

Mechanism of Uptake of Copper-oxidized Low Density Lipoprotein in Macrophages Is Dependent on Its Extent of Oxidation*

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Several investigators have reported nonreciprocal cross-competition between unlabeled acetyl low density lipoprotein (LDL) and oxidized LDL for the degradation of the corresponding labeled LDLs. The failure of acetyl LDL to compete fully for the degradation of oxidized LDL has been interpreted as evidence for additional receptor(s) specific for oxidized LDL. In the present study, it is demonstrated that the ability of oxidized LDL to compete for the degradation of acetyl LDL is determined largely by its extent of oxidation. Extensively oxidized LDL competed for 90% of acetyl LDL degradation in murine macrophages, and hence there appears to be no pathway in these cells that is specific for acetyl LDL but not oxidized LDL. The reciprocal situation (competition by acetyl LDL for uptake and degradation of oxidized LDL) proved to be more complicated. Oxidized LDL is known to be susceptible to aggregation, and less than half of the aggregates found in the present experiments were large enough to be removed by filtration or centrifugation at $10,000 \times g$. When oxidized LDL was prepared under conditions that resulted in minimal aggregation, acetyl LDL competed for greater than 80% of oxidized LDL degradation. With more extensive oxidation and aggregation of LDL, acetyl LDL only competed for about 45% of oxidized LDL degradation, while polyinosinic acid remained an effective competitor. Individual preparations of oxidized LDL that differed in degree of oxidation were separated into aggregated and nonaggregated fractions, and it was shown that both fractions were competed to a similar degree by acetyl LDL in mouse peritoneal macrophages and in Chinese hamster ovary cells transfected with human scavenger receptor type I cDNA. Hence, aggregation by itself did not alter the apparent rate of uptake by the scavenger receptor pathway. These results indicate that the extent of oxidation of LDL affects its mechanism of uptake and that about half of the uptake of very extensively oxidized LDL appears to be via a pathway distinct from the scavenger receptor type I/II. The uptake of very extensively oxidized LDL was not affected by cytochalasin D, an inhibitor of phagocytosis. As well, it was not affected by an antibody to CD36 in human monocyte-derived macrophages or in THP-1 cells, suggesting that this alternate pathway does not involve CD36.

potentially atherogenic actions and properties (1–12). There is considerable evidence that at least a mild degree of oxidation of LDL occurs in arterial lesions *in vivo* (13–17). The possibility that oxidation of LDL might play a causal role in atherogenesis was suggested by several studies showing that antioxidant drugs can slow the progression of atherosclerosis in experimental animals (18–21).

One of the properties associated with extensively oxidized LDL is the ability to interact with scavenger receptors on phagocytic cells (22, 23). It has been postulated that this could lead to unregulated delivery of LDL to macrophages, resulting in the formation of foam cells (1). Scavenger receptor cDNA of bovine, murine, rabbit, and human origin have been cloned and sequenced (24–30). The scavenger receptor has six structural domains, including a collagen-like domain (V) that is believed to mediate ligand binding. The human scavenger receptor gene is located on chromosome 8, and two forms of the receptor (termed type I and type II) are produced from this gene through alternate splicing of mRNA (31). The cysteine-rich extracellular domain VI is deleted in the type II receptor, without evident effect on binding of most ligands (32), although recently it has been shown that bacterial lipopolysaccharide binds less well to type I than to type II receptors (30). Various substances that are structurally unrelated except that they have domains with high negative charge bind to the receptor, including polyinosinic acid, fucoidan, dextran sulfate, maleylated albumin, and acetyl LDL (33). This suggests that ligand binding may be mediated principally by ionic interactions. Freeman and co-workers (28) found that although both acetyl LDL and oxidized LDL were internalized and degraded by Chinese hamster ovary cells transfected with type I or type II bovine scavenger receptors, and acetyl LDL competed efficiently for degradation of oxidized LDL, oxidized LDL did not compete well for acetyl LDL degradation. Very similar results were reported by De-jager and colleagues (34) in both scavenger receptor-transfected Chinese hamster ovary cells and phorbol ester-treated rabbit smooth muscle cells. It was proposed that the failure of oxidized LDL to compete for acetyl LDL degradation might reflect nonidentical binding sites for the two ligands on the same receptor molecule (28). Additional evidence for this was provided by Doi and colleagues (35), who analyzed the effect of deletion mutations on the human type I scavenger receptor, and concluded that a cluster of lysine residues within the C-terminal 22 amino acids of the collagen-like domain of the receptor were essential for ligand binding. There were differences in sensitivity to individual lysine mutations between acetyl LDL and oxidized LDL, consistent with the notion that the binding sites for these ligands may not be identical.

