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Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins

(cell surface receptors/atherosclerosis)

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ABSTRACT **Human low density lipoprotein (LDL) was incubated with an established line of rabbit aortic endothelial cells. Density gradient fractionation showed a time-, concentration-, and temperature-dependent increase in the average density of the LDL (from about 1.036 to as high as 1.070 g/ml). Incubation without cells or with other types of cultured cells (fibroblasts, hepatocytes, 3T3-L1 cells) caused no significant change in density. ^{125}I-Labeled LDL (^{125}I-LDL) recovered after incubation with endothelial cells (EC-modified LDL) was taken up and degraded 3 to 4 times more rapidly than control LDL by resident mouse peritoneal macrophages and by an established tumor line of mouse macrophages (J774 cells). Macrophage degradation of EC-modified ^{125}I-LDL exhibited saturation kinetics (>85% inhibited by excess unlabeled EC-modified LDL). Degradation was also inhibited by unlabeled acetylated LDL and, conversely, unlabeled EC-modified LDL inhibited degradation of acetylated ^{125}I-LDL. Incubation of LDL with conditioned medium removed from endothelial cell cultures modified neither its density nor its rate of degradation by macrophages. These studies show that endothelial cells have the potential to metabolically modify the LDL molecule, generating a form that is more rapidly degraded by macrophages and that is recognized by the macrophage receptor for acetylated LDL. This process may play a significant role in the pathogenesis of atherosclerosis.**

Low density lipoproteins (LDL) are strongly implicated in the pathogenesis of atherosclerosis. Much of the cholesterol accumulating in lesions derives from plasma LDL, the major carrier of plasma cholesterol. Arterial smooth muscle cells and endothelial cells take up and degrade LDL in part by adsorptive endocytosis mediated by the LDL receptor (1–4). Another prominent feature of atheromata are the lipid-laden foam cells, believed to arise in part from macrophages (5, 6). Mouse peritoneal macrophages degrade native LDL at a low rate and primarily by nonspecific pathways (7). However, certain chemically modified forms of LDL (derivatized by acetylation, acetoacetylation, or treatment with malondialdehyde) are degraded much more rapidly by macrophages via receptor-mediated pathways (7–9). Incubation of macrophages with acetylated LDL (acetyl-LDL) leads to gross accumulation of lipids and generation of cells with the general appearance of foam cells (7). To date it has not been possible to identify a biologically generated form of LDL that might behave like acetyl-LDL.

In preliminary experiments it was observed that human LDL after incubation in the presence of cultured human umbilical vein endothelial cells demonstrated a higher average density and greater mobility on agarose gel electrophoresis as compared to control LDL incubated in the absence of cells. The present studies show that human LDL incubated with an established line of cultured rabbit aortic endothelial cells is consistently converted to a form with increased density in a time- and temperature-dependent fashion. This biologically modified LDL is degraded by macrophages several times more rapidly than is native LDL. Its uptake and degradation is receptor-mediated and competitive with degradation of acetyl-LDL.

METHODS

Cell Cultures. Endothelial cells, an established line derived from rabbit thoracic aorta, were a gift from V. Buonassisi (Department of Biology, University of California, San Diego, CA). The general properties of these cells (10, 11) and their metabolism of lipoproteins (4) have been described. Cells were grown in F-10 medium (GIBCO) supplemented with 15% fetal calf serum (Irvine Scientific, Irvine, CA) and epidermal growth factor [gift of Gordon Sato (Department of Biology, University of California, San Diego)] at 10 ng/ml. Mouse resident peritoneal macrophages were obtained and cultured as described (12). J774 cells, an established line of macrophage-like cells (13), were a gift of J. Unkeless and H. Plutner (The Rockefeller University, New York). They were grown in suspension culture in α minimal essential medium (GIBCO) containing 5% heat-inactivated newborn calf serum. Cells were plated in 35 × 10 mm dishes (1.5×10^5 per dish) 2 days before use and covered 50–70% of the dish on the day of the experiment. The methods used for culturing human skin fibroblasts (14) and 3T3 mouse cells (15) have been described.

