Regulation of the metabolism of lipoprotein–proteoglycan complexes in human monocyte-derived macrophages

Parakat VIJAYAGOPAL

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

Studies were performed to evaluate the effect of several factors on the metabolism of lipoprotein–proteoglycan complexes in human monocyte-derived macrophages. *In vivo* apoB-lipoprotein–proteoglycan complex was isolated from human aorta fibrous-plaque lesions and low-density lipoprotein (LDL)–proteoglycan complex was formed *in vitro*. Degradation of LDL–proteoglycan complex and cholesteryl ester synthesis mediated by the *in vivo* and *in vitro* complexes were lowest in freshly isolated monocytes. With the maturation of monocytes into macrophages, there was a dramatic rise in both. The degradation of the complex and the resultant stimulation of cholesterol esterification increased significantly with increasing cell density. Preincubation of macrophages in medium containing

lipoprotein cholesterol did not down-regulate the subsequent degradation of LDL–proteoglycan complex. Macrophage-conditioned medium had a profound stimulatory effect on the degradation of LDL–proteoglycan complex and cholesterol esterification by mature macrophages and freshly isolated monocytes. The conditioned medium lost its stimulatory activity after boiling, dialysis and trypsin digestion. Macrophage activation with phorbol ester and bacterial lipopolysaccharide resulted in a marked suppression of the binding and degradation of the complex, as well as the complex-mediated cholesteryl ester synthesis. These results demonstrate that several factors regulate the metabolism of lipoprotein–proteoglycan complexes in human monocyte-derived macrophages.

INTRODUCTION

Lipid-laden foam cells are the hallmarks of early atherosclerotic lesions, and foam cells originate predominantly from monocyte-derived macrophages [1–4]. The lipids of foam cells are predominantly cholesteryl esters, which are derived from plasma low-density lipoproteins (LDL). However, the precise cellular mechanism responsible *in vivo* for cholesteryl ester accumulation in macrophages is not known. *In vitro*, the uptake of LDL by macrophages via the LDL receptor does not cause intracellular cholesteryl ester accumulation. In contrast, the uptake of several modified forms of LDL through the scavenger receptor(s) of macrophages results in massive cholesteryl ester accumulation [5].

We and other workers have shown that macrophages accumulate large amounts of cholesteryl ester when incubated with complexes of LDL and artery-wall proteoglycans [6–9]. More recently we have isolated intact apoB-lipoprotein–proteoglycan complexes from human atherosclerotic lesions and showed that these complexes produced *in vivo* promoted foam-cell formation in human monocyte-derived macrophages [10]. Further studies indicated that the human cells bound LDL–proteoglycan complexes by a receptor which was different from the LDL receptor [11]. The present study was conducted to determine the factors regulating the metabolism of lipoprotein–proteoglycan complexes in human monocyte-derived macrophages.

MATERIALS AND METHODS

Materials

[1-¹⁴C]Oleic acid (50 mCi/mmol) and Na¹²⁵I were purchased from ICN Biochemicals (Costa Mesa, CA, U.S.A.). RPMI-1640 medium was obtained from GIBCO BRL (Gaithersburg, MD,

U.S.A.) and cell culture dishes from Costar (Cambridge, MA, U.S.A.). All other chemicals used in this study were purchased from Sigma (St. Louis, MO, U.S.A.).

Lipoproteins

LDL ($d = 1.019$ – 1.055 g/ml) was isolated from pooled human serum by preparative ultracentrifugation [12]. EDTA (0.05%), butylated hydroxytoluene (BHT) (10 μ M) and phenylmethylsulphonyl fluoride (PMSF) (2 mM) were added to the serum before centrifugation. The purity of LDL was determined by agarose-gel electrophoresis. Acetyl-LDL and lipoprotein-deficient serum were prepared as described previously [10] and LDL and acetyl-LDL were radiolabelled with carrier-free ¹²⁵I as described by Bilheimer et al. [13]. Lipoproteins were stored at 4 °C in 0.15 M NaCl/0.01% EDTA, pH 7.4, and used within 3 weeks. They were sterilized by membrane filtration just before being used in cell-culture studies.

