A Macrophage Receptor That Recognizes Oxidized Low Density Lipoprotein but Not Acetylated Low Density Lipoprotein*

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The formation of cholesterol-loaded macrophage foam cells in arterial tissue may occur by the uptake of modified lipoproteins via the scavenger receptor pathway. The macrophage scavenger receptor, also called the acetylated low density lipoprotein (Ac-LDL) receptor, has been reported to recognize Ac-LDL as well as oxidized LDL species such as endothelial cell-modified LDL (EC-LDL). We now report that there is another class of macrophage receptors that recognizes EC-LDL but not Ac-LDL. We performed assays of 0 °C binding and 37 °C degradation of 125I-Ac-LDL and 125I-EC-LDL by mouse peritoneal macrophages. Competition studies showed that unlabeled Ac-LDL could compete for only 25% of the binding and only 50% of the degradation of 125I-EC-LDL. Unlabeled EC-LDL, however, competed for >90% of 125I-EC-LDL binding and degradation. Unlabeled Ac-LDL was >90% effective against 125I-Ac-LDL; EC-LDL competed for about 50% of 125I-Ac-LDL binding and degradation. Copper-oxidized LDL behaved the same as EC-LDL in all the competition studies. Copper-mediated oxidation of Ac-LDL produced a superior competitor which could now displace 90% of 125I-EC-LDL binding. After 5 h at 37 °C in the presence of ligand, macrophages accumulated six times more cell-associated radioactivity from 125I-EC-LDL than from 125I-Ac-LDL, despite approximately equal amounts of degradation to trichloroacetic acid-soluble products, which may imply different intracellular processing of the two lipoproteins. Our results suggest that 1) there is more than one macrophage “scavenger receptor” for modified lipoproteins; and 2) oxidized LDL and Ac-LDL are not identical ligands with respect to macrophage recognition and uptake.

Macrophage-derived foam cells are an important feature of the early atherosclerotic lesion (1, 2). Native LDL† taken up through the apoB/E receptor is not an effective cholesterol donor for foam cell formation in vitro (8). Various modifications of LDL, however, produce lipoprotein particles that can cause foam cell formation from cultured macrophages. These modifications include acetylation (4), acetateacetylation (5), conjugation with malondialdehyde (6), and oxidative modification, whether induced by endothelial cells (7) or by copper ions (8). Each of these modified forms of LDL is taken up at least in part by way of the so-called scavenger receptor, or Ac-LDL receptor, of macrophages (9, 10). Scavenger receptors have been found on the surface of mouse peritoneal macrophages, human monocyte-derived macrophages, rat liver sinusoidal endothelial cells (10, 11), and bovine aortic endothelial cells (12).

In our studies of endothelial cell modification of LDL, we have shown that this complicated process is oxidative in nature (13) and can be mimicked by copper ions in tissue culture medium (8). We have recently shown that the oxidative modification of LDL can be also be accomplished by incubation with two purified enzymes: soybean lipoproteinase and phospholipase A2 (14). Results we obtained while characterizing the receptor specificity of enzymatically modified LDL (14) led us to re-evaluate the recognition of EC-LDL by the mouse peritoneal macrophage scavenger receptor. In this paper we present evidence that implies the existence of at least two classes of saturable receptors for EC-LDL: one that recognizes both EC-LDL and Ac-LDL, and another that recognizes EC-LDL but not Ac-LDL.

MATERIALS AND METHODS

Cells and Culture Media—Cell culture media and supplies were from Gibco Laboratories. A line of rabbit aortic endothelial cells was obtained from Dr. Vincent Buonassisi (15) and maintained in Ham's F-10 medium containing 15% fetal bovine serum, 10 ng/ml epidermal growth factor (Collaborative Research Inc., Lexington, MA), and 0.05 mg/ml gentamycin. Cells were used at confluence and were washed three times in F-10 medium prior to addition of LDL. Mouse resident peritoneal macrophages were isolated by peritoneal lavage, and 5–8 × 10⁶ cells were plated into each well of 24-well dishes in RPMI 1640 medium containing 10% fetal bovine serum, 50 μg/ml gentamycin, and 2.5 μg/ml Fungizone. For experiments involving time courses, approximately 15 × 10⁶ cells were plated in 60-mm dishes. After overnight incubation, nonadherent cells were removed by washing three times with Dulbecco's modified Eagle's medium.

