Human monocyte-derived macrophages bind low-density-lipoprotein–proteoglycan complexes by a receptor different from the low-density-lipoprotein receptor

Parakat VIJAYAGOPAL,*† Sathanur R. SRINIVASAN,*‡§ Bhandaru RADHAKRISHNAMURTHY§ and Gerald S. BERENSON§||

Departments of *Medicine, †Anatomy and ‡Biochemistry, Louisiana State University Medical Center, New Orleans, LA 70112, U.S.A.,

and §Department of Applied Health Sciences, Tulane University Medical Center, School of Public Health and Tropical Medicine, New Orleans, LA 70112, U.S.A.

We have shown recently that lipoprotein–proteoglycan complexes isolated from human atherosclerotic lesions stimulated cholesteryl ester synthesis in human monocyte-derived macrophages [Vijayagopal, Srinivasan, Radhakrishnamurthy and Berenson (1992) *Arterioscler. Thromb.* 12, 237–249]. The present study was conducted to determine the mechanism of cellular uptake of the complexes. A chondroitin sulphate–dermatan sulphate proteoglycan was isolated from normal human aorta and complexed to ^{125}I -labelled human low-density lipoprotein (LDL). The binding and degradation of ^{125}I -LDL–proteoglycan complex were then studied in human monocyte-derived macrophages. The specific binding and degradation of the complex showed saturability and concentration-dependency. The K_d for binding was 1.5×10^{-8} M, which was greater than that reported for LDL in monocyte-derived macrophages. Binding of the complex was not subject to down-regulation. Chloroquine inhibited degradation of the complex and the resultant stimulation of

cholesteryl ester synthesis. Limited treatment of macrophages with proteolytic enzymes abolished binding and degradation of the complex significantly. Macrophages bound ^{125}I -methyl-LDL–proteoglycan complex to the same extent as ^{125}I -LDL–proteoglycan complex. Excess LDL and proteoglycan did not compete against the binding of the complex; however, excess acetyl-LDL competed for 61 % of the binding. Likewise, excess LDL–proteoglycan complex inhibited the binding of ^{125}I -acetyl-LDL by 64 %. Polyinosinic acid and cytochalasin D inhibited the binding of ^{125}I -LDL–proteoglycan complex by 60 % and 36 % respectively. Compared with that of acetyl-LDL, the degradation of LDL–proteoglycan complex was retarded in human macrophages. The results indicate that the uptake of LDL–proteoglycan complex in human monocyte-derived macrophages is not mediated through binding to the LDL receptor; but occurs predominantly via the scavenger receptor, with phagocytosis playing a minor role in the process.

INTRODUCTION

Accumulation of lipids, notably cholesteryl ester in resident macrophages of the artery wall, is an important early event in atherosclerosis. However, the *in vivo* mechanism of cholesteryl ester accumulation in macrophages, which transforms these cells into foam cells, is not clear. *In vitro*, several modified forms of low-density lipoproteins (LDL) induce cholesteryl ester accumulation in macrophages. These include acetyl-LDL [1], malondialdehyde (MDA)-modified LDL [2], oxidized LDL [3], LDL isolated from atherosclerotic lesions [4] and aggregated LDL [5]. Native LDL, on the other hand, does not convert macrophages into foam cells due to efficient feed-back control of its uptake.

Another mechanism *in vitro* of foam-cell formation involves the uptake by macrophages of LDL complexed to artery-wall proteoglycans [6–9]. The isolation of such lipoprotein–proteoglycan complexes from human arteriosclerotic lesions [10] has led to the hypothesis that foam-cell formation could result, *in vivo*, from the uptake of the complexes by macrophages. In a recent study we showed that lipoprotein–proteoglycan complexes isolated from fatty streaks and fibrous plaques of human aorta induced cholesteryl ester synthesis and accumulation in human monocyte-derived macrophages *in vitro* [11].

Human monocyte-derived macrophages, unlike mouse peritoneal macrophages, express the LDL receptor and the scavenger receptor. In a recent study, Hurt et al. [12] reported that LDL modified by reversible association with artery-wall proteoglycan was metabolized via the apolipoprotein (apo) B,E receptor in

human monocyte-derived macrophages. Our previous study in mouse peritoneal macrophages [13] and a recent preliminary study in human monocyte-derived macrophages [11] indicated that lipoprotein–proteoglycan complexes were metabolized via the scavenger-receptor pathway. The present study was therefore carried out to resolve the discrepancy between our result and that of Hurt et al. [12] regarding the mechanism of uptake of apo B-lipoprotein–proteoglycan complexes by human monocyte-derived macrophages. Since the specific radio-labelling of the apo B component of the complexes isolated from atherosclerotic lesions was not feasible, we used *in vitro* complex of ^{125}I -LDL and a chondroitin sulphate–dermatan sulphate proteoglycan from human aorta as an alternative model system. The results indicate that LDL–proteoglycan complex is metabolized predominantly by a receptor-mediated pathway which is different from the LDL receptor.

MATERIALS AND METHODS

Materials

Tissue-culture plasticware was obtained from Costar (Cambridge, MA, U.S.A.) and RPMI 1640 medium from Gibco (Grand Island, NY, U.S.A.). Na^{125}I and $[1\text{-}^{14}\text{C}]\text{joleic acid}$ (50 mCi/mmol) were obtained from ICN Biomedicals (Costa Mesa, CA, U.S.A.). Histopaque, trypsin (bovine pancreas), Pronase, polyinosinic acid and cytochalasin D came from Sigma (St. Louis, MO, U.S.A.).