Proteoglycan

A high-molecular-mass human aorta proteoglycan was isolated as described previously [11,14]. Briefly, proteoglycans were extracted from autopsied intima-media segments of lesion-free human aortas obtained within 12 h of death. The extraction media contained 4 M guanidine hydrochloride, 0.05 M sodium acetate and a variety of protease inhibitors. The extract was dialysed against 0.5 M guanidine hydrochloride and centrifuged in a CsCl gradient. We then removed the bottom two-fifths of the gradient and fractionated it on a column of Sepharose CL-2B. The proteoglycan fraction used in the study was excluded from the column and contained 72% chondroitin sulphate, 9% dermatan sulphate and 19% hyaluronic acid.

Lipoprotein–proteoglycan complexes

In vivo complex

ApoB-lipoprotein–proteoglycan complexes were isolated from human aorta fibrous-plaque lesions as described previously [10]. Briefly, lesioned human aortas were obtained during autopsy, which was performed within 12 h of death. Fibrous-plaque lesions were collected and individually extracted with 0.15 M NaCl/0.05 M Tris/HCl (pH 7.4) containing protease inhibitors and antioxidants. The extracts from several aortas were pooled and fractionated on a column of Bio-Gel-A-50m. The fraction eluting in the inclusive volume, ahead of human LDL, was further purified by anti-apoB-affinity chromatography to obtain the lipoprotein–proteoglycan complex used in the present study. In addition to apoB-lipoproteins, the complex contained fibronectin, hyaluronic acid, chondroitin 6-sulphate, dermatan sulphate and heparin. Before its addition to macrophage cultures, the complex was dialysed against the appropriate medium and sterilized by membrane filtration. The cholesterol/protein ratio of the *in vivo* complex was 1.6.

In vitro complex

In vitro complex of LDL or ^{125}I -LDL and human aorta proteoglycan was prepared as described before [6]. LDL (150 μg of cholesterol) and proteoglycan solution (0.1 ml, 60 μg of glycosaminoglycan) were mixed and CaCl_2 (final concentration of 30 μM) was added to the mixture, followed by 0.001 M Tris/HCl (pH 7.4), keeping the final volume to 1.5 ml. After 30 min incubation at room temperature, the precipitated LDL–proteoglycan complex was recovered by centrifugation. The complex was dissolved in the culture medium and sterilized by membrane filtration before being added to macrophage cultures. Cholesterol and uronic acid contents of the complex were used as measures of LDL and proteoglycan respectively. Almost all the LDL and about 85% of the proteoglycan were recovered in the complex. The cholesterol/protein ratio of the *in vitro* complex was 1.3.

We have shown recently that both the *in vivo* and *in vitro* complexes remained intact when incubated in the macrophage culture medium for up to 40 h [15].

Macrophage culture and macrophage-conditioned medium

Human monocyte-derived macrophages were isolated from heparinized human blood by the Ficol–Hypaque method [16]; details of the procedure have been described previously [10]. The cells were plated at a density of 2×10^6 cells/ml in RPMI-1640 medium containing 20% (v/v) human serum, 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Unless otherwise indicated, the cells were routinely cultured for 8 days before the initiation of metabolic studies. The cells were classified as macrophages by previously described criteria [10]. Cell viability, as determined by Trypan Blue exclusion, averaged 93%.

To prepare macrophage-conditioned medium, cells were cultured for 8 days as above. The medium was removed and the cells were washed with RPMI-1640 without serum. Fresh RPMI-1640 containing 0.1% BSA was added and the cells were incubated at 37 °C for 24 h. The conditioned medium was then collected and centrifuged to remove cellular debris; the supernatant was stored at –20 °C and used within 2 weeks. Conditioned medium was also prepared from activated macrophages. To this end, 8-day-old macrophages were incubated in the presence of phorbol 12-

myristate 13-acetate (PMA; 100 ng/ml). After 24 h, the medium was removed and discarded. The activated cells were then washed and incubated in fresh RPMI-1640 containing 0.1% BSA. After 24 h, the conditioned medium was harvested.

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

Binding studies were conducted at 4 °C as described previously [17]. After 8 days of culture, the macrophages were preincubated for 30 min in ice-cold RPMI-1640 medium containing 10 mM Hepes, at 4 °C, before the addition of various concentrations of ^{125}I -LDL–proteoglycan complex. Binding data were corrected for non-specific adsorption of the complex to plastic.

Lysosomal degradation of ^{125}I -labelled ligands was determined as described previously [6]. Macrophages were incubated in RPMI-1640 medium containing ^{125}I -labelled ligands. After 6 h at 37 °C, trichloroacetic acid-soluble (non-iodide) radioactivity in the medium was assayed. Results were corrected for small amounts of acid-soluble radioactivity formed in parallel cell-free incubations.

