivity from labeled LDL-PG (data not shown).

Lipoprotein binding and internalization

In our previous work, the uptake of LDL-PG by human macrophages was demonstrated by using fluorescent DiI-labeled-LDL-PG (16). In the present study, we decided to quantify the difference in uptake between LDL-PG and native LDL by labeling the lipoproteins with ^{125}I-labeled tyramine linked to cellobiose, a nonhydrolyzable disaccharide. Upon internalization and degradation of the labeled protein, the labeled disaccharide is not degraded and is thus retained in the cells as a cumulative marker of the uptake (22). Because neither free iodine nor iodoamino acids are release to the medium, labeled lipoproteins allow a more precise measurement of LDL and LDL-PG uptake. However, to measure degradation we also had to use conventionally labeled ^{125}I-lipoproteins (21). **Fig. 2** presents the results obtained by measuring the uptake of ^{125}I-TC-LDL and ^{125}I-TC-LDL-PG as a function of their concentration in the medium. As can be observed in Fig. 2, the interaction of LDL with arterial PG stimulates both the trypsin-releasable cell surface-bound LDL and its internalization 2- to 3-fold above the values measured with native LDL. LDL and LDL-PG binding appear biphasic but did not reach saturation. There was an initial high affinity process up to 20 μg/ml and a nonsaturable one from 20–100 μg/ml. Subtraction of the low affinity slope ordinate intersection (20–100 μg/ml) allowed an estimate of near-saturation values for binding and internalization of LDL and internalization of LDL-PG (23). However, for LDL-PG binding, the curve retained nonsaturability after subtraction.

Lipoprotein uptake in the presence of proteoglycan

To test whether addition of the arterial CSPG affects the lipoprotein uptake and could explain the differences in uptake and binding between native LDL and LDL-PG, the following experiment was conducted. HMDM were incubated with 20 μg/ml of ^{125}I-TC-native LDL (100 cpm/ng) and increasing amounts of arterial CSPG was added (2 to 20 μg/ml in culture medium). The results presented in **Fig. 3** indicate that the presence of CSPG in the medium, in the concentrations that are used to prepare LDL-PG, if anything, reduced the lipoprotein uptake. Therefore, this effect cannot be responsible for the increased uptake observed for the proteoglycan-treated LDL when compared with native LDL.

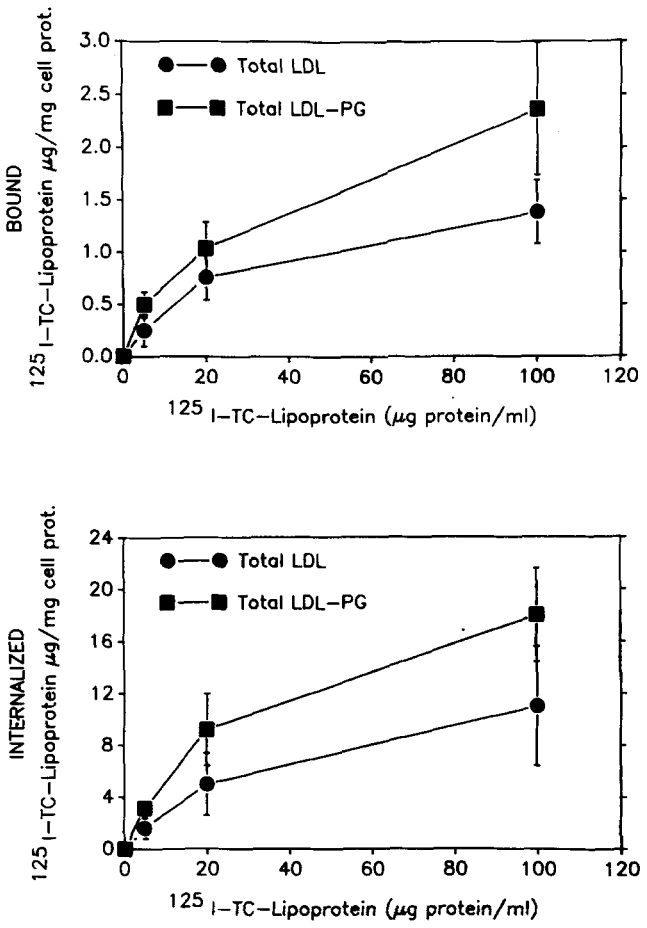

Fig. 2. Uptake of ^{125}I-TC-LDL-PG and ^{125}I-TC-LDL. After 24 h preincubation in medium B, macrophages were incubated in 1 ml of medium B containing the indicated concentrations of ^{125}I-TC-LDL (●) or ^{125}I-LDL-PG (■). After incubation for 6 h, the amount of labeled lipoprotein bound to the cell surface (top) and internalized by the macrophages (bottom) was determined as described in Materials and Methods. The values are the average of four determinations ± SD.