Isolation of proteoglycan aggregate

A high-molecular-mass proteoglycan was prepared from pooled human aortas as described by Oegema et al. [14]. Briefly, aortas were obtained at autopsy within 12 h of death from the Pathology Service, Charity Hospital, New Orleans. Areas of normal intima-media tissue from several aortas were pooled and extracted twice with 4 M guanidine hydrochloride, 0.05 M sodium acetate, pH 5.8, containing protease inhibitors [0.1 M ϵ -aminohexanoic acid, 0.005 M benzamidine hydrochloride, 0.01 M EDTA and 0.01 M phenylmethanesulphonyl fluoride (PMSF)] for 24 h at 4 °C. The combined extract was dialysed against 0.4 M guanidine hydrochloride, 0.05 M sodium acetate, pH 5.8, containing protease inhibitors and centrifuged in a CsCl gradient (1 g of CsCl/g of extract). We then removed the bottom two-fifths of the gradient (fraction A1), and fractionated it on a Sepharose CL-2B column (0.9 cm $\times$ 100 cm). Fractions were monitored for uronic acid and protein content and those eluting at the void volume of the column were pooled, dialysed against distilled water and freeze-dried. This pooled fraction was used for complexing with lipoproteins.

Lipoproteins and lipoprotein-deficient serum

LDL ($d = 1.019\text{--}1.055$ g/ml) was prepared from pooled human serum by ultracentrifugation [15]. Additions of EDTA (0.05%), butylated hydroxytoluene (10 μ M) and PMSF (20 mM) were made to the blood immediately after collection. The purity of LDL was determined by agarose-gel electrophoresis. Apo B was the only apolipoprotein identified in LDL. Acetyl-LDL [16], methyl-LDL [17] and lipoprotein-deficient serum [18] were prepared as described in the indicated references. Acetylation and methylation of LDL modified 93% and 89% of available free amino groups respectively. Aggregated LDL was prepared by vortexing LDL for 30 s [5]. The lipoproteins were radio-iodinated with ^{125}I according to the procedure of McFarlane [19] as modified by Bilheimer et al. [20]. All preparations of lipoproteins and lipoprotein-deficient serum were dialysed against 0.15 M NaCl, 0.01% EDTA at pH 7.4, and sterilized by membrane filtration.

Lipoprotein–proteoglycan complexes

Complexes formed *in vitro*

Unlabelled LDL, ^{125}I -LDL or ^{125}I -methyl-LDL was complexed to human aorta proteoglycan as described previously [21]. Briefly, proteoglycan solution (0.1 ml, 60 μ g of glycosaminoglycan) was added to the appropriate lipoprotein (150 μ g of cholesterol) followed by the addition of CaCl_2 (final concentration of 30 mM) and appropriate volumes of 0.001 M Tris/HCl (pH 7.4) keeping the final volume to 1.5 ml. The mixture was left at room temperature for 30 min and the precipitated LDL–proteoglycan complex was recovered by centrifugation (8000 g for 5 min). The complex was solubilized in culture medium and filtered through a 0.45- μ m filter before use in various experiments. Lipoprotein and proteoglycan contents of the complex were determined by measuring cholesterol and uronic acid concentrations respectively. The complex contained nearly all of the lipoprotein and 80% of proteoglycan.

Complexes formed *in vivo*

Apo B–lipoprotein–proteoglycan complexes were isolated from human aorta fibrous-plaque lesions as described previously [11]. Briefly, human aorta fibrous-plaque lesions were extracted with 0.15 M NaCl/0.05 M Tris/HCl (pH 7.4), containing protease

inhibitors and antioxidants. The extract was fractionated on a column of Bio-Gel A-50m. The fraction eluting near the elution volume of LDL was further purified by anti-(apo B) affinity chromatography to obtain the lipoprotein–proteoglycan complex used in this study. We have previously reported the characterization of these complexes [11]. In addition to apo B lipoproteins, the complex contained hyaluronic acid, chondroitin 6-sulphate, dermatan sulphate and heparin. The fibrous-plaque complex was dialysed against the appropriate medium and sterilized by membrane filtration (0.45 μ m pore size) before addition to macrophage cultures.

Macrophage cultures

Monocyte-derived macrophages were isolated from heparinized human blood by the Ficol–Hypaque method [22]. The method has been described in detail previously [11]. The cells were cultured in RPMI 1640 medium containing 20% (v/v) human serum, 100 units/ml penicillin and 100 μ g/ml streptomycin (2×10^6 cells/ml) for 8 days before they were used for experiments. The culture medium was changed every 3 days. The cells were classified as macrophages by previously described criteria [11]. After 8 days of culture an average of 92% of the cells were viable as determined by Trypan Blue exclusion.

Binding, uptake and degradation of ^{125}I -labelled ligands

Macrophages were cultured in medium containing 20% (v/v) human serum for 7 days. The cells were then either maintained in the same medium or, after washing twice in RPMI 1640 medium, switched to RPMI 1640 medium containing 5% (v/v) lipoprotein-deficient serum (medium A), in order to induce LDL receptors. After 24 h at 37 °C, the cells were washed twice with RPMI 1640 medium and used for lipoprotein binding and degradation studies on day 9 as described before [13]. For binding studies the macrophage monolayers were pre-incubated for 30 min in ice-cold RPMI 1640 medium containing 10 mM Hepes (medium B) at 4 °C before adding various concentrations of ^{125}I -labelled ligands. Binding results were corrected for unspecific absorption of ligands to plastic. The kinetics of binding were determined by the Scatchard data analysis [23].

For uptake studies macrophages were incubated in RPMI 1640 medium containing ^{125}I -labelled ligands for 6 h at 37 °C. The cells were washed as described previously [13] and cell pellets were counted for radioactivity.

Lysosomal degradation of ^{125}I -labelled ligands was determined by assaying the trichloroacetic acid-soluble non-iodide radioactivity in the medium [24].