Cholesteryl ester synthesis

Cholesteryl ester synthesis was measured as described previously [10]. Briefly, macrophages were incubated in RPMI-1640 medium containing 0.2 mM [^{14}C]oleate–albumin and various ligands for 16 h at 37 °C. Cellular synthesis of cholesteryl [^{14}C]oleate was then assayed. Control incubations were performed without cells and these values were subtracted from the results.

Analytical methods

Protein content was determined by a modified Lowry procedure [18] and cholesterol with a commercial enzymic reagent kit (Autoflow cholesterol 236691), Boeringer–Mannheim Diagnostics, Houston, TX, U.S.A.). Uronic acid was assayed by the method of Blumenkrantz and Asboe-Hansen [19]. The extent of oxidation of lipoprotein–proteoglycan complexes was determined by a thiobarbituric acid assay using malondialdehyde as a standard [20].

RESULTS

Macrophage maturation

Figure 1(a) compares the effect of macrophage maturation in culture on the degradation of LDL, LDL–proteoglycan complex and acetyl-LDL. In these experiments, the cells were cultured for the indicated duration and then incubated for 6 h with ^{125}I -labelled ligands to determine their degradation. For the cholesterol esterification studies, the duration of incubation with various ligands was 16 h. During the first 2 days of culture, the degradation of all three ligands was low, while the degradation of ^{125}I -LDL by macrophages showed a slight increase with maturation. In contrast, the degradation of both ^{125}I -LDL–proteoglycan complex and ^{125}I -acetyl-LDL increased almost linearly from the second day of culture until maximal degradation of both ligands occurred on the eighth day. Thereafter, the degradation of LDL–proteoglycan complex continued to rise slowly as a function of time in culture, while the degradation of acetyl-LDL declined gradually. The stimulation of cholesteryl ester synthesis, mediated by LDL–proteoglycan complex and apoB-lipoprotein–proteoglycan complex from fibrous plaques in macrophages at different stages of maturation, also followed a pattern similar to the degradation of ^{125}I -LDL–proteoglycan complex (Figure 1b). At all times, the fibrous-

Figure 1 Effect of macrophage maturation on (a) the degradation of various ligands and (b) cholesteryl-ester synthesis mediated by fibrous-plaque complex and LDL-proteoglycan complex

(a) Monocytes were cultured in RPMI-1640 medium containing 20% (v/v) human serum. At the indicated time, the cells were washed and incubated with ^{125}I -LDL (Δ), ^{125}I -LDL-proteoglycan complex (^{125}I -LDL-PG complex) ($\bullet$) or ^{125}I -acetyl-LDL ($\circ$) ($20 \mu\text{g}$ of cholesterol/ml) in the presence and absence of a 25-fold concentration of the corresponding unlabelled ligand. After 6 h at 37°C , the degradation of each ligand was assayed. Specific degradation was calculated by subtracting the degradation in the presence of excess unlabelled ligand from that in its absence. Each data point is the mean $\pm$ S.E.M. of eight different experiments. For LDL-proteoglycan complex and acetyl-LDL, degradation from day 4 was significantly greater than that of day 1 cultures ($P < 0.005$). (b) Monocytes were cultured as in (a). At the indicated time, cells were incubated in RPMI-1640 medium containing 0.2 mM [^{14}C]oleate-albumin and $100 \mu\text{g}$ of cholesterol/ml of fibrous-plaque (FP) complex ($\bullet$) or LDL-proteoglycan (LDL-PG) complex ($\circ$). After 16 h at 37°C , the cellular content of cholesteryl [^{14}C]oleate was determined. Each data point is the mean $\pm$ S.E.M. of six experiments. Cholesteryl ester synthesis from day 4 was significantly greater than that of day 1 cultures for both FP complex and LDL-PG complex ($P < 0.005$).

plaque complex was more potent than the *in vitro* LDL-proteoglycan complex in its ability to stimulate cholesteryl ester synthesis.

Figure 2 Stimulation of cholesteryl synthesis in macrophages by fibrous-plaque complex and LDL-proteoglycan complex as a function of ligand concentration

Monocyte-derived macrophages were cultured for 8 days and then incubated with 0.2 mM [^{14}C]oleate-albumin and the indicated concentrations of either fibrous-plaque complex or LDL-proteoglycan complex. After 16 h at 37°C , the formation of cholesteryl [^{14}C]oleate was determined. Each data point is the mean $\pm$ S.E.M. of three separate experiments.