Several groups have reported evidence of a receptor for oxidatively modified LDL distinct from the scavenger receptor on

Oxidatively modified LDL¹ has been shown to have many

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¹ The abbreviations used are: LDL, low density lipoprotein; CHO,

Chinese hamster ovary; PBS, phosphate-buffered saline; diI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate.

macrophages (36–38) or Kupffer cells (39–41). In several of these studies, the evidence for multiple receptors was the finding that acetyl LDL was able to compete for only about 40% of oxidized LDL uptake (36, 37, 39, 40). More direct evidence for a separate receptor for oxidized LDL was the finding that in rats, intravenously injected oxidized LDL was cleared preferentially by Kupffer cells whereas acetyl LDL was cleared by sinusoidal endothelial cells (39). However, there are several potential explanations for this observation, including the possibility that aggregation affecting only the oxidized LDL preparations in this study might have caused increased Kupffer cell uptake. Endemann and colleagues (38, 42) undertook the identification of new receptors for oxidized LDL using a mouse cDNA library and an expression cloning strategy with fluorescent-labeled oxidized LDL. They identified two cell surface proteins that could mediate the binding and internalization of oxidized LDL but not acetyl LDL. The first was found to be the murine FcγRII-B2 receptor (38). However, blocking antibodies to the FcγRII-B2 receptor failed to inhibit the uptake of oxidized LDL in mouse peritoneal macrophages, and hence it is questionable if this receptor accounts for a significant component of oxidized LDL uptake in these cells. The second protein was shown to be the murine homologue of CD36 (42). Studies in 293 cells transiently transfected with CD36 showed an increase in binding of oxidized LDL, but degradation was very low, and hence it was unclear from this experiment if CD36 could account for the scavenger receptor-independent uptake and degradation of oxidized LDL (42). It was found that only a mild degree of oxidation of LDL was sufficient to permit binding to this protein, in contrast to the extensive oxidation of LDL required for receptor-mediated uptake and degradation in mouse peritoneal macrophages (23). Monoclonal antibody OKM5 against human CD36 reduced the binding of oxidized LDL to THP-1 human monocytic leukemia cells by 52%, but the effect of this antibody on LDL degradation was not reported. More recently, Nicholson and colleagues (43) reported that anti-CD36 antibody 8A6 inhibited the degradation of oxidized LDL by human monocyte-macrophages by 22%. More direct evidence for a role for CD36 in oxidized LDL uptake was the finding that monocyte-macrophages from subjects with inherited deficiency of CD36 have a reduced rate of uptake and degradation of oxidized LDL (44). Hence, it is possible that CD36 is a mediator of oxidized LDL uptake and degradation, at least in human macrophages.

There are a number of issues that require clarification before the results in the above studies can be reconciled. In particular, an explanation is needed for the finding in some studies that acetyl or acetoacetylated LDL competed fully for oxidized LDL uptake and degradation in macrophages (23, 34, 41), while in others it competed only partially (36, 38, 40). The failure of acetyl LDL to compete for oxidized LDL degradation in macrophages cannot adequately be accounted for simply on the basis of nonidentical binding sites on scavenger receptors type I or II for these two ligands, because acetyl LDL was found to compete well for oxidized LDL degradation in cells expressing only type I or type II scavenger receptors (28, 34).

The objectives of the present study were to determine if the uptake of oxidized LDL by macrophages involved one or more than one major pathway, and if the apparent non-reciprocal cross-competition between oxidized LDL and acetyl LDL (and some of the discrepancies between previous publications) might be due to ligand heterogeneity of oxidized LDL rather than heterogeneity or multiplicity of receptors. Specifically, we wished to determine if heterogeneity in terms of the extent of oxidation of LDL and/or the degree of aggregation in oxidized LDL might account for these findings.