Lipoprotein degradation

The degradation of internalized LDL and LDL-PG by HMDM was studied. **Fig. 4** shows the high affinity degradation as a function of LDL and LDL-PG concentration in the medium. ^{125}I-LDL-PG was degraded to a higher extent than ^{125}I-LDL at protein concentration of 5 to 20 μg of protein/ml. Fig. 4 also presents the amount of internalized LDL-PG and native LDL remaining intracellularly at the end of the incubation time. These results show that LDL-PG was also more internalized, as compared with native LDL. In these experiments the ratio between degradation and what remains internalized after trypsin treatment of the cells was consistently higher for LDL-PG than for LDL.

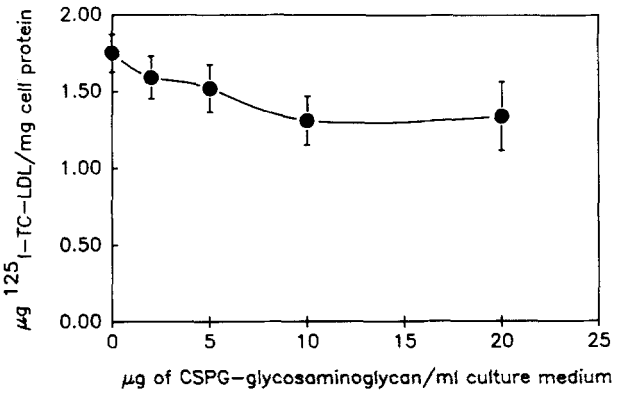

Fig. 3. Effect of addition to the medium of arterial proteoglycans on macrophage uptake of ^{125}I-TC-LDL. After 24 h incubation in medium B, macrophages were incubated for 16 h in 1 ml of lipoprotein-deficient medium containing the indicated concentrations of arterial CSPG-glycosaminoglycan and 20 μg/ml of total native ^{125}I-TC-LDL protein (100 cpm/ng). The values are the average of four determinations ± SD.

Effect of unlabeled native LDL, LDL-PG, and Ac-LDL on the uptake of labeled native LDL and LDL-PG

Once the uptake and degradation experiments had shown that LDL-PG appeared to be internalized by HMDM, competition experiments were performed to determine whether LDL-PG uptake was mediated through the apoB,E(LDL) receptor or through the scavenger receptor for modified lipoproteins. **Fig. 5** shows the results of trypsin-releasable cell surface-bound and internalization measurements of ^{125}I-TC-LDL-PG and ^{125}I-TC-LDL in the presence of 40-fold excess of unlabeled LDL, Ac-LDL, and LDL-PG. Native LDL, but not Ac-LDL, inhibited the binding and internalization of LDL-PG. These results clearly indicated that LDL-PG uptake in human macrophages was mediated mainly by the apoB,E(LDL) receptor and not by the scavenger receptor for modified lipoproteins.

Regulation of lipoprotein receptors on human macrophages

Previous studies have demonstrated that the apoB,E(LDL) receptor, but not the scavenger receptor, are down-regulated by extracellular cholesterol in human macrophages (28, 29). To determine whether LDL-PG-derived cholesterol was able to down-regulate the apoB,E(LDL) receptor, we assessed the uptake of ^{125}I-TC-LDL-PG, ^{125}I-TC-LDL, and ^{125}I-TC-Ac-LDL in macrophages that were preincubated with medium B containing either LDL, LDL-PG, or Ac-LDL. Although preexposure of cells to 4 μg/ml of LDL protein was sufficient to substantially inhibit subsequent binding of the lipoprotein, pre-exposure to 50 μg/ml of LDL protein was included in order to cover the range of values for maximal binding reported for HMDM (29).

The results of cell surface-bound lipoprotein and the internalized lipoprotein are presented in **Fig. 6** and in **Fig. 7**, respectively. Several experiments indicated that preincubation with LDL and Ac-LDL significantly decreased the uptake of both native LDL and LDL-PG ($P < 0.010$), which in turn suggests that this process is subject to down-regulation. However, preincubation with LDL-PG did not decrease ^{125}I-TC-LDL-PG and native ^{125}I-TC-LDL uptake as efficiently as LDL or Ac-LDL did. The amount of ^{125}I-TC-LDL-PG internalized after preincubation with unlabeled LDL-PG was significantly higher (22.5 ± 0.5 μg/mg cell protein) than the uptake after preincubation with unlabeled LDL (11.45 ± 0.04 μg/mg