Cholesterol esterification

Cholesterol esterification was determined by measuring the incorporation of [^{14}C]oleate into cholesteryl [^{14}C]oleate as described in [6]. Macrophages were incubated with lipoprotein–proteoglycan complexes and [^{14}C]oleate–albumin. After 16 h at 37 °C, cellular synthesis of cholesteryl [^{14}C]oleate was determined.

Analytical methods

Uronic acid was determined by the method of Blumenkrantz and Asboe-Hansen [25] and protein by a modified Lowry procedure [26] using BSA as a standard. Cholesterol was assayed by a commercial enzymic reagent kit (Autoflo cholesterol 236691, Boehringer–Mannheim Diagnostics, Houston, TX, U.S.A.).

Relative proportions of individual glycosaminoglycans in the proteoglycan were determined as follows: the glycosamino-

glycans were released from the proteoglycan by digestion with alkaline borohydride [27]. After neutralization, the glycosaminoglycans were recovered by chromatography on Dowex 50W-X2 (H^+ form) and isomeric chondroitin sulphates were quantified based on their susceptibility to chondroitinases [28]. The relative proportion of hyaluronic acid was determined by digestion with leech hyaluronidase.

The extent of oxidation of LDL-proteoglycan complex was determined by a thiobarbituric acid assay using MDA as a standard [29].

RESULTS

Characterization of proteoglycans

In the native state, proteoglycans in aorta and other tissues exist as large aggregates containing proteoglycan monomers, hyal-

uronic acid and link protein [30,31]. The proteoglycan aggregates can be isolated under associative conditions using 0.4 M guanidine hydrochloride. The proteoglycan fraction (A1), prepared under associative conditions in this study, resolved into two peaks on a column of Sepharose CL-2B, one eluting at the void volume and the other in the inclusive volume of the column (results not shown). The high-molecular-mass proteoglycan in the void-volume fraction, representing proteoglycan aggregate, was used in the study. This accounted for 45% of the proteoglycans in the A1 fraction. The glycosaminoglycan composition of the proteoglycan aggregate was 72% chondroitin sulphate, 9% dermatan sulphate and 19% hyaluronic acid.

LDL-proteoglycan complex

Both LDL and the proteoglycan used in the study are high-molecular-mass compounds. Therefore, if both of these macromolecules are in association with each other in solution, it is to be expected that the molecular size of LDL-proteoglycan complex will be larger than that of uncomplexed LDL. In order to test this we compared the elution profiles of ^{125}I -LDL and ^{125}I -LDL-proteoglycan complex solubilized in culture medium on a column of Bio-Gel A-50m. As shown in Figure 1, the ^{125}I -LDL-proteoglycan complex eluted in the inclusive volume of the column, with an elution volume higher than that of ^{125}I -LDL. Previously, we reported a similar elution profile for the lipoprotein-proteoglycan complex from the human atherosclerotic fibrous-plaque lesions used in the study [11].

We investigated the possibility of potential oxidation of LDL-proteoglycan complexes when incubated in RPMI 1640 medium. LDL-proteoglycan complex was formed, and incubated in 1 ml of the medium for 8 h at 37 °C and the extent of oxidation of LDL in the complex before and after incubation was compared. The values in terms of nmol of MDA formed/ml were: before incubation, 0.53 ± 0.01 ; after incubation, 0.52 ± 0.01 (mean $\pm$ S.D.). Thus, the medium did not induce any oxidation of the complex.

Binding and degradation of LDL-proteoglycan complex and stimulation of cholesteryl ester synthesis

Figure 2 shows the specific binding at 4 °C of ^{125}I -LDL and ^{125}I -LDL-proteoglycan complex to macrophages as a function of time. The binding of both ligands reached equilibrium by 2 h. At that point, the binding of LDL-proteoglycan complex was almost 6-fold greater than that of LDL. In these experiments, we assayed the binding of the ligands to macrophages without any pre-incubation. However, in parallel experiments we also measured binding after the cells were pre-incubated in medium A for 24 h (to induce LDL receptor). While the specific binding of ^{125}I -LDL showed a 30% increase as compared with the results in Figure 2, the binding of ^{125}I -LDL-proteoglycan complex showed no difference (results not shown). These results suggested that the LDL receptor is not involved in the binding of LDL-proteoglycan complex to macrophages.

Figure 3 illustrates the specific binding of ^{125}I -LDL-proteoglycan complex to macrophages as a function of ligand concentration in the medium. Binding was concentration dependent and saturable. Scatchard analysis of the binding data gave a K_d of 1.5×10^{-8} M (16 μ g of LDL cholesterol/ml). This was 1.75-fold higher than the K_d reported for LDL in human monocyte-derived macrophages [32]. At receptor saturation, the membrane-bound LDL-proteoglycan complex contained 509 ng of LDL-protein per mg of cell protein. Assuming the molecular mass of LDL to be 3000 kDa, of which the protein represents

Figure 1 Bio-Gel A-50m chromatography of ^{125}I -LDL and ^{125}I -LDL-proteoglycan complex

^{125}I -LDL and ^{125}I -LDL-proteoglycan complexes were eluted on a Bio-Gel A-50m column (1.5 cm $\times$ 60 cm) with 0.15 M NaCl/0.05 M Tris/HCl, pH 7.4. Fractions (2 ml) were monitored for ^{125}I radioactivity.

Figure 2 Time course of binding of ^{125}I -LDL-proteoglycan complex and ^{125}I -LDL to monocyte-derived macrophages

Monocyte-derived macrophages were cultured for 8 days. On day 9, the cells were washed and incubated in ice-cold medium B for 30 min at 4 °C. The medium was replaced with fresh cold medium B containing 10 μ g of cholesterol/ml of ^{125}I -LDL-proteoglycan complex or ^{125}I -LDL in the presence and absence of unlabelled LDL-proteoglycan complex or LDL (400 μ g of cholesterol/ml), and the cells were incubated at 4 °C. At the indicated interval, the amount of ^{125}I -labelled ligand bound to the cells was determined. Specific binding was calculated by subtracting the binding in the presence of excess unlabelled ligand from that in its absence. Each data point is the average of two experiments which were performed in duplicate.