Lipoprotein Isolation and Modification—LDL was isolated from the plasma of normal volunteers in the density range 1.019–1.063 by ultracentrifugation as described (16). EDTA (0.01%) was added to the syringe prior to blood drawing and was then present throughout the isolation and dialyses to prevent oxidation. LDL was radioiodinated using carrier-free Na125I (Amersham Corp.) and IODO-GEN (16). The specific activity of the 125I-LDL was approximately 200 cpm/ng. LDL concentrations are given as mass of protein/ml. When modified 125I-LDL was required, the iodination was always done prior to the modification. LDL was acetylated using acetic anhydride as described by Goldstein et al. (4) except that butylated hydroxytoluene was added to 0.02 mM to prevent oxidation of the lipoprotein. The extent of modification of LDL lysines by acetylation was determined as described (17); for the Ac-LDL preparations used in this work the mean ± S.D. of the degree of modification was 80% ± 4.7. EC-LDL was produced by incubating LDL (100 μg/ml) with confluent endothelial cells in Ham's F-10 medium for 24 h. In most cases EC-LDL was resisolated by ultracentrifugal flotation and then dialyzed and

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†The abbreviations used are: LDL, low density lipoprotein; Ac-LDL, acetylated LDL; EC-LDL, endothelial cell-modified LDL; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
sterilized prior to use. Essentially the same results were obtained whether or not the EC-LDL particles were reisolated.

**Cellular Binding and Degradation Assays**—Macrophages cultured as described above were washed three times with Dulbecco’s modified Eagle’s medium and then used immediately. Degradation assays of 125I-labeled lipoproteins were performed in Dulbecco’s modified Eagle’s medium containing human lipoprotein-deficient serum (1 mg of protein/ml). The cells were incubated for 5 h at 37°C, and then the medium was assayed for trichloroacetic acid-soluble, silver nitrate-soluble radioactivity as described (18). Control incubations in wells containing no cells were performed, and these control values were subtracted from the experimental values. The macrophage protein in the wells was determined after dissolving the cells in 0.2 N NaOH. Data are expressed as micrograms of apoB degraded during the 5 h per mg of macrophage protein. Assays of the binding of 125I-labeled lipoproteins to the surface of macrophages were performed at 0°C on ice in Dulbecco’s modified Eagle’s medium containing 20 mM HEPES, pH 7.4, and 1 mg/ml human lipoprotein-deficient serum, but free of bicarbonate. The cells were precooled for 30 min prior to the start of the assay. After 4 h of incubation with the 125I-lipoproteins, the medium was removed, and the cells were washed three times with phosphate-buffered saline containing 1 mg/ml albumin. The last wash was left on the cells for 10 min following which the cells were washed rapidly with phosphate-buffered saline and then dissolved in 0.2 N NaOH. The radioactivity in the dissolved cells was determined, and then the total protein was measured by the modified “Lowry” method described by Peterson (19). Data are expressed as nanograms of apoB bound per mg of macrophage protein. When competition studies were performed, the 125I-labeled ligands and the unlabeled competitors were preincubated with medium and human lipoprotein-deficient serum prior to incubation with the cells. Most experiments were performed with duplicates of each point; the duplicates were usually less than 10% different. The absolute amounts of binding and degradation of 125I-Ac-LDL and 125I-EC-LDL varied somewhat from preparation to preparation; the data shown are entirely representative of our results.

**RESULTS**

**Saturation Analysis of Binding and Degradation**—Recognition of Ac-LDL and EC-LDL by mouse peritoneal macrophages was studied by performing binding assays at 0°C and degradation assays at 37°C. Saturation curves for both assays and both ligands are presented in Fig. 1. Binding constants were determined from the data in Fig. 1B by computer-aided nonlinear regression analysis in which the degree of nonspecific binding was treated as one of the variables (20). The constants obtained were as follows (value ± asymptotic S.D.).