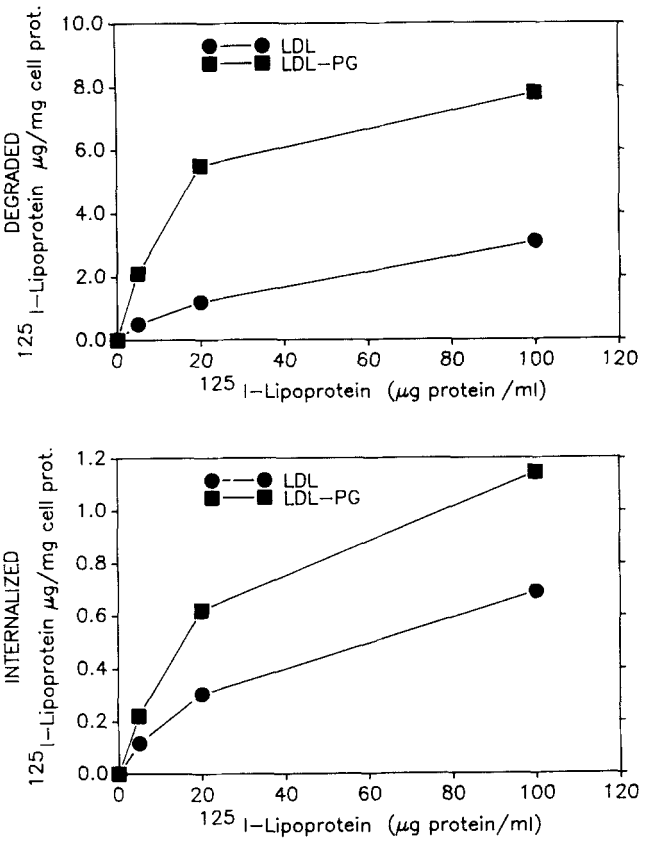

Fig. 4. Degradation of ^{125}I-labeled LDL-PG and ^{125}I-labeled LDL. After 24 h preincubation in medium B, macrophages were incubated in 1 ml of medium B containing the indicated concentrations of ^{125}I-labeled LDL-PG (■) or ^{125}I-labeled LDL (●). After 16 h at 37°C, the medium from each culture dish was removed and the extent of lipoprotein degradation was determined (top graph) by measuring the content of ^{125}I-labeled acid-soluble monotyrosine. The cells were trypsinized and the amount of labeled lipoprotein left intracellularly (bottom graph) was measured as described in Materials and Methods. The values represent the average of duplicate determinations.

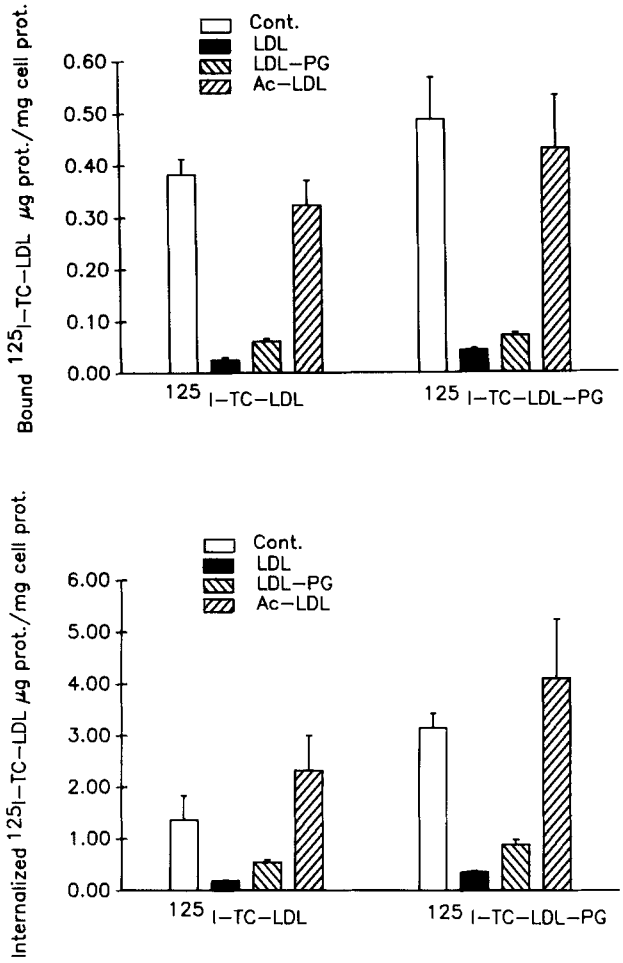

Fig. 5. Competition experiment. After 24 h preincubation in medium B, macrophages were incubated in 1 ml of medium B containing 5 μg protein/ml of ^{125}I-TC-LDL or ^{125}I-LDL-PG in the absence (open bars) or presence of 40-fold excess (200 μg/ml) unlabeled LDL (■), LDL-PG (▨) or Ac-LDL (▨). After 6 h, the amount of labeled lipoprotein bound to the cell surface (top graph) and internalized by the macrophages (bottom graph) was determined as described in Materials and Methods and compared with the values obtained in the absence of competitors (□). The values represent the average of four experiments ± SEM.