Figure 2 shows the stimulation of cholesteryl ester synthesis by the *in vivo* and *in vitro* complexes in 8-day-old macrophage cultures as a function of ligand concentration. Both complexes stimulated cholesterol esterification in a dose-dependent manner. At all ligand concentrations, the *in vivo* fibrous-plaque complex was more potent than the *in vitro* LDL-proteoglycan complex. Similar results were obtained when the experiments were performed using macrophages cultured for 16 days (results not shown).

Cell density

We have previously reported that the degradation of LDL, acetyl-LDL and LDL-proteoglycan complex in mouse peritoneal macrophages decreased with increasing cell density [21]. Cell density has also been reported to influence LDL-receptor activity in rat aortic smooth-muscle cells [22], human fibroblasts [23], and bovine aortic endothelial cells [24], and scavenger-receptor activity in human monocyte-derived macrophages [25]. The effect of cell density on the metabolism of lipoprotein-proteoglycan complexes by human macrophages was investigated. The degradation of ^{125}I -LDL-proteoglycan complex increased significantly with increasing cell density (results not shown). In similar experiments, we found that the degradation of ^{125}I -acetyl-LDL also increased substantially with increasing cell density, while the degradation of ^{125}I -LDL increased only slightly (results not shown). Cholesteryl ester synthesis mediated by the *in vivo* fibrous-plaque complex and the *in vitro* LDL-proteoglycan complex also increased significantly with increasing cell density (results not shown). Once again, the fibrous-plaque complex produced a greater stimulation of cholesteryl ester synthesis than the LDL-proteoglycan complex.

To determine whether the difference between the fibrous-plaque complex and the LDL-proteoglycan complex in the stimulation of cholesterol esterification was due to a difference in

Table 1 Effect of preincubation with various ligands on the degradation of ^{125}I -LDL, ^{125}I -LDL-proteoglycan complex and ^{125}I -acetyl-LDL by macrophages

Macrophages were cultured for 8 days in RPMI-1640 containing 20% (v/v) human serum. Some of the cultures were then maintained in the same medium. Others were switched to either RPMI-1640 containing 5% (v/v) lipoprotein-deficient serum (LPDS; medium A) or medium A containing 200 μg of cholesterol/ml of LDL, LDL-proteoglycan complex (LDL-PG complex) or acetyl-LDL. After 24 h at 37 °C, the media were removed and the cells washed twice with RPMI-1640. The cells were then incubated with ^{125}I -LDL, ^{125}I -LDL-proteoglycan complex or ^{125}I -acetyl-LDL (20 μg of cholesterol/ml) for 6 h. Degradation of each ligand was then assayed. Each value is the mean $\pm$ S.E.M. of six cultures. * Significantly different from cells incubated in 20% serum; $P < 0.05$ to < 0.005 .

Ligand present during preincubation	Degradation ($\mu\text{g}/\text{mg}$ of protein)		
	^{125}I -LDL	^{125}I -LDL-proteoglycan complex	^{125}I -acetyl-LDL
5% LPDS	$2.2 \pm 0.3^*$	4.2 ± 0.4	4.9 ± 0.6
20% Human serum	1.3 ± 0.2	4.0 ± 0.5	4.6 ± 0.4
5% LPDS + LDL	$0.6 \pm 0.2^*$	3.9 ± 0.6	4.8 ± 0.6
(200 μg of cholesterol/ml)			
5% LPDS + LDL-PG complex	$0.4 \pm 0.3^*$	4.1 ± 0.4	4.5 ± 0.8
(200 μg of cholesterol/ml)			
5% LPDS + acetyl-LDL	$0.5 \pm 0.2^*$	4.0 ± 0.3	5.1 ± 0.7
(200 μg of cholesterol/ml)			

the degree of oxidation, we compared the extent of lipid peroxidation of the complexes. As we reported earlier [10], the degree of oxidation of the fibrous-plaque complex was 2.5-fold greater than that of the LDL-proteoglycan complex [thiobarbituric acid-reactive substance (TBARS): fibrous-plaque complex, 1.18 ± 0.15 nmol/mg of protein; LDL-proteoglycan complex, 0.47 ± 0.06 nmol/mg of protein, mean $\pm$ S.E.M.]. Incubation of the two complexes with macrophages for 6 h did not increase their TBARS content. In contrast, previously we have observed an increase in the TBARS value of both complexes during prolonged incubation with macrophages [15].