Lipoproteins. Human LDL (density = 1.019–1.063 g/ml) was prepared and radioiodinated as described (2), using carrier-free Na^{125}I (Amersham/Searle, Arlington Heights, IL). Lipoprotein-deficient human serum (LDS) was prepared as described (2).

Endothelial cell-modified LDL (*EC-modified LDL*) was prepared by incubating ^{125}I-labeled LDL (^{125}I-LDL) with endothelial cells at the indicated concentrations (generally 200 μg of protein per ml, rinsed twice) in 2 ml of F-10 medium (with or without LDS). At the end of the incubation the medium was removed sterilely, centrifuged to remove cells and debris, and stored at 4°C. *Control LDL* was prepared by incubating in the same way in the absence of cells. Acetyl-LDL was prepared by reaction with acetic anhydride as described by Basu *et al.* (16).

Degradation of ^{125}I-LDL. Medium containing EC-modified ^{125}I-LDL was diluted with fresh F-10 medium to obtain the desired final concentrations and incubated at 37°C with macrophages or fibroblasts (1.5 ml of medium per dish) for the times

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Abbreviations: LDL, low density lipoprotein; acetyl-LDL, LDL modified by acetylation with acetic anhydride; EC-modified LDL, LDL modified by previous incubation with cultured endothelial cells; LDS, lipoprotein-deficient serum.

indicated. Fibroblasts were incubated 20 hr in 5% human LDS before incubation with EC-modified ^{125}I-LDL. Dishes were placed on ice, medium was removed, and the cells were washed four times with cold phosphate-buffered saline and dissolved in 0.25 M NaOH for protein determination (17).

^{125}I-Labeled degradation products in the medium were determined by adding bovine serum albumin (1 mg/ml) and trichloroacetic acid (final concentration, 10%). Silver nitrate was added to the supernate (10 mg/ml) to precipitate free iodide (18). After centrifugation the supernate was drawn through a 0.22-μm-pore-diameter filter and ^{125}I was measured by using a Searle model 1197 automatic gamma system.

Some ^{125}I-labeled degradation products were produced during the preparation of EC-modified ^{125}I-LDL. To correct for this, equal concentrations of EC-modified ^{125}I-LDL were incubated in cell-free dishes parallel to the incubations with macrophages or fibroblasts. The noniodide, trichloroacetic acid-soluble ^{125}I in these control incubations was subtracted from the experimental values; it never exceeded 20% of the total.

Characterization of EC-Modified LDL. Medium containing EC-modified LDL was layered either at the top or at the bottom of a stepwise sucrose gradient (2 ml each at densities 1.20, 1.095, 1.075, 1.045, and 1.006 g/ml). Tubes were centrifuged for 48–64 hr at 170,000 × *g* at 12°C in a Beckman SW-41 swinging-bucket rotor. Tube contents were collected in 20 0.5-ml fractions by draining from the bottom and the density of each fraction was determined by refractometry. ^{125}I-LDL in each fraction was measured by adding carrier bovine serum albumin (1 mg/ml) and trichloroacetic acid (10%) and radioassaying the precipitate; less than 0.05% of ^{125}I was recovered in the supernates. In some experiments protein in each fraction was determined (17) after precipitating with trichloroacetic acid and deoxycholic acid (19).

Electrophoretic mobility was determined by agarose gel electrophoresis (20).

RESULTS

^{125}I-LDL previously incubated with rabbit aorta endothelial cells (EC-modified LDL) showed a clear increase in its average hydrated density (Fig. 1). The shift in density occurred either in the absence or in the presence of LDS. ^{125}I-LDL incubated under identical conditions in the absence of cells or in the presence of other cell types showed no significant shift in density. While the 48-hr centrifugation time should yield equilibrium density distribution, the possibility that the apparent increase in density might be due to changes in molecular shape or to other changes altering migration through the sucrose gradient had to be considered. However, the shift in density was similar whether the medium LDL was layered initially at the top of the gradient, as in Fig. 1, or at the bottom, as in Fig. 2. Moreover, LDL incubated with human umbilical vein endothelial cells and reseparated by sequential flotation in KBr showed a similar density shift.