cell protein) ($P < 0.002$, n = 2). The uptake of ^{125}I-TC-LDL-PG after preincubation of HMDM with unlabeled LDL-PG was not significantly different from its uptake after preincubation in lipoprotein-depleted medium, where the expression of apoB,E(LDL) receptor is induced (31 ± 3.0, n = 2). These results indicate that LDL-PG did not down-regulate the apoB,E(LDL) receptors, as did native LDL. LDL-PG can be internalized through the apoB,E(LDL) receptors, but once internalized, it appears to be processed by human macrophages differently than the native LDL.

Based on the work of Goldstein and Brown (28) neither the pre-incubation with native LDL, LDL-PG, or Ac-LDL would be expected to down-regulate the scanvenger receptor. As predicted, none of these lipoproteins decreased the uptake of ^{125}I-TC-Ac-LDL (data not shown).

Lipoprotein specific binding

The specific binding of ^{125}I-labeled LDL-PG and ^{125}I-labeled LDL to the surface of human macrophages at 4°C was both saturable and concentration-dependent (**Fig. 8**). Analysis of the data according to the method of Scatchard (24) demonstrated that human macrophages bind ^{125}I-labeled with a K_d of 8.6 × 10^{-9} M, which is similar to the level reported for human fibroblast and human monocyte-macrophages (30). The K_d obtained for ^{125}I-labeled LDL-PG was 9.3 × 10^{-9} M , which is similar to the LDL equilibrium dissociation constant. At receptor saturation, the B_{max} for LDL was 1.2 × 10^{-10} mol and 1.8 10^{-10} mol for LDL-PG, given a ratio of LDL-PG to native LDL bound to the cells of 1.5. Using 500,000 for the molecular weight of an apoB-100 molecule, this ratio indicates that at both saturation and 4°C, the cells bound 7.2 × 10^{10}

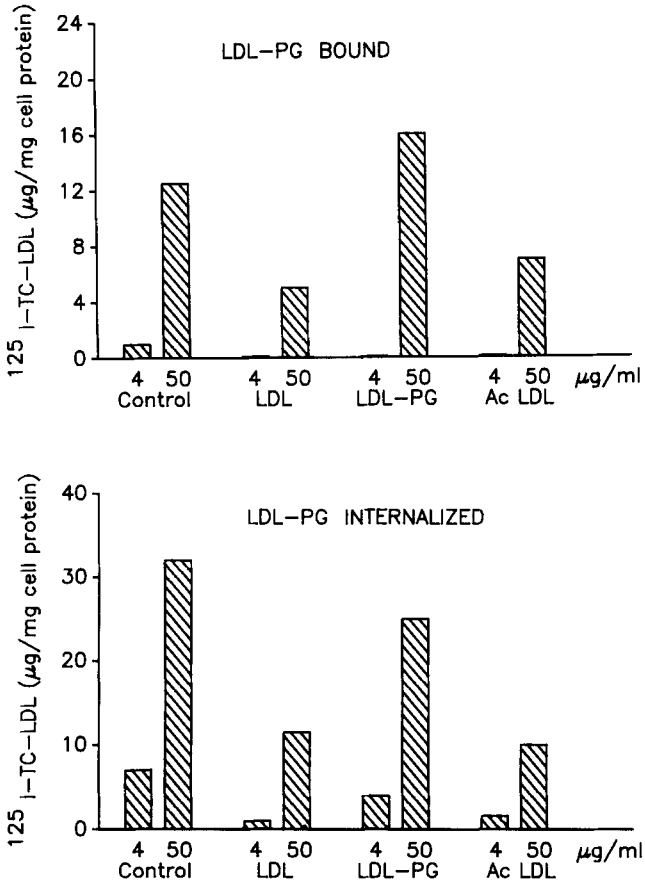

Fig. 6. Effect of preincubation with unlabeled lipoproteins on subsequent uptake of ^{125}I-TC-labeled LDL-PG. Macrophages were preincubated in 1 ml medium B (control) or in medium B containing 100 μg protein/ml of unlabeled LDL, LDL-PG, or Ac-LDL. After 24 h incubation, the macrophages were washed three times with PBS-BSA and then three times with PBS. The cells were then incubated in 1 ml medium B containing 4 or 50 μg protein/ml of ^{125}I-TC-LDL-PG. After 6 h incubation, the amount of labeled lipoprotein bound to the cell surface (top graph) and internalized by the cells (bottom graph) was determined as described in Materials and Methods. The values represent the average of duplicate assays.