Figure 3 Specific binding of ^{125}I -LDL-proteoglycan complex by monocyte-derived macrophages as a function of ligand concentration

Monocyte-derived macrophages were cultured for 8 days. Following pre-incubation in medium B as described for Figure 2, the cells were incubated in fresh ice-cold medium B containing the indicated concentrations of ^{125}I -LDL-proteoglycan complex in the presence and absence of the unlabelled complex (500 μg of cholesterol/ml). After 5 h at 4 °C, specific binding of ^{125}I -LDL-proteoglycan complex was determined. Each data point represents the average of three experiments, each of which was conducted in duplicate.

about 20% [33], we calculated that at 4 °C each macrophage bound 51 000 LDL molecules in the complex.

Similar to the binding data, the degradation of ^{125}I -LDL-proteoglycan complex also showed saturability and concentration dependency (Figure 4a). Analysis of the kinetics of degradation by a double-reciprocal plot demonstrated that the apparent K_m for degradation was 18 μg of LDL cholesterol/ml. Degradation of the LDL-proteoglycan complex did not occur at 4 °C, indicating that internalization was a prerequisite. Chloroquine, a lysosomotropic agent, completely inhibited degradation of the complex at a concentration of 20 μM (results not shown).

We also determined the cellular consequence of LDL-proteoglycan-complex uptake. Macrophages exhibited a concentration-dependent stimulation of cholesteryl ester synthesis when incubated with the complex (Figure 4b). At equivalent concentrations of cholesterol in the medium, the stimulation of cholesterol esterification that was mediated by the LDL-proteoglycan complex was less than that mediated by the lipoprotein-proteoglycan complex isolated from human aorta fibrous plaques (Figure 4b). Chloroquine also inhibited cholesteryl ester synthesis mediated by the complexes (results not shown).

Nature of the binding sites

Enzyme treatment

The binding and degradation results described above strongly suggest that human monocyte-derived macrophages metabolize LDL-proteoglycan complex by a receptor pathway. This means that the uptake of the complex is mediated through its binding to specific sites on the cell surface. Therefore, destruction of the cell-surface binding sites should abolish binding and degradation of the complex. In order to verify this, we studied the binding and degradation of ^{125}I -LDL-proteoglycan complex following a brief exposure of macrophages to the proteolytic enzymes trypsin and Pronase. As shown in Table 1, both enzymes significantly inhibited the specific binding and degradation of the complex. The inhibition of binding was greater than that of degradation. However, as we reported earlier for mouse peritoneal macrophages [13], the enzymes did not abolish binding and degradation of the complex completely. There was no cell detachment or loss of cell viability in enzyme-treated cultures.

Figure 4 (a) Specific degradation of ^{125}I -LDL-proteoglycan complex by macrophages and (b) stimulation of cholesteryl ester synthesis in macrophages by fibrous-plaque complex and LDL-proteoglycan complex

(a) Monocyte-derived macrophages were incubated in RPMI 1640 medium containing the indicated concentrations of ^{125}I -LDL-proteoglycan complex in the presence and absence of the unlabelled complex (500 μg of cholesterol/ml). After 6 h at 37 °C the degradation of ^{125}I -LDL-proteoglycan complex was assayed and the specific degradation calculated. Each data point is the average of duplicate assays and is representative of two experiments. (b) Monocyte-derived macrophages were cultured for 8 days and then incubated in RPMI 1640 medium containing 0.2 mM [^{14}C]oleate-albumin, and the indicated concentrations of fibrous-plaque complex (FP complex) or *in vitro* LDL-proteoglycan complex (LDL-PG complex). After 16 h at 37 °C, the cellular content of cholesteryl [^{14}C]oleate was determined. Values are the averages of triplicate assays.

Binding of ^{125}I -methyl-LDL-proteoglycan complex

The above results confirmed that LDL-proteoglycan complex binds to cell-surface binding sites on the macrophages. Further studies were, therefore, conducted to determine the nature of the binding sites. Since pre-incubation of macrophages in medium containing lipoprotein-deficient serum failed to increase the binding of LDL-proteoglycan complex, the involvement of the apo B,E(LDL) receptor in the binding of the complex was less likely. This was further investigated in binding studies using methyl-LDL-proteoglycan complex. The lysine and arginine residues of LDL are essential for receptor binding, as well as for interaction with the proteoglycan [17,21]. Reductive methylation of lysine residues of LDL abolishes its binding to the receptors on fibroblasts [17]; however, it does not affect the LDL-proteoglycan interaction [21]. Therefore, if the methyl-LDL-proteoglycan complex binds to macrophages to the same extent as the LDL-proteoglycan complex, this would be strong evidence against the participation of macrophage LDL receptors in the

Table 1 Effect of treatment with trypsin and Pronase on the binding and degradation of ^{125}I -LDL-proteoglycan complex in monocyte-derived macrophages

Monocyte-derived macrophages were cultured for 8 days. On day 9, the cells were incubated in RPMI 1640 medium either alone, or in the presence of the indicated concentrations of trypsin or Pronase. After 1 h at 37 °C, the medium was removed and each monolayer was washed twice with medium A and once with RPMI 1640 medium. For binding studies, the cells were pre-incubated in medium B for 30 min at 4 °C. The medium was then replaced with fresh medium B containing ^{125}I -LDL-proteoglycan complex (10 μg of cholesterol/ml) in the presence and absence of unlabelled LDL-proteoglycan complex (400 μg of cholesterol/ml). The cells were incubated at 4 °C for 5 h and binding was assayed as described in the Materials and methods section. For degradation studies, the cells were incubated in RPMI 1640 medium containing the same concentrations of ^{125}I -labelled and unlabelled ligand as used for binding studies at 37 °C for 6 h. Degradation of ^{125}I -LDL-proteoglycan complex was then assayed. Specific binding and degradation values were calculated by subtracting the binding or degradation in the presence of excess unlabelled complex from those in the absence of the unlabelled complex. Each value represents the average of two experiments, each of which was performed in duplicate.