MATERIALS AND METHODS

Carrier-free ^{125}I was purchased from DuPont NEN (Lachine, Quebec, Canada). α -Minimal essential medium, fetal bovine serum, and gentamicin were from Life Technologies, Inc. (Burlington, Ontario, Canada). Monoclonal antibody to human CD36 (CLB-703) was purchased from Cedarlane Laboratories, Hornby, Ontario, Canada. Purified human platelet thrombospondin was a gift from Dr. Dana Devine, Department of Pathology, University of British Columbia. Female CD-1 mice were supplied by the University of British Columbia Animal Care Centre. Formaldehyde was from J. B. EM Services (Dorval, Quebec, Canada). Butylated hydroxytoluene was purchased from J.T. Baker (Toronto, Ontario, Canada). DiI, (1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate) was obtained from Molecular Probes (Eugene, OR). Other chemicals and solvents were purchased from Fisher Scientific (Vancouver, British Columbia, Canada) or BDH (Toronto).

Analytic Procedures—Protein determination was done by the method of Lowry (45) in the presence of 0.05% sodium deoxycholate to minimize turbidity. Bovine serum albumin was used as the standard. Lipoprotein electrophoresis was done using a Corning apparatus and Universal agarose film in 50 mM barbital buffer (pH 8.6). Bovine albumin was added to dilute lipoprotein samples to ensure reproducible migration distances. Lipoprotein bands were visualized by staining with fat red, and albumin was seen as a clear band against the background staining of the gel. Migration of albumin in this system was typically 24 mm, and was used to standardize migration distances of lipoproteins by expressing these as the ratio of migration distance of lipoprotein divided by that of albumin on the same gel. Neutral lipids in LDL were extracted with chloroform:methanol according to the method of Bligh and Dyer (46). Fatty acid methyl esters were prepared using a direct transesterification procedure as described by Lepage and colleagues (47). Two ml of methanol:benzene (4:1) was added to 50 μg of LDL in 0.1 ml of phosphate-buffered saline (PBS) containing 10 μM EDTA. Heptadecanoic acid (20 μg) dissolved in ethanol was added as an internal standard. 200 μl of acetyl chloride was then added with continuous vortexing over a period of 1 min. Tubes were tightly sealed using Teflon-lined caps and subjected to methanolysis at 100 °C for 1 h. After the tubes had been allowed to cool, the samples were neutralized by the addition of 5 ml of 6% K_2CO_3 , shaken vigorously and centrifuged at 3,000 rpm for 10 min. Aliquots of the benzene upper phase were injected onto a Hewlett-Packard 5880A gas chromatograph equipped with a 0.53 mm \times 30-meter DB-WAX fused silica capillary column (J & W Scientific) and a flame ionization detector. Helium was used as carrier gas at a flow rate of 6 ml/min. Injector and detector temperatures were 230 °C and 250 °C, respectively. The column temperature was maintained at 180 °C for 12 min and then increased at a rate of 20 °C/min to a maximum temperature of 220 °C. Retention times of commercially available lipid standards (Sigma) were used to identify fatty acid methyl esters. The amount of fatty acid present was calculated by multiplying the peak area by the mass/peak area of heptadecanoate added to each sample. A correction factor was applied to compensate for the lower ionization detector response to unsaturated fatty acids relative to the corresponding saturated fatty acid (48).

Lipoprotein Isolation and Labeling—LDL ($d = 1.019\text{--}1.063$) was isolated by sequential ultracentrifugation of EDTA-anticoagulated fasting plasma obtained from healthy normolipidemic volunteers (49). Radioiodination was performed using a modification of the iodine monochloride method of MacFarlane (50). Specific radioactivities were 100–150 cpm/ng. Iodination was performed before oxidation or acetylation of LDL.