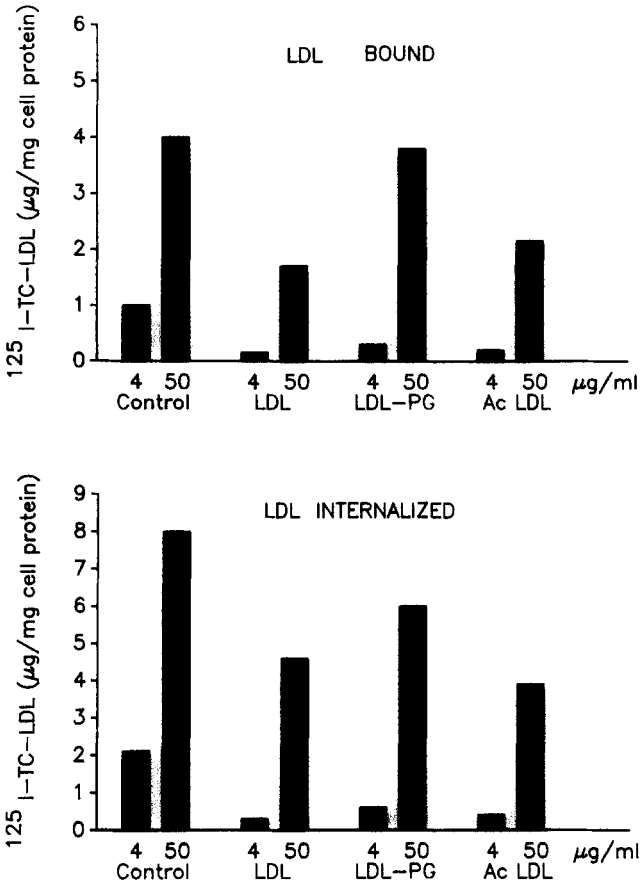

Fig. 7. Effect of preincubation with unlabeled lipoproteins on subsequent internalization of ^{125}I-TC-LDL. Macrophages were preincubated in 1 ml of medium B (control) or in medium B containing 100 μg protein/ml of unlabeled LDL, LDL-PG, or Ac-LDL. After 24 h incubation the macrophages were washed three times with PBS-BSA and then three times with PBS. The cells were then incubated in 1 ml medium B containing 4 or 50 μg protein/ml of ^{125}I-TC-LDL. After 6 h incubation, the amount of labeled lipoprotein bound to the cell surface (top graph) and internalized by the cells (bottom graph) was determined as described in Materials and Methods. The values represent the average of duplicate assays.

apoB molecules/mg cell protein when incubated with LDL-PG. The Hill coefficient for LDL was approximately 1.0, which suggests a homogeneous ligand-receptor system. However, with the LDL-PG binding data, a slightly nonlinear tendency of the Scatchard curve was observed, with a Hill coefficient of 1.3, which is indicative of a complex ligand-receptor reaction (31). As found for other cells (32), there was substantially more binding at 37°C than at 4°C. The binding values at receptor saturation for LDL were similar to those reported in the literature for HMDM (30).

HMG-CoA reductase assay

In tissue-cultured cells, the apoB,E(LDL) receptor and HMG-CoA reductase are both subject to end-product feedback regulation by cholesterol (33). The results presented until now led us to postulate the following hypothesis: if LDL-PG was partially internalized through apoB,E(LDL) receptors, but failed to down-regulate the expression of the receptors, then LDL-PG-derived cholesterol may not suppress the activity of HMG-CoA reductase, as compared with native LDL-derived cholesterol. The results of the HMG-CoA reductase activity measurements presented in **Fig. 9** show that the basal activity level of the enzyme was 200 pmol mevalonate/mg cell protein per min, when the macrophages were cultured in the presence of exogenous cholesterol (medium A), but increased to 500 pmol mevalonate/mg cell protein per min when they were cultured in the absence of exogenous cholesterol (medium B). Similar results have previously been reported with other cells (34). The effect of LDL, LDL-