Treatment	Specific binding		Specific degradation	
	($\mu\text{g}/\text{mg}$ of protein)	(% of control)	($\mu\text{g}/\text{mg}$ of protein)	(% of control)
None	215	100	1.3	100
Trypsin ($\mu\text{g}/\text{ml}$)				
50	65	30	0.6	46
75	26	12	0.4	31
100	22	10	0.4	31
Pronase ($\mu\text{g}/\text{ml}$)				
10	47	22	0.4	31
20	17	8	0.3	23
30	15	7	0.3	23

Figure 5 Binding of ^{125}I -methyl-LDL-proteoglycan complex, ^{125}I -LDL-proteoglycan complex, ^{125}I -methyl-LDL and ^{125}I -LDL to macrophages

Monocyte-derived macrophages were incubated in medium B containing the indicated concentrations of ^{125}I -methyl-LDL-proteoglycan complex (^{125}I -methyl-LDL-PG), ^{125}I -LDL-proteoglycan complex (^{125}I -LDL-PG), ^{125}I -methyl-LDL or ^{125}I -LDL in the presence or absence of the corresponding unlabelled ligand (500 μg of cholesterol/ml). After 5 h at 4 °C, the specific binding of each ligand was determined. Values are the average of two experiments, each performed in duplicate.

binding of LDL-proteoglycan complex. Figure 5 shows that the binding of ^{125}I -methyl-LDL-proteoglycan complex to macrophages was virtually identical to that of ^{125}I -LDL-proteoglycan complex. In contrast, methylation abolished the binding of ^{125}I -LDL to macrophages by 87%. We also found that the degra-

Table 2 Effect of various ligands on the binding of ^{125}I -LDL-proteoglycan complex to monocyte-derived macrophages

Monocyte-derived macrophages were cultured for 8 days. The cells were precooled in medium B and then incubated in medium B containing 10 μg of cholesterol/ml of ^{125}I -LDL-proteoglycan complex with or without a 40-fold excess concentration of the various unlabelled competitor ligands. After 5 h at 4 °C, binding was assayed. Each value is the average of two experiments, each of which was performed in triplicate.

Unlabelled competitor ligand	Bound ^{125}I -LDL-proteoglycan complex	
	($\mu\text{g}/\text{mg}$ of protein)	(% of control)
None	1.8	100
LDL	1.7	94
LDL-proteoglycan complex	0.3	17
Fibrous-plaque complex	0.2	11
Acetyl-LDL	0.7	39
Proteoglycan	1.9	105

dation of ^{125}I -LDL-proteoglycan complex by macrophages was unaffected by LDL methylation (results not shown).

Competition experiments

In an attempt to further explore the nature of LDL-proteoglycan interaction with macrophages, we investigated the binding of ^{125}I -labelled complex in the presence of a 40-fold excess concentration of several unlabelled ligands. As shown in Table 2, binding was not suppressed by LDL and proteoglycan. As expected, excess unlabelled LDL-proteoglycan complex and fibrous-plaque complex inhibited the binding of the radiolabelled complex almost completely. Excess acetyl-LDL, on the other hand, competed for 61 % of the binding of LDL-proteoglycan complex.

Similar results were obtained when the binding of ^{125}I -acetyl-LDL was studied using excess unlabelled LDL-proteoglycan complex as the competitor (Figure 6). A 40-fold excess of the complex reduced the binding of ^{125}I -acetyl-LDL by 64 %, while excess unlabelled acetyl-LDL inhibited the binding completely. The results from the above cross-competition experiments indicated that the acetyl-LDL receptor was involved in the binding of ^{125}I -LDL-proteoglycan complex to macrophages.

This was further confirmed in competition experiments using polyinosinic acid, which is a specific inhibitor of the acetyl-LDL receptor. Figure 7 shows that polyinosinic acid inhibited 60 % of the binding of ^{125}I -LDL-proteoglycan complex, while it completely inhibited the binding of ^{125}I -acetyl-LDL.

Effect of cytochalasin D

To determine whether phagocytosis of LDL-proteoglycan complex played a role in its uptake by macrophages, we studied the effect of cytochalasin D, an inhibitor of phagocytosis on the process. As shown in Table 3, cytochalasin D at a concentration of 2 $\mu\text{g}/\text{ml}$ decreased the degradation of ^{125}I -LDL-proteoglycan complex by 36 %. In contrast, the drug at similar concentrations inhibited the degradation of aggregated LDL by 84 % (results not shown). However, cytochalasin D did not affect the degradation of ^{125}I -LDL and ^{125}I -acetyl-LDL (results not shown).

Uptake and degradation of ^{125}I -LDL-proteoglycan complex and ^{125}I -acetyl-LDL

To assess whether differences exist in the metabolism of LDL-

Figure 6 Effect of unlabelled acetyl-LDL and LDL-proteoglycan complex on the binding of ^{125}I -acetyl-LDL to macrophages

Macrophages, after 8 days of culture, were incubated in medium B containing $10\text{ }\mu\text{g}$ of cholesterol/ml of ^{125}I -acetyl-LDL and the indicated concentrations of unlabelled acetyl-LDL or LDL-proteoglycan complex (LDL-PG complex). After 5 h at $4\text{ }^{\circ}\text{C}$, cellular binding of ^{125}I -acetyl-LDL was assayed. Each point is the average of triplicate assays. The 100% value for binding was $320\text{ ng }^{125}\text{I}$ -acetyl-LDL/mg of cell protein.