For EC-LDL, the total specific binding capacity was 1493 ± 168 ng of LDL protein bound per mg, and the dissociation constant was 3.3 ± 0.8 μg/ml; for Ac-LDL, the total specific binding capacity was 671 ± 66 ng of LDL protein bound per mg, and the dissociation constant was 4.8 ± 0.9 μg/ml.

It should be noted from Fig. 1 that macrophage degradation of 125I-Ac-LDL was more rapid than that of 125I-EC-LDL. However, macrophage binding of 125I-Ac-LDL was less than that of 125I-EC-LDL (Fig. 1B). Possible reasons for this difference are discussed below.

**Competition Analysis of Binding and Degradation**—To determine the extent to which Ac-LDL and EC-LDL are degraded by the same receptor, competition experiments were performed. Both binding and degradation were measured for both 125I-Ac-LDL and 125I-EC-LDL. Unlabeled Ac-LDL, EC-LDL, and native LDL were tested for their ability to compete for the binding and degradation of the labeled ligands. The results are presented in Fig. 2.

Fig. 2A shows that the binding of 125I-Ac-LDL was effectively inhibited by either unlabeled Ac-LDL or unlabeled EC-LDL and to about the same extent. Fig. 2B shows that the degradation of 125I-Ac-LDL was similarly inhibited by either Ac-LDL or EC-LDL. Ac-LDL was somewhat more effective than EC-LDL in both cases.

The results with 125I-EC-LDL as the ligand, however, were strikingly different. Unlabeled EC-LDL at a 60-fold excess reduced the total binding of 125I-Ac-LDL by 93%, showing that almost all the binding is saturable. In contrast, unlabeled Ac-LDL could only compete for approximately 25% of the total binding of 125I-EC-LDL even at a 125-fold excess (Fig. 2C). Degradation assays of 125I-EC-LDL gave similar results, i.e. unlabeled EC-LDL competed for 94% of the total degradation, whereas unlabeled Ac-LDL only competed for 50% of the total (Fig. 2D). In all cases, native LDL was an ineffective competitor (Fig. 2, A–D). These results show that for both ligands tested, greater than 90% of both the binding and degradation measured at 4 μg/ml is saturable since the cognate ligand competed for more than 90% of the total.

In a similar series of experiments, copper-oxidized LDL (8) proved to be comparable to EC-LDL as a ligand using all the same criteria (data not shown). Thus, the results relate to oxidized LDL in general and exclude the possibility that proteins secreted by the endothelial cells play any role in generating the new receptor recognition site(s).

The data in Fig. 2 are consistent with the following hypothesis: there exist at least two classes of macrophage receptors that bind EC-LDL. One receptor, the Ac-LDL receptor, binds both Ac-LDL and EC-LDL. The other receptor (which will be referred to here provisionally as the “oxidized LDL receptor”) binds EC-LDL but not Ac-LDL.

**Comparison of Macrophage Binding at 0 and 37°C**—Above we referred to the apparent anomaly in the data of Fig. 2, C

![Graph](image-url)
and D, i.e. unlabeled Ac-LDL inhibited 53% of the 37 °C macrophage degradation of 125I-EC-LDL (Fig. 2D) but only 26% of the 0 °C macrophage binding of 125I-EC-LDL (Fig. 2C). The possibility was considered that this difference was caused by a change in receptor behavior between 0 and 37 °C. Therefore, as shown in Table I, the macrophage binding of 125I-EC-LDL and its competition by unlabeled Ac-LDL were measured at both temperatures. To prevent significant internalization of the ligands at 37 °C, ligand was incubated with the cells for only 10 min. The results presented in Table I show that the total binding of 125I-EC-LDL was slightly lower at 37 °C than at 0 °C, and the percentage competed by unlabeled Ac-LDL was greater. Very similar results were obtained from measurements of 37 °C binding after only 5 min of incubation. Taken together, the data of Fig. 2 and Table I imply that at 0 °C approximately 25% of the total binding of 125I-EC-LDL is to the Ac-LDL receptor, whereas at 37 °C that fraction is approximately 50%. The latter value agrees with the results of Fig. 2D which show that unlabeled Ac-LDL competed for about half of the degradation of 125I-EC-LDL.