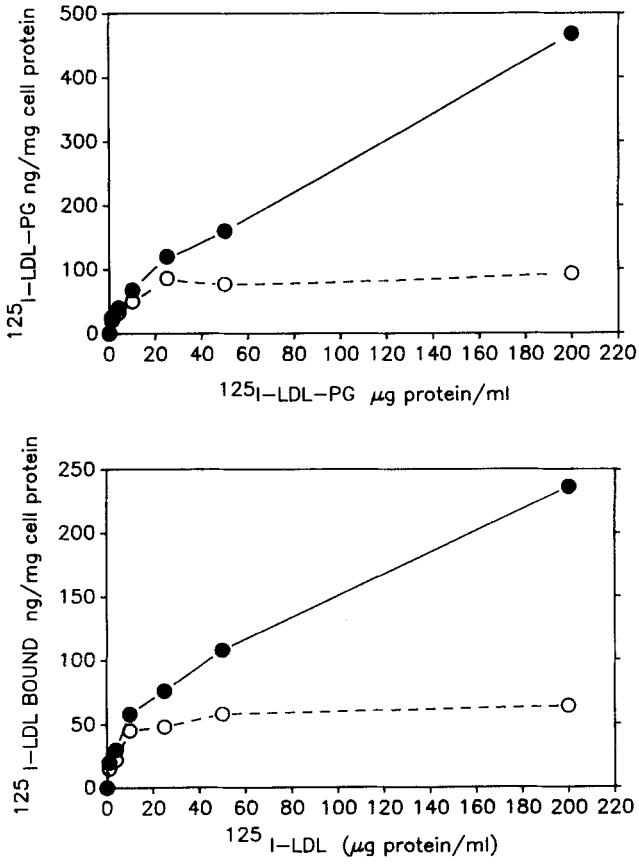

Fig. 8. Total and specific binding of ^{125}I-labeled LDL-PG and ^{125}I-labeled LDL by human macrophages as a function of its concentration. Macrophages, preincubated for 24 h in medium B, were incubated in 1 ml of medium B containing the indicated concentrations of ^{125}I-labeled LDL-PG (top graph) or ^{125}I-labeled LDL (bottom graph) in the presence or absence of the corresponding unlabeled LDL-PG or LDL (200 or 400 μg protein/ml) Equilibrium binding assays were performed at 4°C for 6 h. The amount of bound ^{125}I-labeled lipoprotein was measured. Specific binding (dashed line) for each type of lipoprotein, LDL-PG and LDL, was calculated by substracting the amount of labeled lipoprotein bound in the presence of excess unlabeled lipoprotein from that obtained in the absence of unlabeled lipoprotein (solid line). The values represent the average of duplicate determinations.

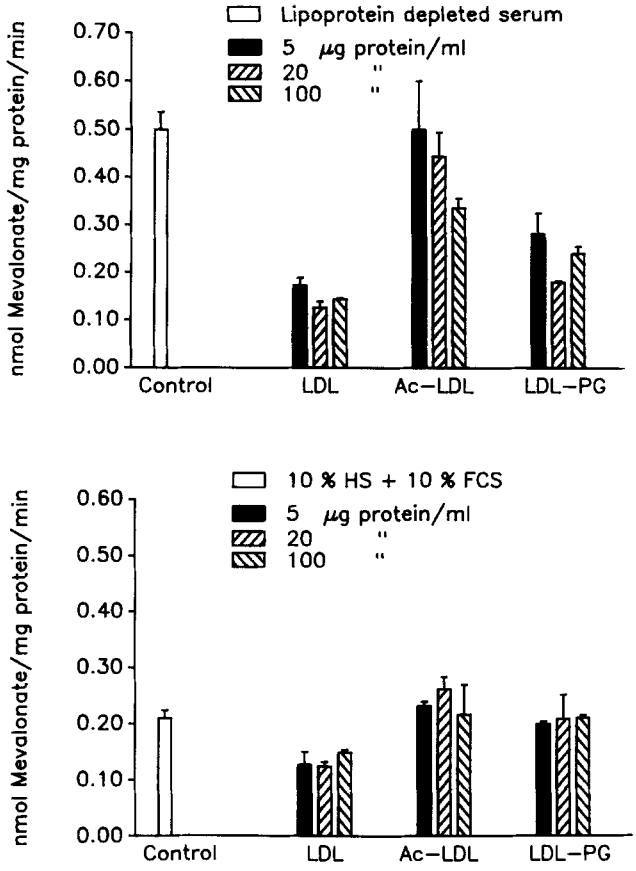

Fig. 9. Effect of LDL-PG, LDL, and Ac-LDL on HMG-CoA reductase activity. Macrophages were preincubated in medium A (lipoprotein-containing medium, top graph) or in medium (lipoprotein-depleted medium, bottom graph). After 24 h, the cells were washed and 1 ml of medium B containing the indicated amounts of LDL, LDL-PG, or Ac-LDL was added. After 16 h the cells' medium was removed and the cells were processed as indicated in Materials and Methods. The open bars represent the enzyme activity before adding the lipoproteins in both culture conditions. The values represent the average of HMG-CoA reductase activity in macrophages from quadruplicate dishes. Standard error is indicated.