Figure 7 Effect of polyinosinic acid on the binding of ^{125}I -LDL-proteoglycan complex and ^{125}I -acetyl-LDL to macrophages

Macrophages were cultured for 8 days and then incubated in medium B containing $10\text{ }\mu\text{g}$ of cholesterol/ml of ^{125}I -LDL-proteoglycan complex or ^{125}I -acetyl-LDL and the indicated concentrations of polyinosinic acid. After 5 h at $4\text{ }^{\circ}\text{C}$, the binding of each ligand was assayed. Values represent the average of triplicate assays.

proteoglycan complex and acetyl-LDL, we investigated the uptake and degradation of the two ligands in macrophages following incubation of the cells at $37\text{ }^{\circ}\text{C}$. The results in Table 4 indicate that the ratio of cell associated to degraded ligand for ^{125}I -acetyl-LDL was 3-fold greater than that for ^{125}I -LDL-proteoglycan complex. This means that as compared with acetyl-LDL, the degradation of LDL-proteoglycan complex is retarded in macrophages.

Regulation of LDL-proteoglycan complex uptake

We investigated whether the binding sites for the LDL-proteoglycan complex are subject to down-regulation by extracellular lipoprotein cholesterol. The binding of ^{125}I -LDL-

Table 3 Effect of cytochalasin D on the degradation of ^{125}I -LDL-proteoglycan complex in monocyte-derived macrophages

Monocyte-derived macrophages were cultured for 8 days and then incubated with ^{125}I -LDL-proteoglycan complex ($20\text{ }\mu\text{g}$ of cholesterol/ml) in the presence or absence of either the indicated concentrations of cytochalasin D in $10\text{ }\mu\text{l}$ dimethyl sulphoxide (DMSO) or $10\text{ }\mu\text{l}$ DMSO alone. After 6 h at $37\text{ }^{\circ}\text{C}$ cellular degradation of the complex was assayed. Each value is the average of triplicate assays.

Treatment	^{125}I -LDL-proteoglycan complex degraded ($\mu\text{g}/\text{ml}$ of protein)
None	3.3
Cytochalasin D ($\mu\text{g}/\text{ml}$)	
0.5	2.8
1.0	2.3
2.0	2.1

Table 4 Relative uptake and degradation values of ^{125}I -LDL-proteoglycan complex and ^{125}I -acetyl-LDL in monocyte-derived macrophages

After 8 days of culture, macrophages were incubated in RPMI 1640 medium containing either ^{125}I -LDL-proteoglycan complex or ^{125}I -acetyl-LDL ($50\text{ }\mu\text{g}$ of cholesterol/ml of each ligand) at $37\text{ }^{\circ}\text{C}$ for 6 h. The cells were washed and the cellular uptake of each ligand was assayed as described in the Materials and methods section. The medium was assayed to determine cellular degradation of each ligand. Each value represents the average of triplicate assays.

^{125}I ligand	Uptake ($\mu\text{g}/\text{mg}$ of protein)	Degradation ($\mu\text{g}/\text{mg}$ of protein)	Ratio (uptake/degradation)
LDL-proteoglycan complex	4.7	5.2	0.9
Acetyl-LDL	3.7	12.2	0.3

proteoglycan complex to monocyte-derived macrophages was determined after pre-incubation of the cells for 48 h with a 40-fold excess of unlabelled LDL-proteoglycan complex, LDL and acetyl-LDL. Binding of ^{125}I -LDL-proteoglycan complex did not show down-regulation after pre-incubation of macrophages with the three ligands (results not shown).

DISCUSSION

Previously we have shown that complexes of human plasma LDL and a bovine aorta proteoglycan were taken up and degraded by mouse peritoneal macrophages [6,13]. As a consequence of this, the cells accumulated large amounts of cholesteryl ester. We have now extended the studies to the human monocyte-derived macrophages using complexes of LDL and a human aorta proteoglycan. The results demonstrate that like the mouse cells, the human cells also metabolize the LDL-proteoglycan complexes. This in turn stimulated cholesteryl ester synthesis by the cells.

The metabolic studies indicate that the cells bind and degrade LDL-proteoglycan complex by a saturable process of moderate affinity of $10\text{ }\mu\text{g}$ of LDL-protein/ml ($16\text{ }\mu\text{g}$ of cholesterol/ml). This suggests that the uptake of the complex is mediated through binding to specific cell-surface binding sites. This is further supported by the demonstration that brief exposure of macrophages to proteolytic enzymes abolished significantly the binding and degradation of the complex, presumably due to the destruction of the membrane receptors.

A comparison of the receptors for LDL–proteoglycan complex on mouse peritoneal macrophages reported in our earlier study [13], and human monocyte-derived macrophages in the present study reveals two major differences. First, the human cells bind the complex with a 2.5-fold higher affinity than the mouse cells ($K_d = 1.5 \times 10^{-8}$ M versus 3.8×10^{-8} M). Secondly, the human macrophages express more receptors per cell than the mouse cells (51000 versus 32000). This could be due to an increase in the receptor number in the human macrophages as the cells mature. Earlier, Fogelman et al. [34] observed a dramatic rise in the scavenger-receptor activity with the maturation of human blood monocytes into macrophages.