Lipoprotein cholesterol in medium

Studies were conducted to determine whether the lipoprotein cholesterol concentration of the culture medium affected the degradation of various ligands by macrophages. The cells were cultured for 8 days. Some of the cultures were then maintained in the same medium, while others were switched to either RPMI-1640 medium containing 5% (v/v) lipoprotein-deficient serum (medium A), or medium A containing 200 μg of cholesterol/ml of either LDL, LDL-proteoglycan complex or acetyl-LDL. After 24 h, the medium was removed and the cells washed twice with serum-free RPMI-1640. The degradation of ^{125}I -LDL, ^{125}I -LDL-proteoglycan complex and ^{125}I -acetyl-LDL (20 μg of cholesterol/ml of each ligand) was then assayed, following a 6 h incubation. The results are presented in Table 1. Compared with the degradation of ^{125}I -LDL by macrophages maintained throughout in medium containing 20% (v/v) serum, the degradation of the lipoprotein by cells preincubated in medium containing lipoprotein-deficient serum increased by 69% ($P < 0.001$). On the other hand, degradation of ^{125}I -LDL was depressed by 54%, 69% and 62% by preincubation in medium containing 200 μg of cholesterol/ml of LDL, LDL-proteoglycan complex and acetyl-LDL respectively ($P < 0.001$). In contrast, the degradations of ^{125}I -LDL-proteoglycan complex and ^{125}I -acetyl-LDL were unaffected by preincubations in medium devoid of, or containing, lipoproteins.

Macrophage-conditioned medium

It has been reported that macrophage-conditioned medium has a stimulatory effect on the degradation of LDL by vascular smooth-muscle cells and fibroblasts and acetyl-LDL degradation

in human monocytes [26,27]. Our earlier studies indicated that macrophage-conditioned medium also stimulated the degradation of LDL-proteoglycan complex in mouse peritoneal macrophages [21]. Therefore, we investigated the effect of macrophage-conditioned medium on the metabolism of lipoprotein-proteoglycan complexes by human monocyte-derived macrophages. Conditioned medium stimulated the degradation of ^{125}I -LDL-proteoglycan complex by macrophages in a dose-dependent manner (Figure 3a). Stimulation of degradation by the conditioned medium ranged between 1.7-fold and 4.3-fold. Degradation of LDL-proteoglycan complex by freshly isolated monocytes was also stimulated significantly by macrophage-conditioned medium, although the absolute values were much less than those observed with mature macrophages (results not shown). In cell-free incubations, macrophage-conditioned medium did not stimulate the degradation of LDL-proteoglycan complex. Cholesteryl ester synthesis that is mediated by the lipoprotein-proteoglycan complexes was also stimulated by incubating macrophages with increasing concentrations of the conditioned medium (Figure 3b). Conditioned medium also stimulated cholesterol esterification in freshly isolated monocytes (results not shown). Macrophage-conditioned medium had a significant stimulatory effect on the degradation of ^{125}I -acetyl-LDL by macrophages and a slight stimulatory effect on the degradation of ^{125}I -LDL (results not shown).

The macrophage-conditioned medium lost its ability to stimulate the degradation of LDL-proteoglycan complex in macrophages when it was boiled for 5 min. Similarly, digestion of the conditioned medium with trypsin and dialysis through a membrane with a molecular-mass cutoff of 25000 also resulted in the loss of stimulatory activity.

Macrophage stimulation

Activation of macrophages has been reported to influence their lipoprotein metabolism [21,28–31]. Therefore, we investigated the effect of macrophage activation on the degradation of ^{125}I -LDL-proteoglycan complex, ^{125}I -LDL and ^{125}I -acetyl-LDL. Macrophages were activated with PMA, which directly activates protein kinase C [32]. PMA suppressed the degradation of LDL-proteoglycan complex and acetyl-LDL in a dose-dependent manner (Figure 4a). PMA also decreased the degradation of

Figure 3 Stimulation of the degradation of ^{125}I -LDL–proteoglycan complex and cholesteryl ester synthesis mediated by the complex in macrophages by macrophage-conditioned medium