Lipoprotein Modification—The concentration of EDTA in LDL preparations was reduced prior to oxidation by dialysis against Dulbecco's PBS containing 10 μM EDTA. Standard conditions for LDL oxidation were: 200 $\mu\text{g}/\text{ml}$ LDL in Dulbecco's PBS containing 5 μM CuSO_4 incubated at 37 °C for 20 h (23). This typically resulted in electrophoretic mobility 0.85 relative to albumin. "Very extensively oxidized" LDL was obtained after 33 h of incubation under the same conditions, and typically resulted in electrophoretic mobility 1.03 relative to albumin. To promote formation of LDL aggregates during oxidation, some incubations were done with a high concentration of LDL (1 mg/ml), and this required increasing the copper concentration to 20 μM , and extending the incubation time to 72 h to give electrophoretic mobility about 0.85 relative to albumin. In some experiments, LDL aggregation was also induced by vortexing LDL for 15–30 s in a 15-ml conical tube with a benchtop mixer at medium speed setting. Extent of aggregation (turbidity) was monitored by absorbance at 680 nm (51). Acetylation or malondialdehyde modification of LDL or of albumin was performed as

described previously (52). Acetylation resulted in derivatization of more than 75%, and malondialdehyde modification of more than 62% of free amino groups.

Separation of Aggregated and Nonaggregated Fractions of Oxidized LDL—Both chromatography over Sepharose CL4B and ultracentrifugation were evaluated for separating aggregated from nonaggregated LDL. We obtained somewhat better separation and higher recovery by ultracentrifugation, and so the following ultracentrifugal procedure was used in the experiments described here. Extensively oxidized LDL was spun at $10,000 \times g$ for 15 min to remove very large aggregates and then mixed with NaBr to adjust the solution density to 1.10, overlaid with 1–2 ml of NaBr solution of the same density, and centrifuged for 50 min at 38,000 rpm in a 50 Ti rotor. The top (aggregated) fraction was removed, and the remaining (nonaggregated) fraction was floated by further centrifugation for 16 h at 38,000 rpm. Analysis by agarose gel electrophoresis showed 71% aggregation in the “aggregated” and 11% in the “non-aggregated” fraction.

Cell Culture—Resident peritoneal macrophages were obtained from female CD-1 mice by peritoneal lavage with ice-cold Ca^{2+} -free Dulbecco's PBS. Cells were suspended in α -minimal essential medium with 10% fetal bovine serum and plated in 12-well plastic culture plates at a density of 1×10^6 cells/well.

Human monocytes were isolated from freshly obtained citrate-anticoagulated blood using Ficoll-Hypaque. Multiple tubes containing 30 ml of blood layered over 15 ml of Ficoll-Hypaque were centrifuged at $440 \times g$ for 25 min at 10°C , and the mononuclear cells at the interface were collected and pooled. Platelets were removed by differential centrifugation in RPMI 1640. Purified mononuclear cells were added to 10-cm diameter Costar plastic culture dishes at a density of 2×10^7 cells/ml and incubated undisturbed for 1 h at 37°C . Nonadherent cells were then removed by three washes with prewarmed RPMI 1640 medium with 100 units/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin. Loosely adherent monocytes were scraped off in cold RPMI with a sterile cell lifter and counted. The viability was $>95\%$ as judged by trypan blue exclusion. To permit differentiation to macrophages, $1\text{--}2 \times 10^6$ monocytes/well were plated in 24-well culture plates with 1 ml of RPMI 1640 containing 20% autologous serum. Cells were used for experiments between 9 and 12 days after seeding. For each experiment only cells derived from one donor were used. Autologous serum was prepared from blood that had been allowed to clot at 37°C for 2 h by centrifugation at $3200 \times g$ for 15 min at room temperature. The differentiation of monocytes into macrophages under these conditions was assessed by morphology and level of expression of scavenger receptor activity, which increased 10-fold as judged by uptake and degradation of acetyl LDL. THP-1 human monocytic leukemia cells were obtained from American Type Culture Collection (Rockwell, MD) and were cultured in RPMI 1640 medium with 10% FBS. THP-1 cells were induced to differentiate to a macrophage-like phenotype by 72 h of exposure to 200 ng/ml phorbol myristate acetate.

Transfection of CHO Cells with Human Scavenger Receptor cDNA—The expression vector pRC/CMV containing a full-length insert of human scavenger receptor type I cDNA was generously provided by Dr. T. Kodama, University of Tokyo. The plasmid was purified by alkaline lysis and ultracentrifugal banding in CsCl, and showed only the predicted 2028-kilobase pair insert band on agarose electrophoresis after digestion with *Hind*III and *Xba*I. The plasmid was transfected into CHO K1 cells using the calcium precipitation method. Several colonies that survived selection in medium containing G418 were cloned by limiting dilution, and then screened for uptake of diI-labeled acetyl LDL.