The possibility that the shift in density of ^{125}I-LDL might not reflect distribution of LDL mass was tested. As shown in Fig. 2, the distribution of LDL protein in the density gradient paralleled that of ^{125}I-LDL. The overlap of the density distributions was minimal, and the width of the density distribution was similar for control and EC-modified LDL, suggesting that most of the LDL in the dish (400 μg total) was altered. Furthermore, the EC-modified LDL migrated as a single discrete band on agarose gel electrophoresis but with a mobility distinctly greater than that of control LDL. The relative mobility of EC-modified LDL increased with time of incubation and by 24 hr was twice that of control LDL. Other studies using only native unlabeled LDL showed similar results (data not shown).

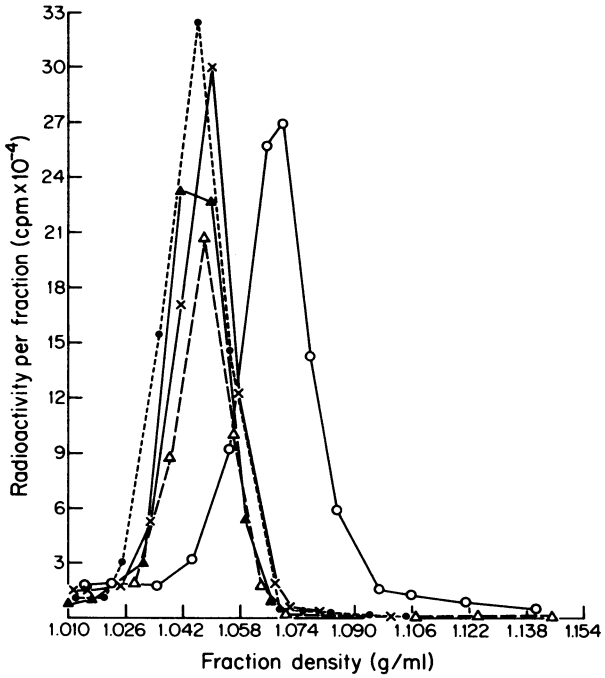

FIG. 1. Density gradient fractionation of ^{125}I-LDL after a 20-hr incubation in the absence of cells (●), with rabbit endothelial cells (○), with normal human skin fibroblasts (△), with fibroblasts from a patient with homozygous familial hypercholesterolemia (▲), or with mouse 3T3-L1 cells (×). Cells (about 400 μg of protein per dish) were incubated at 37°C in F-10 medium containing human ^{125}I-LDL (200 μg of protein per ml). At the end of the incubation, medium was centrifuged at low speed to remove cells and debris, and the supernate was layered over a sucrose density gradient and centrifuged for 48 hr. The gradient was divided into 20 0.5-ml fractions. Density and radioactivity precipitated by trichloroacetic acid were determined for each fraction.

The extent of the shift in LDL density was dependent on temperature, time, and concentration. After a 24-hr incubation, there was virtually no shift at 4°C, whereas at 37°C the peak of the distribution shifted from 1.036 to 1.061 g/ml. A shift in peak density (using 200 μg of LDL protein per ml) was evident as early as 4 hr, but the extent of the shift was greater at 10 hr and reached a maximum at 24 hr—i.e., there was no further increase at 48 hr (data not shown). When lower concentrations of LDL were used, the magnitude of the shift in density was even greater. For example, at 50 μg of LDL protein per ml the peak of the density distribution after a 48-hr incubation was shifted to 1.095 g/ml (data not shown).

The rate of degradation of EC-modified LDL was examined in normal resident mouse peritoneal macrophages and in J774 cells. As shown in Fig. 3, both types of macrophages degraded EC-modified LDL 3–4 times faster than LDL preincubated in the absence of cells (control LDL). The enhanced degradation of EC-modified LDL by macrophages was dependent on prior exposure of LDL to the endothelial cells themselves, because EC-conditioned medium devoid of LDL did not affect macrophage degradation of native ^{125}I-LDL. In contrast, normal human skin fibroblasts did not degrade EC-modified LDL more rapidly than native or control LDL (data not shown). The curvilinearity of the relationship between the concentration of EC-modified LDL and its degradation rate indicates that the uptake process includes a saturable component. Indeed, the degradation of EC-modified ^{125}I-LDL (6 μg/ml) by fresh resident mouse peritoneal macrophages was inhibited by more than 85% in the presence of excess unlabeled EC-modified LDL (115 μg/