Macrophages were cultured for 8 days. The cells were washed and incubated in RPMI-1640 containing the indicated volumes of macrophage-conditioned medium or an equivalent volume of medium that had been incubated in the absence of cells. After 24 h: (a) ^{125}I -LDL–proteoglycan complex was added (20 μg of cholesterol/ml) and the incubation continued for an additional 6 h. Degradation of ^{125}I -LDL–proteoglycan complex was then assayed. (b) [^{14}C]Oleate–albumin (0.2 mM) and fibrous-plaque complex or LDL–proteoglycan complex (100 μg of cholesterol/ml of each) were added and the incubation continued for an additional 16 h. The amount of cholesteryl [^{14}C]oleate formed was then determined. Each bar in (a) represents the mean $\pm$ S.E.M. of eight experiments. Each data point in (b) is the mean $\pm$ S.E.M. of six different experiments. * $P < 0.05$; ** $P < 0.005$.

LDL (Figure 4a). Figure 4(b) shows that when macrophages were preincubated with 100 ng/ml of PMA and then incubated with increasing concentrations of ^{125}I -LDL–proteoglycan complex, the degradation of the complex was reduced by 36–40%, in comparison with the degradation of the complex by cells not exposed to PMA. Preincubation with 100 ng/ml of PMA also reduced the subsequent binding of ^{125}I -LDL–proteoglycan complex to macrophages by 46% (results not shown). Similarly, activation of macrophages significantly suppressed the cholesteryl ester synthesis mediated by both fibrous-plaque complex and LDL–proteoglycan complex (results not shown). Earlier, Leake et al. [28] reported that macrophage stimulation with PMA decreased the binding of ^{125}I -acetyl-LDL.

Since, as reported above, macrophage activation by PMA suppressed the degradation of ^{125}I -LDL–proteoglycan complex, we determined whether the conditioned medium from PMA-activated macrophages also decreased the degradation of the complex in macrophages. However, the conditioned medium showed the same stimulatory effect of ^{125}I -LDL–proteoglycan complex degradation as that observed with the conditioned medium prepared with unstimulated macrophages (results not shown). In contrast, conditioned medium from unstimulated and stimulated macrophages failed to stimulate the degradation of ^{125}I -LDL–proteoglycan complex in washed, PMA-stimulated macrophages (results not shown).

Figure 4 Effect of macrophage activation on the degradation of ^{125}I -LDL, ^{125}I -LDL–proteoglycan complex and ^{125}I -acetyl-LDL by macrophages

(a) Macrophages were cultured for 8 days. The cells were then incubated with the indicated concentrations of PMA. After 24 h, the cells were washed and incubated with ^{125}I -LDL, ^{125}I -LDL–proteoglycan complex or ^{125}I -acetyl-LDL (20 μg of cholesterol/ml of each) in the presence and absence of the corresponding unlabelled ligand (500 μg of cholesterol/ml). After 6 h, the degradation of each ligand was assayed and specific degradation calculated. Each data point is the mean $\pm$ S.E.M. of eight experiments. (b) This shows specific degradation of ^{125}I -LDL–proteoglycan complex by stimulated and unstimulated macrophages as a function of ligand concentration. Macrophages (8 days old) were incubated in the presence and absence of PMA (100 ng/ml). After 24 h, the cells were washed and incubated with the indicated concentrations of ^{125}I -LDL–proteoglycan complex in the presence and absence of unlabelled ligand (600 μg of cholesterol/ml). Degradation of the complex was assayed after 6 h and specific degradation calculated. Data represent the means $\pm$ S.E.M. of six experiments. All data points for PMA-treated cultures were significantly different from control values (no PMA); * $P < 0.05$; ** $P < 0.005$.

We also investigated the effect of macrophage activation with bacterial lipopolysaccharide (LPS), a very potent stimulator of macrophage activation, on the degradation of ^{125}I -LDL–proteoglycan complex and ^{125}I -acetyl-LDL. The range of LPS concentrations in these experiments is similar to that used by others [30]. As shown in Table 2, preincubation of macrophages

Table 2 Effect of bacterial LPS on the degradation of ^{125}I -LDL-proteoglycan complex and ^{125}I -acetyl-LDL by macrophages

Macrophages were cultured for 8 days. The medium was removed and the cells were incubated with or without the indicated concentrations of bacterial LPS. After 24 h, the cells were washed three times with RPMI-1640 and then incubated with ^{125}I -LDL-proteoglycan complex (^{125}I -LDL-PG complex) or ^{125}I -acetyl-LDL (20 μg of cholesterol/ml of each). After 6 h at 37 °C, degradation of the ligands was assayed. Values are the mean $\pm$ S.E.M. of four wells. Abbreviation: ND, not determined.