**Table I**

Comparison of 125I-EC-LDL macrophage binding at 0 and 37 °C

Macrophage binding of 4 μg/ml 125I-EC-LDL was measured at 0 °C after 4 h of incubation as described under "Materials and Methods" or at 37 °C after 10 min of incubation followed by the standard washing procedures. Numbers given are means ± 1 S.D. (n = 3).

<table>
<thead>
<tr>
<th>Unlabeled lipoprotein added</th>
<th>Binding of 4 μg/ml 125I-EC-LDL</th>
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<tbody>
<tr>
<td></td>
<td>0 °C</td>
</tr>
<tr>
<td></td>
<td>37 °C</td>
</tr>
<tr>
<td>None</td>
<td>743 ± 5 (100)</td>
</tr>
<tr>
<td>100 μg/ml Ac-LDL</td>
<td>567 ± 23 (76)</td>
</tr>
<tr>
<td>100 μg/ml EC-LDL</td>
<td>45 ± 1 (6)</td>
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**Fig. 2.** Competition of macrophage binding and degradation of 125I-Ac-LDL and 125I-EC-LDL by unlabeled LDL, Ac-LDL, and EC-LDL. Competition studies were performed using 4 μg/ml of either 125I-Ac-LDL (A and B) or 125I-EC-LDL (C and D) for either binding (A and C) or degradation (B and D) assays. The unlabeled lipoproteins Ac-LDL (A), EC-LDL (D), or native LDL (E) were added at the indicated concentrations. Each point is the mean of duplicate determinations.

Macrophage Receptor for Oxidized LDL
The rate of cellular uptake of EC-LDL is underestimated by Ac-LDL appears to be degraded more rapidly than $^{125}$I-EC-LDL. After washing with phosphate-buffered saline containing $0.2 \text{ mg/ml}$ albumin, once with phosphate-buffered saline alone, and finally dissolved in $0.2 \text{ N NaOH}$, the radioactivity associated with the dissolved cells was measured and assayed for degradation products as described under "Materials and Methods." The cells were washed once with medium, once with phosphate-buffered saline containing $1 \text{ mg/ml}$ albumin, and finally dissolved in $0.2 \text{ N NaOH}$. The radioactivity associated with the dissolved cells was measured and then total cell protein determined.

LDL in the endosomal and lysosomal compartments of the cells. Similar data for Ac-LDL have been reported previously (4). In dramatic contrast, however, the macrophages incubated with $^{125}$I-EC-LDL showed a steadily increasing amount of cell-associated radioactivity (Fig. 3B). After 5 h, the ratio of degradation products in the medium to cell-associated radioactivity was 8.1 for $^{125}$I-Ac-LDL and only 1.4 for $^{125}$I-EC-LDL. For both ligands, more than 95% of the cell-associated radioactivity was acid-precipitable when the cells were dissolved in detergent at neutral pH. These data show that $^{125}$I-Ac-LDL appears to be degraded more rapidly than $^{125}$I-EC-LDL partly because a smaller percentage of the $^{125}$I-EC-LDL internalized by the cell is degraded to trichloroacetic acid-soluble products during the standard assay. In other words, the rate of cellular uptake of EC-LDL is underestimated by measuring only the degradation products in the medium.

**Oxidation of Ac-LDL Creates a Ligand That Competes More Effectively against $^{125}$I-EC-LDL**—To determine whether oxidation of Ac-LDL would produce a ligand that would compete more effectively against $^{125}$I-EC-LDL, we performed the following experiment. Ac-LDL ($0.2 \text{ mg/ml}$) was incubated for 24 h at $37 \text{ C}$ in F-10 medium in the presence of either $20 \text{ µM}$ copper acetate (Oxidized Ac-LDL) or $50 \text{ µM}$ EDTA (Control Ac-LDL). Native LDL was similarly oxidized with copper (Oxidized LDL). The extent of copper-mediated oxidation of Ac-LDL and native LDL was identical as judged by the level of thiobarbituric acid-reactive substances produced in the two incubations (53 nmol/mg apoB). Macrophage binding of $4 \text{ µg/ml}$ $^{125}$I-EC-LDL was measured in the presence or absence of unlabeled lipoproteins as follows: untreated Ac-LDL (A), control Ac-LDL (B), oxidized LDL (C), and oxidized Ac-LDL (D). Each point is the mean of duplicate determinations.