Assays of LDL Uptake and Degradation—Macrophages or CHO cells were cultured overnight in a humidified CO_2 incubator and then washed with serum-free medium. Lipoproteins were added to the cells in serum-free medium. After 5 h of incubation at 37°C , media were removed and assayed for trichloroacetic acid-soluble noniodide degradation products (22). Cells were then washed three times with Dulbecco's PBS, dissolved in 0.1 N NaOH, scraped from the plates, and assayed for radioactivity and protein content.

RESULTS

It has been proposed that macrophages possess at least two pathways for the uptake of oxidized LDL: one shared with acetyl LDL and mediated by scavenger receptors type I and II, and another pathway or pathways specific for oxidized LDL but not acetyl LDL (36). If this were the case, one would expect that oxidized LDL would compete fully for the degradation of acetyl LDL, but that acetyl LDL would compete for only part of the

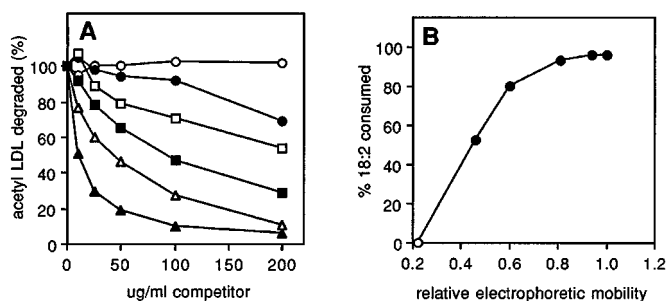


FIG. 1. Effect of extent of oxidation on the ability of oxidized LDL to compete for acetyl LDL degradation. A, macrophages were incubated 5 h with 5 $\mu\text{g}/\text{ml}$ acetyl ^{125}I -LDL together with varying concentrations of unlabeled native LDL (○, electrophoretic mobility 0.22); unlabeled LDL oxidized by exposure to 5 μM Cu^{2+} for 18 h (●, electrophoretic mobility 0.83), 21 h (□, electrophoretic mobility 0.91), 28 h (■, electrophoretic mobility 0.98), 30 h (△, electrophoretic mobility 1.02), or unlabeled acetyl LDL (▲). Acetyl LDL degradation is expressed as a percentage of that in the absence of competitor. Values shown are means of duplicate incubations that varied by less than 10%. B, fatty acid composition was determined for native LDL (○) and LDL oxidized to varying degrees as above (●). The percentage of linoleic acid consumed is plotted as a function of electrophoretic mobility of LDL.

degradation of oxidized LDL. However, depending on the conditions employed, oxidized LDL preparations can differ greatly in their extent of oxidation and degree of aggregation as well as in the nature of lipid peroxidation products present, and the degree of derivatization and fragmentation of apoB. To determine how the extent of oxidation affects binding to the acetyl LDL scavenger receptor, a series of oxidized LDL preparations that differed in their extent of oxidation were compared for their ability to compete for the degradation of ^{125}I -acetyl LDL. It should be noted that all of these oxidized LDL preparations were modified to an extent sufficient for rapid high affinity uptake in macrophages (the “threshold” level of oxidation for scavenger receptor recognition typically occurs at electrophoretic mobility 0.70–0.75 relative to albumin). Results shown in Fig. 1A demonstrate that the effectiveness of oxidized LDL as a competitor for acetyl LDL degradation varies greatly depending on the extent of oxidation. The most extensively oxidized LDL preparation in this experiment competed for about 90% of acetyl LDL degradation, although the apparent affinity was less than that of unlabeled acetyl LDL. Thus, in contrast to a report by Arai and colleagues (37), in mouse peritoneal macrophages we found no evidence of a specific receptor for acetyl LDL that did not recognize oxidized LDL. Although electrophoretic mobility is a convenient and reproducible indicator of the extent of LDL oxidation by copper, it is important to standardize this against a quantitative measure of the amount of fatty acid substrate consumed by lipid peroxidation. Accordingly, LDL samples with varying degrees of oxidation were analyzed for fatty acid composition. Arachidonic acid was consumed very rapidly, but trace amounts were difficult to quantify because oxidation generated many new peaks in that region of the chromatogram. As expected, oleic acid was relatively stable, with only 23% consumption even with extensively oxidized LDL. Fig. 1B describes the correlation between electrophoretic mobility and consumption of linoleic acid (the most abundant unsaturated fatty acid in LDL). Nearly all of the linoleic acid was consumed in LDL with electrophoretic mobility greater than 0.9, indicating that these samples were maximally oxidized.