Concn. of LPS ($\mu\text{g}/\text{ml}$)	Degradation ($\mu\text{g}/\text{mg}$ of protein)	
	^{125}I -LDL-PG complex	^{125}I -Acetyl-LDL
0	4.1 $\pm$ 0.4	4.8 $\pm$ 0.5
2	4.2 $\pm$ 0.3	3.5 $\pm$ 0.2
5	4.0 $\pm$ 0.5	2.9 $\pm$ 0.3
10	3.8 $\pm$ 0.3	2.2 $\pm$ 0.3
20	4.1 $\pm$ 0.5	1.5 $\pm$ 0.2
30	3.3 $\pm$ 0.3	ND
50	2.8 $\pm$ 0.2	ND
100	2.0 $\pm$ 0.2	1.2 $\pm$ 0.2
200	1.7 $\pm$ 0.3	ND

with LPS at concentrations ranging from 2 to 20 $\mu\text{g}/\text{ml}$ had no effect on the degradation of LDL-proteoglycan complex. At concentrations above 20 $\mu\text{g}/\text{ml}$, LPS reduced the degradation of the complex in a dose-dependent manner. At 100 $\mu\text{g}/\text{ml}$ of LPS, the binding at 4 °C of ^{125}I -LDL-proteoglycan complex to macrophages was also inhibited by 38 % (results not shown). As reported by others [30], stimulation of macrophages by LPS produced a dose-dependent inhibition of the degradation of ^{125}I -acetyl-LDL. This inhibition was more than the inhibition of degradation of the ^{125}I -LDL-proteoglycan complex.

DISCUSSION

Our recent studies indicate that human monocyte-derived macrophages metabolize LDL-proteoglycan complexes predominantly via a receptor-mediated pathway that has properties similar to, but not identical with, the acetyl-LDL receptor [11]. The results of the present study show that several factors are capable of regulating the metabolism of lipoprotein-proteoglycan complexes in human macrophages. These include the stage of macrophage maturation, cell density, the lipoprotein cholesterol concentration of the culture medium, macrophage activation and macrophage-conditioned medium. Some of these factors are known to influence the metabolism of LDL and modified LDL in macrophages, smooth-muscle cells, endothelial cells and fibroblasts [21–25,28–31].

A significant increase in the degradation of acetyl-LDL and malondialdehyde-modified LDL, but not LDL, with maturation of monocytes into macrophages in culture has been reported [25]. We now report a similar dramatic increase in the degradation of LDL-proteoglycan complex and consequent cholesteryl ester synthesis with macrophage maturation. Throughout the study period the degradation of the complex continued to rise with maturation. In addition, degradation was not down-regulated by preincubation of macrophages with LDL, acetyl-LDL or LDL-proteoglycan complex. Taken together, these results suggest a dual role for the lipoprotein-proteoglycan-complex-binding sites in macrophages. First, they mediate the uptake of the complexes and thereby facilitate intracellular cholesteryl ester synthesis and accumulation. Secondly, due to the lack of down-regulation,

these binding sites mediate the unabated uptake of the complex and thus help maintain the foam cells in that state. Our recent observation [15] that foam cells isolated from rabbit atherosclerotic lesions metabolized lipoprotein-proteoglycan complexes via receptor-mediated endocytosis and consequently continued to accumulate cholesteryl ester, supports this concept.

Degradation of LDL-proteoglycan complex and synthesis of cholesteryl ester that is mediated by the *in vitro* and *in vivo* complexes increased substantially with increasing cell density. The results are in agreement with the studies of Fogelman et al. [25], who found a significant increase in scavenger-receptor activity in human monocyte-derived macrophages with increasing cell density. However, the results are inconsistent with our earlier observation in mouse peritoneal macrophages [21]. The degradation of LDL-proteoglycan complex by mouse cells decreased with increasing cell density. The reason for the discrepancy between the human and mouse cells is not clear but it may be due to the species difference.