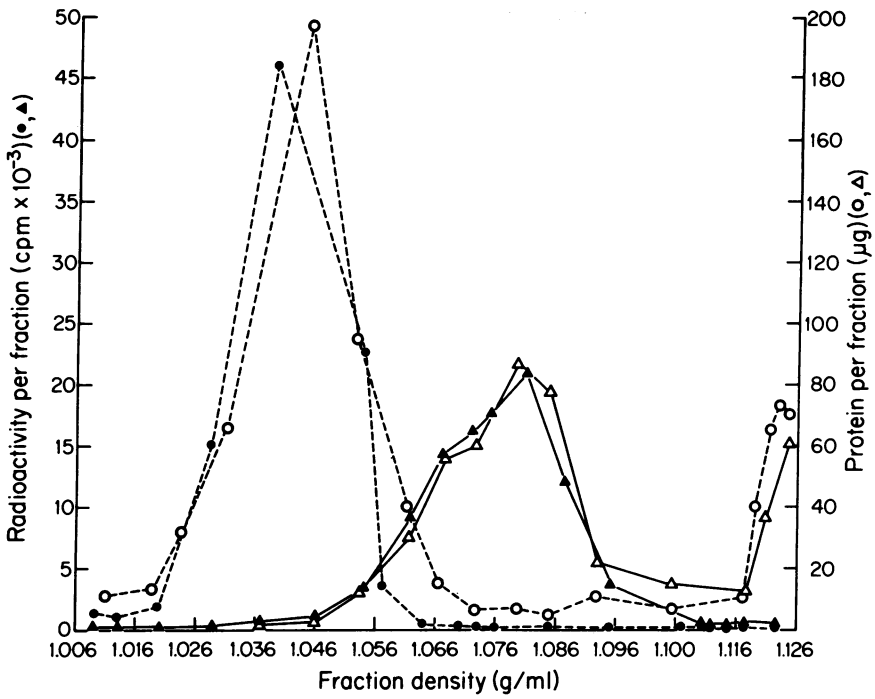

FIG. 2. Distribution of ^{125}I (closed symbols) and of total protein (open symbols) in a sucrose density gradient after incubation of ^{125}I-LDL (200 μg of protein per ml) in the absence of cells (circles) or in the presence of rabbit endothelial cells (triangles; about 400 μg of cell protein per dish). Methods were as described in the legend to Fig. 1 except that here the medium was initially layered at the *bottom* of the sucrose density gradient and centrifuged for 44 hr.

ml). It should be noted that the curve for control LDL was not strictly linear, implying that these macrophages express a small number of receptors for unmodified LDL.