Several lines of evidence indicate that the LDL receptor is not involved in the uptake of LDL–proteoglycan complex in human monocyte-derived macrophages: (1) pre-incubation of macrophages in lipoprotein-deficient medium did not stimulate the binding of 125 I-LDL–proteoglycan complex, whereas it increased the binding of 125 I-LDL by 30 %; (2) the binding and degradation of 125 I-methyl-LDL–proteoglycan complex were indistinguishable from those of 125 I-LDL–proteoglycan complex; in contrast methylation of 125 I-LDL abolished its binding to macrophages; (3) LDL did not compete for the binding of 125 I-LDL–proteoglycan complex; (4) pre-incubation of macrophages with a 40-fold excess concentration of LDL did not suppress the binding of the complex.

Our conclusion that the LDL receptor does not mediate the uptake of LDL–proteoglycan complex in human monocyte-derived macrophages differs from that of Hurt et al. [12]. The difference may be due to the nature of the complexes used in the two studies. Since Hurt et al. [12] used a high ratio of LDL to proteoglycan in complex formation, their complexes contained at most 2–4 μ g of glycosaminoglycans per 100 μ g of apo B protein. In contrast, the LDL–proteoglycan complex in our study contained approx. 55 μ g of glycosaminoglycan per 100 μ g of apo B protein. In addition, under the conditions employed for complex formation by Hurt et al. [12], the proteoglycan did not remain bound to LDL after solubilization of the complex. Therefore the properties of LDL, including surface charge and hydrodynamic size, were not altered as a result of the reversible type of association between LDL and proteoglycan. Consequently, the lipoprotein was taken up through the macrophage LDL receptor. The association of LDL with a relatively large amount of proteoglycan, as in the current study, increases the hydrodynamic size of the lipoprotein (Figure 1). Moreover, since the lysine and arginine residues of LDL are involved in both receptor binding and interaction with proteoglycan [17,21], complex formation with proteoglycan blocks these amino-acid residues of LDL, thereby preventing cellular uptake through the LDL receptor.

Binding of 125 I-LDL–proteoglycan complex to macrophages was not suppressed by proteoglycan. This indicates that the uptake of the complex is not mediated through the ‘piggy back’ mechanism proposed for the uptake of LDL–dextran sulphate complex in mouse peritoneal macrophages [35].

Excess acetyl-LDL inhibited the binding of 125 I-LDL–proteoglycan complex to macrophages by 61 %. Similarly, excess LDL–proteoglycan complex competed for 64 % of the binding of 125 I-acetyl-LDL. These results indicate that the scavenger receptor is involved in the uptake of LDL–proteoglycan complex in macrophages. This is supported by the demonstration that polyinosinic acid, an inhibitor of the scavenger receptor in macrophages, blocked the binding of 125 I-LDL–proteoglycan complex by 60 %. These results are consistent with our recent findings that the uptake of apo B–lipoprotein–proteoglycan complexes isolated from human atherosclerotic lesions by human

monocyte-derived macrophages is also blocked significantly by acetyl-LDL and polyinosinic acid [11].

The results presented here, and in our previous study [11], indicate that an additional minor pathway for the macrophage uptake of LDL–proteoglycan complex *in vitro* and apo B–lipoprotein–proteoglycan complexes *in vivo* is phagocytosis. Cytochalasin D, an inhibitor of phagocytosis, inhibited the degradation of 125 I-LDL–proteoglycan complex by 36 %. Even though the LDL–proteoglycan complex was completely soluble in the culture medium, it is possible that some of the complex remains in the form of aggregates. Internalization of these aggregates probably occurs via phagocytosis. Previous studies have shown that the uptake of aggregated LDL in macrophages is mediated through phagocytosis [5].

Even though acetyl-LDL and LDL–proteoglycan complex are internalized by human monocyte-derived macrophages through similar receptor-mediated endocytosis, there are major differences in the cellular processing of the two ligands. Compared with 125 I-acetyl-LDL, more of the 125 I-LDL–proteoglycan complex accumulated in the cells because of its retarded cellular degradation. Previously we have observed similar differences in the metabolism of LDL–proteoglycan complex, acetyl-LDL, and LDL–heparin complex in mouse peritoneal macrophages [36,37]. The mechanism responsible for this slower lysosomal degradation of LDL–proteoglycan complex is not known. However, it is possible that the complex might somehow interfere with normal lysosomal functions. In this context, it is relevant to mention that glycosaminoglycans are capable of disrupting normal lysosomal function in macrophages [38].

The macrophage receptors for LDL–proteoglycan complex are not subject to down-regulation. Therefore, the unregulated uptake of the complex by the cells could cause massive accumulation of intracellular cholesteryl ester, resulting in the transformation of these cells into foam cells. Consistent with this concept is our recent finding that the uptake of apo B–lipoprotein–proteoglycan complexes by human monocyte-derived macrophages led to stimulation of cholesteryl ester synthesis and accumulation [11].

In summary, these studies show that human monocyte-derived macrophages metabolize LDL–proteoglycan complexes predominantly via a receptor-mediated pathway that has properties similar to the acetyl-LDL-receptor pathway. Since the uptake of the complexes is not regulated, they are potent stimulators of synthesis and accumulation of cholesteryl ester in macrophages. Additionally, proteoglycan aggregates are natural components of the arterial wall and their synthesis by smooth muscle cells is augmented in response to arterial wall injury. Therefore, the binding of proteoglycans to LDL and other apo B-lipoproteins could be a major pathway of entrapment of extracellular lipids in the arterial wall and ultimate intracellular lipid-mediated foam-cell formation. Since the scavenger receptor mediates the uptake of both LDL–proteoglycan complex and oxidized LDL [3], the question unsettled at present is the relative contribution of the two ligands to foam-cell formation *in vivo*.

This study was supported by grants HL-42993 and HL-02942 from the National Institutes of Health.