**DISCUSSION**

Both Ac-LDL and oxidized LDL have been reported to be recognized by the scavenger receptor (9). We have reinvestigated the ligand-binding properties of the mouse peritoneal macrophage scavenger receptor, and we show that unlabeled copper-oxidized Ac-LDL is a much more effective competitor for the binding of $^{125}$I-EC-LDL than is nonoxidized Ac-LDL.

**Polyinosinic Acid and Maleylated Albumin Compete against the Degradation of $^{125}$I-EC-LDL**—The Ac-LDL receptor is almost completely blocked by a variety of polyanionic compounds, including polyinosinic acid and maleylated albumin (3). These two compounds were tested for their ability to block macrophage degradation of $^{125}$I-EC-LDL and were found to be comparably effective (Fig. 5). Thus the apparent second receptor for EC-LDL, like that for Ac-LDL, is positively charged and blocked by polyanions.
Ac-LDL competes only partially against the binding and degradation of $^{125}$I-EC-LDL. In contrast, unlabeled EC-LDL competes effectively and almost completely against both the binding and degradation of $^{125}$I-EC-LDL and the binding and degradation of $^{125}$I-Ac-LDL. Our evidence is consistent with the hypothesis that mouse peritoneal macrophages have at least two classes of receptors that recognize modified LDL: 1) an acetyl-LDL receptor which recognizes both Ac-LDL and oxidized LDL with similar affinities; and 2) an oxidized LDL receptor which recognizes oxidatively modified LDL but not Ac-LDL. We cannot, of course, rule out the possibility that there are more than two classes of receptors involved. Oxidized LDL is a heterogeneous ligand, and therefore different receptors may recognize different epitopes present in the population of oxidized LDL particles. In this view, acetylation of LDL produces a ligand that mimics only a fraction of the various epitopes of EC-LDL.

It might be suggested that the incomplete competition by unlabeled Ac-LDL against $^{125}$I-EC-LDL is due to a lower affinity of the common receptor for Ac-LDL. There are two arguments against this. First, mathematical analysis of the data of Fig. 1 indicates similar affinities for the two ligands. Second, and more important, the competition curves for unlabeled Ac-LDL against $^{125}$I-EC-LDL reach a plateau level after an initial steep phase. If the ineffectiveness of Ac-LDL were due to a lower affinity, the competition curve should show a steady decline and not a plateau.

Our laboratory has previously reported that EC-LDL is degraded by the Ac-LDL receptor (9). In that study and in subsequent papers (21, 22) emphasis was placed on the recognition by the Ac-LDL receptor and the fact that the competition was incomplete was neglected. The data, however, are not inconsistent with the present data. Fig. 2 shows that unlabeled Ac-LDL competes for 53% of the degradation of $^{125}$I-EC-LDL; we have previously reported values of 57% (9), 60% (21), and 69% (22). The significance of this partial competition was not previously appreciated. The competition of oxidized LDL species for EC-LDL has been variable in different reports (compare the present work with Refs. 9 and 14). This is probably due to the complex and variable nature of the oxidation process. The critical test of competition, however, is the extent of competition at high levels of competitor; when this has been determined, the consistent answer has been that EC-LDL competes well against Ac-LDL, but Ac-LDL can only compete partially for EC-LDL.

There are alternative models that could explain our findings of incomplete competition by Ac-LDL against $^{125}$I-EC-LDL binding. A single receptor molecule could have multiple binding sites with more sites available for EC-LDL than for Ac-LDL. Another possibility is that the mouse peritoneal macrophages that we used are a heterogeneous population and that differences among cells explain the partial competition against $^{125}$I-EC-LDL. Previous work, however, has shown that greater than 80% of the macrophages in culture become foam cells during incubation with Ac-LDL (23), implying that the vast majority of the cells express the Ac-LDL receptor.