PG, and Ac-LDL-derived cholesterol was measured in parallel cultures of HMDM pre-incubated in medium A or in medium B. Under both conditions, LDL-PG-derived cholesterol suppressed the activity of HMG-CoA reductase to a lesser extent than native LDL-derived cholesterol. The addition of 5 μg of protein/ml (9 μg of cholesterol/ml) of native LDL suppressed 66% of the HMG-CoA reductase activity, whereas LDL-PG reduced the activity by 44%. Maximal suppression reached 75% with LDL, compared with 52% obtained with LDL-PG at 100 μg of protein/ml (189 μg of cholesterol). The levels of HMG-CoA reductase activity in HMDM incubated with LDL-PG were significantly higher than after incubation with native LDL at any of the three concentrations used ($P < 0.030$–0.001). Even in the presence of higher concentrations of LDL in the medium, however, repression of the enzyme was never complete (35). Ac-LDL-derived cholesterol was the least efficient suppressor of HMG-CoA reductase activity in both cell culture conditions. Maximal suppression of the enzyme reached 33% with 100 μg of protein/ml of Ac-LDL.

DISCUSSION

The interaction of apoB-containing lipoproteins with the vascular extracellular matrix components and their subsequent handling by the tissue appears to play an important role in atherogenesis (9, 10, 36). The hypothesis that the interaction of apoB-containing lipoproteins with arterial proteoglycans may lead to transformation of blood monocyte-derived macrophages into foam cells, the hallmark cells of atherosclerosis, is supported by the following: *a*) there is evidence for complex formation and co-localization of apoB-containing lipoproteins and chondroitin sulfate-rich proteoglycans (37–40); *b*) LDL-proteoglycan complexes induce intracellular accumulation of lipids in macrophages (7, 16, 28); and *c*) macrophage foam cells are present in proteoglycan-rich regions of intima in humans (41) and in experimental animals (10).

Chondroitin sulfate-rich arterial proteoglycans can react in vitro with apoB lipoproteins, forming both soluble and insoluble complexes (18). Incubation of LDL with proteoglycans in the presence of 150 mM NaCl induces formation of only soluble complexes that also induce cholesterol accumulation in mouse peritoneal macrophages and in HMDM (8, 16). The irreversible interaction of LDL with arterial proteoglycan leads to the formation of insoluble aggregates, which have been reported by several groups to induce lipid accumulation in mouse peritoneal macrophages and subendothelial cells (7, 8, 12, 13). Recently, a specific receptor-mediated pathway for the metabolism of LDL-proteoglycan insoluble complexes was reported in mouse peritoneal macrophages (42). The reversible type of association between LDL and arterial PG used in our experiments induces structural modifications in LDL; however, the lipoproteins once solubilized are monomeric and show no alteration in their surface charge (16).

Human monocyte-derived macrophages, contrary to mouse peritoneal macrophages, express high-affinity apoB,E(LDL) receptors. One of the proteoglycan-binding peptide sequences in apoB has recently been reported to be located within the putative ligand for the cells' apoB,E(LDL) receptor (14, 15). Therefore, it is possible that the irreversible binding of LDL to the proteoglycan might affect or block the interaction between LDL and the human macrophage apoB,E(LDL) receptor. The reversible type of interaction appeared to be a more plausible model for studying cell-receptor interactions with PG-modified LDL.

The results obtained in this study for measuring the uptake of ^{125}I-TC-labeled lipoproteins by HMDM indicated

that LDL-PG was internalized through apoB,E(LDL) receptors and another unspecific pathway, which may not be subjected to down-regulation. The following criteria establish that LDL-PG lipoprotein interacts with human macrophages through an apoB,E(LDL) receptor: *a*) ^{125}I-labeled LDL-PG specific binding was a saturable process; *b*) ^{125}I-labeled LDL-PG was bound by a high affinity process with an equilibrium dissociation constant of 10^{-9} M; *c*) native LDL, but not Ac-LDL, inhibits the uptake of ^{125}I-TC-LDL-PG; and *d*) LDL-PG was able to partially suppress the HMG-CoA reductase activity. Nevertheless, more LDL-PG was taken up than native LDL, which suggests that an additional mechanism may perhaps be involved in the increased uptake of LDL-PG and subsequent lipid accumulation previously observed (16).