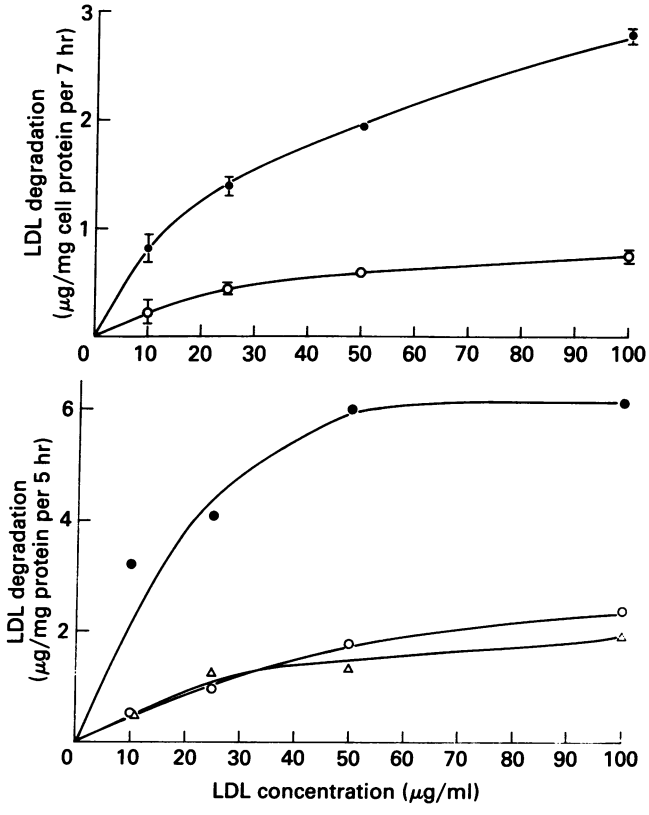

FIG. 3. Rates of degradation of ^{125}I-LDL by J774 cells (*Upper*) and by resident mouse peritoneal macrophages (*Lower*): ○, ^{125}I-LDL preincubated 24 hr in the absence of cells; △, ^{125}I-LDL not previously incubated; ●, ^{125}I-LDL previously incubated 24 hr with rabbit endothelial cells. At the end of the preincubation, medium was centrifuged to remove cells and debris and diluted with fresh F-10 medium to obtain the indicated concentrations of LDL. Degradation by macrophages was determined. Each point represents the mean of duplicate or triplicate determinations. Error bars indicate range.

J774 cells, like resident mouse peritoneal macrophages (7), took up and degraded acetyl-LDL by a saturable specific pathway and at a rate as much as 10 times that for uptake and degradation of native LDL (results to be reported in detail elsewhere). The degradation of EC-modified ^{125}I-LDL in J774 cells was strongly inhibited by unlabeled acetyl-LDL, whereas that of control ^{125}I-LDL was not markedly affected (Fig. 4). In other experiments, however, acetyl-LDL did inhibit degradation of native ^{125}I-LDL but to a lesser extent than that of EC-modified

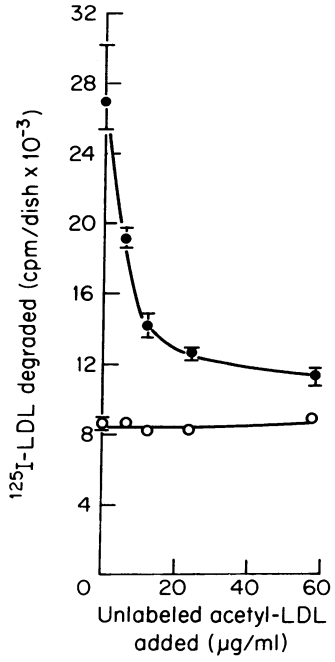

FIG. 4. Effect of increasing concentrations of unlabeled acetyl-LDL on the rates of degradation of different forms of ^{125}I-LDL (5 μg of protein per ml; specific activity, 206 cpm/ng) by J774 cells: ○, ^{125}I-LDL previously incubated 48 hr in the absence of cells; ●, ^{125}I-LDL previously incubated 48 hr with rabbit endothelial cells. Methods were as described in the legend to Fig. 3. Each point represents the mean of duplicate determinations.