Consistent with earlier reports on human and mouse macrophages [21,27], we found that macrophage-conditioned medium stimulated the degradation of acetyl-LDL by human monocyte-derived macrophages. In addition, the conditioned medium also significantly enhanced the degradation of LDL-proteoglycan complex. Furthermore, the conditioned medium stimulated cholesteryl ester synthesis mediated by the *in vivo* and *in vitro* lipoprotein-proteoglycan complexes. In view of the similarities between the binding sites for lipoprotein-proteoglycan complexes and acetyl-LDL in macrophages [11], it is plausible that the same factor in the conditioned medium stimulates the uptake of both ligands. Since macrophages secrete several products with varied functions [33], one or more of these secretory products in the conditioned medium could cause the observed effect. The stimulatory activity was lost during trypsin treatment, dialysis and boiling of the conditioned medium, suggesting that the active factor may be a heat-labile protein. Likely candidates include macrophage-derived growth factor and cytokines. Additional studies are needed to determine the identity of the macrophage secretory product that regulates the metabolism of lipoprotein-proteoglycan complexes.

Several studies have reported decreased scavenger-receptor activity associated with macrophage activation [28–31]. In these studies agents such as PMA, bacterial LPS, bacteria, zymosan, etc. were used to stimulate macrophages. The results described here show that activation of macrophages with PMA and bacterial LPS also causes a significant suppression of the degradation of LDL-proteoglycan complex. This is probably due to a reduction in the number of binding sites, as shown by a decrease in the binding of the complex to stimulated cells. However, the mechanism responsible for reduced receptor expression is not known. Even though macrophage activation increases cellular free cholesterol [34], this cannot account for the reduction in the number of binding sites for the LDL-proteoglycan complex because, as reported in this study, the binding sites for the complex are not down-regulated by cellular free cholesterol. Other probable mechanisms for the decrease in receptors on activated macrophages include an increased degradation or a decreased synthesis of the receptors as well as their inactivation [28].

Similar to the conditioned medium from unstimulated macrophages, the conditioned medium from PMA-stimulated cells was able to stimulate the degradation of LDL-proteoglycan complex by macrophages. Thus, the activated cells still secrete factors that stimulate LDL-proteoglycan complex degradation in unstimulated macrophages. However, the conditioned medium from unstimulated and stimulated macrophages could not reverse

the suppression of LDL–proteoglycan complex degradation in PMA-stimulated macrophages. Collectively, these results indicate that (1) direct stimulation of macrophages by PMA is required for the suppression of LDL–proteoglycan complex degradation, and (2) this suppression is irreversible.

In agreement with our earlier results [15], the *in vivo* fibrous-plaque complex was more effective than was the *in vitro* LDL–proteoglycan complex in stimulating cholesterol esterification in macrophages. As reported earlier [10], and confirmed here, the difference could be due partly to the increased oxidation of the fibrous-plaque complex compared with the *in vitro* LDL–proteoglycan complex. We have reported recently that although both complexes are further oxidized during prolonged incubation with macrophages, the extent of oxidation was greater for the *in vivo* complex compared with the *in vitro* complex [15]. This could also contribute to the higher potency of the fibrous-plaque complex. Also, unlike the *in vitro* complex, the fibrous-plaque complex contains heparin and fibronectin. We observed previously that heparin-containing lipoprotein complexes are more recognizable by macrophages than are complexes that lack this glycosaminoglycan [10]. Similarly Falcone and Salisbury [35] have reported that fibronectin stimulates the uptake of LDL–heparin–collagen complexes by macrophages.

In a previous paper it was reported that an increase occurred in the degradation of LDL–proteoglycan complex in mouse peritoneal macrophages when stimulated with thioglycollate [21]. This is in conflict with the current results in human monocyte-derived macrophages, which could be due either to species differences or a difference in the stimuli used in the two studies (thioglycollate versus PMA and bacterial LPS).

The current results, together with our earlier observations in mouse peritoneal macrophages [21], indicate that some of the factors influence the metabolism of lipoprotein–proteoglycan complexes in human and mouse macrophages in a similar manner. However, other factors, such as cell density and macrophage stimulation, have opposing effects on the two types of cells. Although the reasons for the discrepancy are not evident, this re-emphasizes the perils of extrapolating the results of the animal study to humans, and vice versa.

In summary, the results show that several factors regulate the metabolism of lipoprotein–proteoglycan complexes in human macrophages. Some of these factors might play a role in the pathophysiology of atherosclerosis by facilitating macrophage lipid accumulation.

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