Although Ac-LDL can compete for only a portion of the macrophage binding of $^{125}$I-EC-LDL, oxidation of Ac-LDL produces a lipoprotein that can inhibit essentially all of the binding of $^{125}$I-EC-LDL (see Fig. 4). This implies that there exist recognition epitopes on EC-LDL that are not present on Ac-LDL but that can be produced by oxidizing Ac-LDL. For example, conjugation of lysine amino groups (24) with some of the many fatty acid fragments generated during oxidation (25) may create epitopes recognized differently than is apoB substituted only with acetyl groups. Another possibility is that oxidation affects amino acid residues in addition to lysine. Thus, amino acid analysis of oxidized LDL has shown that there is a loss of histidine residues (18); the oxidation process that destroys the histidines may also generate the epitope necessary for recognition by the receptor for oxidized LDL. Alternatively, scavenger receptor recognition may depend on the exposure of critical site(s) on apoB that are normally hidden, and oxidation exposes more or different sites than does acetylation.

During uptake and degradation of lipoproteins by cells there is usually a small steady-state level of intact lipoprotein that is cell-associated (4). We found such a small pool during the degradation of $^{125}$I-Ac-LDL. The size of this pool was, however, dramatically larger for $^{125}$I-EC-LDL (Fig. 3). There are a number of models that could explain this observation. For example, $^{125}$I-EC-LDL may be degraded more slowly (or less completely) by lysosomal enzymes, leading to a larger pool of intracellular material. Alternatively, oxidized LDL may interact with some endosomal or lysosomal component and thus disrupt the normal processing pathway; this could be mediated by the abnormal oxidized lipids of the particle. Deleterious effects of oxidation on macrophage endocytotic receptors have been reported (26). Whatever the mechanism, the inability of cells to degrade oxidized LDL rapidly after internalization may be important both to the observed cytotoxicity of oxidized LDL (27) and also to the fact that EC-LDL, but not Ac-LDL, induces ceroid formation in mouse peritoneal macrophages (28).

Macrophages and liver sinusoidal endothelial cells have been reported to express a number of distinguishable receptors for modified proteins of various sorts. Sinusoidal cells have an Ac-LDL receptor (11) that also recognizes maleylated albumin (29), although there are additional binding sites for maleylated albumin not blocked by Ac-LDL (29). The data were interpreted as evidence for a complex receptor with more sites for maleylated albumin than for Ac-LDL. Sinusoidal cells also express a receptor specific for aldehyde-conjugated albumin, and this receptor is believed to be distinct from the Ac-LDL receptor (30). These two receptors share the property of being almost completely inhibited by polyinosinic acid (10, 30), a finding similar to our present findings with respect to the receptor for Ac-LDL and oxidized LDL. Human monocyte/macrophages have two different receptors for maleylated albumin, one of which recognizes malondialdehyde-conjugated LDL and one which does not (31). The "maleylated albumin receptor" is not blocked by polyinosinic acid but is blocked by bovine α-casein (31).

It appears that there may be a collection of "scavenger receptors" that take up various modified proteins. Thus it may be preferable to use the term "Ac-LDL receptor" rather than scavenger receptor. The fact that many of these receptors are blocked by polyanions such as polyinosinic acid may mean that they share some structural similarities; perhaps there is a basic structure that is shared and a variable binding or "active site" region in analogy with antibodies. Perhaps the role of these receptors is to recognize and assist in the degradation of aberrant or damaged proteins. Thus the scavenger receptors present on liver sinusoidal endothelial cells clear damaged proteins from the plasma, whereas macrophage scavenger receptors clear them from the extracellular matrix. The observation that monocytes express increased scavenger receptors as they differentiate into macrophages (32) is evidence that phagocytes require scavenger receptors in tissue but not in blood. The removal of oxidized lipoproteins might protect the endothelium from their demonstrated toxicity (27) and prevent thrombosis.
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REFERENCES