We previously reported our inability to measure LDL-PG degradation and that addition of LDL did not reduce LDL-PG cell association (16). However, no quantitative data were presented because our blanks, as stated, were close to the experimental points. We not attribute these difficulties to the use of cell-culture plastic dishes with high unspecific binding for labeled LDL-PG. Similar problems have been reported by other investigators working with proteoglycan-LDL complexes (7). With the dishes used in the present experiments, we consistently obtained blanks with less than 2% of the radioactivity present in the dishes with cells.

The specific binding assays suggest the existence of other receptors for LDL-PG besides the apoB,E(LDL) ones. This receptor may not be subjected to down-regulation, which may explain the lipid accumulation reported before in HMDM incubated with LDL-PG (16). The specific binding data for LDL-PG suggest the possibility of a positive cooperativity site-site interaction. However, to explore these possibilities, an analysis of the kinetics of binding with purified receptors would be required.

Subtle PG-induced modifications on LDL, which remain after the lipoprotein has been resolubilized, may be responsible for divergence in the cell processing of LDL-PG and native LDL. This difference may be particular for HMDM, since the uptake of LDL-PG by human smooth muscle cells has been reported to down-regulate the apoB,E(LDL) receptor (43). Glycosaminoglycans have been shown to inhibit the function of lysosomes in macrophages (44). Although we do not have evidence for the presence of proteoglycans tightly associated to LDL-PG preparations in the cell culture medium, the possibility exists that small amounts of soluble PG taken up by the macrophages could affect the intracellular processing of LDL in macrophages and smooth muscle cells differentially. Experiments to establish the subcellular distribution of TC-labeled lipoproteins might help us understand the differences in the cellular pathway of LDL-PG and native LDL.

Measurements of HMG-CoA reductase activity, the rate-controlling enzyme in cholesterol biosynthesis, indicate that LDL-PG-derived cholesterol did not suppress the enzyme activity as efficiently as native LDL-derived cholesterol at the same concentrations. Since LDL-PG uptake, degradation, and specific binding were higher than for native LDL, even at the lowest concentrations in the medium, the results suggest that native LDL taken up through the apoB,E(LDL) receptor has a greater effect on HMG-CoA reductase activity than LDL-PG. Even in the presence of higher concentrations of LDL in the medium, however, repression of the enzyme was never complete (35). The less efficient suppressor effect on HMG-CoA reductase observed with LDL-PG, as well as its failure to down-regulate the apoB,E(LDL) receptor, also support the possibility that other mechanisms are involved in the high uptake of LDL-PG. However, both observations could be the result of a different intracellular processing of the LDL-PG by human macrophages.

Ac-LDL was the least efficient suppressor of HMG-CoA reductase activity. However, Ac-LDL has been reported by another group to essentially abolish HMG-CoA reductase activity in HMDM that were cultivated for 7 or more days (45). Our experiments were performed with HMDM cultivated for 3 days. The activites of the apoB,E(LDL) and Ac-LDL scanvenger receptors on human monocyte-derived macrophages change depending on the stage of development of the cells, the conditions of culture, and the activation of macrophages (29, 46). During the first week of culture, apoB,E(LDL) receptor activity remains either constant or increases, but scavenger receptor activity surpasses apoB,E(LDL) receptor activity by the 6th day and increases until it is fully expressed during the second week (29). Therefore, after 7 days in culture, the suppressor effect of Ac-LDL on HMG-CoA reductase activity is expected to be higher than after 3 days in culture. Another factor to be considered when comparing data is the phase of macrophage activation.

In conclusion, the results indicate that a reversible interaction of LDL with human chondroitin-6-sulfate-rich arterial proteoglycans induces structural modifications on the LDL which, in turn, causes an increased uptake of the lipoprotein by human macrophages.

ApoB lipoproteins in their passage through the arterial intima may interact with arterial proteoglycans. It is possible that such interactions, both irreversible and reversible, cause structural modifications of the lipoprotein that are partially responsible for the extracellular accumulation of apoB lipoproteins, and which, in addition, may contribute to the transformation of monocyte-derived macrophages into foam cells.

The authors thank Aira Lidell, Ulla-Britt Rignel, Flor López, and Mutjaba Siddiqui for technical assistance. Kyro EOB detergent

was provided by Dr. Elba Marina Bello, Procter and Gamble, Cincinnati, Ohio. Tyramine-cellobiose was a generous gift from Dr. Ray C. Pittman, Department of Medicine, University of California, San Diego, La Jolla, California. 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. no. 4531, 7142, 7515), The Swedish Association Against Heart and Chest Diseases, AB Hässle, the King Gustaf V and Queen Victoria Foundations, and Proyecto SI-1987 from Consejo Nacional de Investigaciones Científicas y Tecnológicas (CONICIT).

Manuscript received 20 April 1989, in revised form 2 August 1989, and in re-revised form 2 October 1989.

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