REFERENCES

- Goldstein, J. L., Ho, Y. K., Basu, S. K. and Brown, M. S. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 333–337
- Fogelman, A. M., Shecter, I., Seager, J., Hokom, M., Child, J. S. and Edwards, P. A. (1980) Proc. Natl. Acad. Sci. U.S.A. **77**, 2214–2218
- Parthasarathy, S., Printz, D. J., Boyd, D., Joy, L. and Steinberg, D. (1986) Arteriosclerosis **6**, 505–510

- 4 Clevidence, B. A., Morton, R. E., Dusek, D. M. and Hoff, H. F. (1984) *Arteriosclerosis* **4**, 196–207
- 5 Khoo, J. C., Miller, E., McLaughlin, P. and Steinberg, D. (1988) *Arteriosclerosis* **8**, 348–358
- 6 Vijayagopal, P., Srinivasan, S. R., Jones, K. M., Radhakrishnamurthy, B. and Berenson, G. S. (1985) *Biochim. Biophys. Acta* **837**, 251–261
- 7 Salisbury, B. G. J., Falcone, D. J. and Minick, C. R. (1985) *Am. J. Pathol.* **120**, 6–11
- 8 Hurt, E. and Camejo, G. (1987) *Atherosclerosis* **67**, 115–126
- 9 Yla-Herttula, S., Jaakola, O., Solakivi, T., Huivaniemi, H. and Nikkari, T. (1986) *Atherosclerosis* **62**, 73–80
- 10 Srinivasan, S. R., Dolan, P., Radhakrishnamurthy, B., Pargaonkar, P. S. and Berenson, G. S. (1975) *Biochim. Biophys. Acta* **388**, 58–70
- 11 Vijayagopal, P., Srinivasan, S. R., Radhakrishnamurthy, B. and Berenson, G. S. (1992) *Arterioscler. Thromb.* **12**, 237–249
- 12 Hurt, E., Bondjers, G. and Camejo, G. (1990) *J. Lipid Res.* **31**, 443–454
- 13 Vijayagopal, P., Srinivasan, S. R., Radhakrishnamurthy, B. and Berenson, G. S. (1988) *Biochim. Biophys. Acta* **960**, 210–219
- 14 Oegema, T. R., Jr., Hascall, V. C. and Eisenstein, R. (1979) *J. Biol. Chem.* **254**, 1312–1318
- 15 Hatch, F. T. and Lees, R. S. (1968) *Adv. Lipid Res.* **6**, 2–63
- 16 Fraenkel-Conrat, H. (1957) *Methods Enzymol.* **4**, 247–269
- 17 Weisgraber, K. H., Innerarity, T. L. and Mahley, R. W. (1978) *J. Biol. Chem.* **253**, 9053–9062
- 18 Brown, M. S., Dana, S. E. and Goldstein, J. L. (1974) *J. Biol. Chem.* **249**, 789–796
- 19 McFarlane, A. S. (1958) *Nature (London)* **182**, 53
- 20 Bilheimer, D. W., Eisenberg, S. and Levy, R. I. (1972) *Biochim. Biophys. Acta* **260**, 212–221
- 21 Vijayagopal, P., Srinivasan, S. R., Radhakrishnamurthy, B. and Berenson, G. S. (1981) *J. Biol. Chem.* **256**, 8234–8241
- 22 Boyum, S. (1976) *Scand. J. Immunol. (Suppl. 5)* **5**, 9–15
- 23 Scatchard, G. (1949) *Ann. N.Y. Acad. Sci.* **51**, 660–672
- 24 Goldstein, J. L. and Brown, M. S. (1974) *J. Biol. Chem.* **243**, 5153–5162
- 25 Blumenkrantz, N. and Asboe-Hansen, G. (1973) *Anal. Biochem.* **54**, 484–489
- 26 Hartree, E. F. (1972) *Anal. Biochem.* **48**, 422–427
- 27 Carlson, D. M. (1968) *J. Biol. Chem.* **243**, 616–626
- 28 Saito, H., Yamagata, T. and Suzuki, S. (1966) *J. Biol. Chem.* **243**, 1536–1542
- 29 Ohkawa, H., Ohishi, N. and Yagi, K. (1978) *J. Lipid Res.* **19**, 1053–1057
- 30 Vijayagopal, P., Radhakrishnamurthy, B., Srinivasan, S. R. and Berenson, G. S. (1985) *Biochim. Biophys. Acta* **839**, 110–118
- 31 Faltz, L. L., Reddi, A. H., Hascall, G. K., Martin, D., Pita, J. C. and Hascall, V. C. (1979) *J. Biol. Chem.* **254**, 1375–1380
- 32 Koo, C., Wernette-Hammond, M. E., Garcia, Z., Malloy, M. J., Uauy, R., East, C., Bilheimer, D., Mahley, R. W. and Innerarity, T. (1988) *J. Clin. Invest.* **81**, 1322–1340
- 33 Fisher, W. R., Hammond, M. G. and Warmke, G. L. (1972) *Biochemistry* **11**, 519–525
- 34 Fogelman, A. M., Haberland, M. E., Seager, J., Hokom, M. and Edwards, P. A. (1981) *J. Lipid Res.* **22**, 1131–1141
- 35 Basu, S. K., Brown, M. S. and Goldstein, J. L. (1979) *J. Biol. Chem.* **254**, 7141–7146
- 36 Vijayagopal, P., Srinivasan, S. R., Radhakrishnamurthy, B. and Berenson, G. S. (1991) *Biochim. Biophys. Acta* **1092**, 291–297
- 37 Srinivasan, S. R., Vijayagopal, P., Eberle, K., Radhakrishnamurthy, B. and Berenson, G. S. (1991) *Biochim. Biophys. Acta* **1081**, 188–196
- 38 Kielian, M. C. and Cohn, Z. A. (1982) *J. Cell Biol.* **93**, 875–882