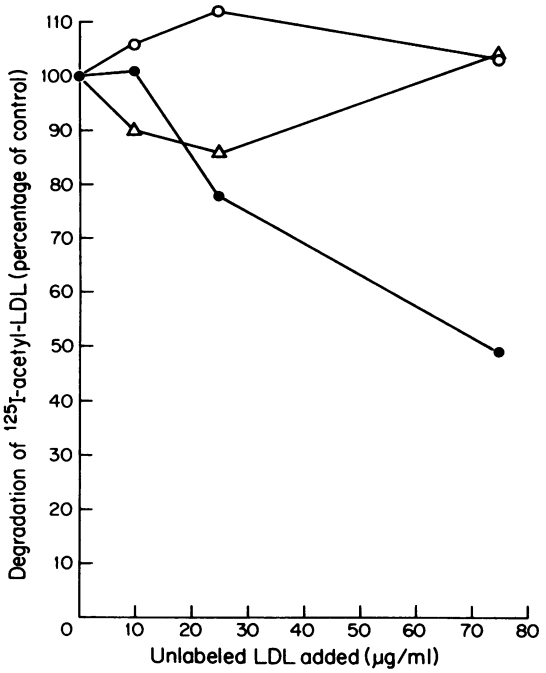

FIG. 5. Effect of increasing concentrations of various forms of unlabeled LDL on the degradation of ^{125}I-labeled acetyl-LDL (2 μg of protein per ml) by J774 cells: ○, unlabeled LDL previously incubated in the absence of cells; △, unlabeled LDL not previously incubated; ●, unlabeled LDL previously incubated with rabbit endothelial cells. Methods were as described in the legend to Fig. 3. Each point represents the mean of duplicate incubations. The degradation rate in the absence of additions was 842 ng/mg of cell protein per 7 hr.

^{125}I-LDL. Reciprocally, the degradation of acetyl-^{125}I-LDL was inhibited by unlabeled EC-modified LDL but not by unlabeled native or control LDL (Fig. 5).

DISCUSSION

These studies show that cultured endothelial cells have the capacity to induce a significant alteration in LDL structure and that such EC-modified LDL is degraded 3 to 4 times more rapidly than control LDL by macrophages. Plasma LDL is constantly exposed to endothelial cells and must normally traverse the endothelium before entering the subendothelial space. Thus, some fraction of the LDL in the subendothelial space may be in a form avidly taken up by developing foam cells and contribute disproportionately to acceleration of the atherogenic process.

The rate at which the cultured endothelial cells modified LDL structure was appreciable. The results suggest that the cells in a single dish (about 400 μg of protein) converted most of the LDL molecules in the medium (400 μg of LDL protein) to a denser form in 24 hr. The entire LDL peak in density gradients was shifted to the right without much change in width, indicating little change in the degree of heterogeneity, and overlap with the distribution of control LDL was minimal. Further, the EC-modified LDL migrated as a single discrete band on agarose gel electrophoresis. Still, the EC-modified preparations may include some unmodified LDL and LDL molecules modified to differing degrees. It is noteworthy that earlier studies showed that the rate at which these rabbit endothelial cells irreversibly degrade LDL presented at 100 μg/ml was less than 0.1 μg/hr per mg of cell protein (4); clearly much more LDL is modified to the higher density form than is degraded to products soluble in trichloroacetic acid.

The EC-modified LDL appears to be recognized and taken up by the acetyl-LDL receptor described by Goldstein *et al.* (7). The same receptor also recognizes acetoacetylated LDL (9). However, these chemically produced forms recognized by the acetyl-LDL receptor thus far do not have a naturally occurring counterpart. The present findings demonstrate a biologically generated form of LDL taken up specifically by the macrophage acetyl-LDL receptor.

Fogelman *et al.* (8) showed that in the presence of aggregating platelets LDL is converted to malondialdehyde-LDL. The latter was rapidly degraded via a specific receptor on monocyte-derived macrophages, but its degradation was not inhibited by acetyl-LDL. Later studies, however, did show competition with acetyl-LDL (21). We have tested our EC-modified LDL for malondialdehyde substitution, using the methods described by Fogelman *et al.* (8), and none was found. β-Migrating very low density lipoprotein, a lipoprotein accumulating in cholesterol-fed animals and in patients with dysbetalipoproteinemia, is also recognized specifically by macrophages but by a receptor distinct from the acetyl-LDL receptor.

The reason for the apparent higher density and increased electrophoretic mobility of EC-modified LDL remains to be determined. It could reflect binding to LDL of materials produced by the endothelial cells (e.g., proteins, glycosaminoglycans). However, incubation with medium removed from cultured cells failed to modify the LDL, suggesting that the result is not simply due to LDL binding of material normally secreted into the medium. It remains possible that LDL must be present to pull some ligand(s) away from the cell surface or that enzymes may be needed to effect the transfer or coupling. Blocking of amino groups by small molecules could effect the change in charge but seems unlikely to shift the density of the LDL. Another set of possibilities relates to losses of lipid from the LDL during incubation (e.g., hydrolysis of lipids or transfer from LDL to cells). Finally, we do not rule out the possibility that LDL molecules are taken into the cell by endocytosis, undergo some processing that changes their structure, and are then returned to the medium.

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