Hoppe and colleagues (53) have reported that oxidized LDL can inactivate lysosomal proteases, and hence the observed inhibition of degradation of acetyl LDL might be due to lysosomal dysfunction rather than to competition for receptors. If this were the case, one would expect to find intracellular accu-

mulation of radioactivity from internalized but undegraded acetyl LDL in the presence of oxidized LDL. To test this possibility, cell-associated radioactivity was measured in parallel with degradation. Even at the highest concentration of oxidized LDL, cell-associated acetyl LDL radioactivity was less than 5% of the amount degraded in the absence of competitor, and therefore most of the inhibition of degradation of acetyl LDL by oxidized LDL was due to competition for uptake and not to lysosomal dysfunction caused by oxidized LDL.

The next experiments were done to determine why acetyl LDL sometimes fails to compete fully for the degradation of oxidized LDL. Fig. 2 shows a typical experiment comparing the ability of acetyl LDL and oxidized LDL to compete for the uptake and degradation of ^{125}I -oxidized LDL. The abrupt drop and subsequent plateau of the competition curve with acetyl LDL suggests that there is more than one class of ligand-receptor interaction between oxidized LDL and the cells, only one of which is efficiently competed by acetyl LDL. Similar results have been reported previously (36) and, as noted above, have been taken as evidence for more than one receptor. However, this finding could also be explained by heterogeneity of the labeled oxidized LDL ligand. A likely source of heterogeneity in oxidized LDL is aggregation, because oxidized LDL is known to be very susceptible to aggregation (54). To test whether aggregation of oxidized LDL might account for the failure of acetyl LDL to compete completely for oxidized LDL uptake, we generated labeled oxidized LDL preparations with differing levels of aggregation and compared the ability of acetyl LDL to compete for their uptake. The extent of aggregation of oxidized LDL was varied in three different ways: by

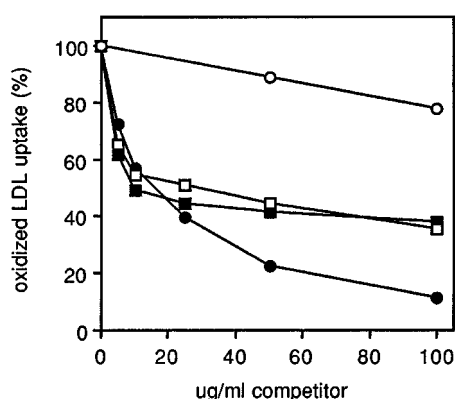


FIG. 2. **Incomplete competition by acetyl LDL for uptake of oxidized LDL.** Macrophages were incubated for 5 h with 3 $\mu\text{g}/\text{ml}$ oxidized ^{125}I -LDL (relative electrophoretic mobility 0.95) together with the indicated concentration of unlabeled native LDL (\circ), acetyl LDL (\blacksquare), malondialdehyde-modified LDL (\square), or oxidized LDL (\bullet). The amount of cell-associated and degraded LDL was determined as described in the methods. Each point is the mean of duplicates that varied by less than 10%. The value for oxidized LDL in the absence of competitor was 18.1 $\mu\text{g}/\text{mg}$. Similar results were obtained in five of five such experiments.

changing the extent of oxidation, by briefly vortexing a "standard" oxidized LDL preparation, or by increasing the concentration of LDL during oxidation. The extent of aggregation was estimated by agarose gel electrophoresis and by centrifugation at $10,000 \times g$ (Table I, Fig. 3). With electrophoresis, aggregation was calculated as the amount of radioactivity recovered in the gel lane from the origin to the lower edge of the main LDL band divided by total radioactivity in that lane. This method demonstrated aggregates that were evidently too small to sediment at $10,000 \times g$. Results shown in Fig. 4 indicate that acetyl LDL competed for more than 80% of the degradation of "standard" oxidized LDL (11% aggregates by electrophoresis) but only about 50% of the degradation of vortexed oxidized LDL (36% aggregates by electrophoresis). The extent of aggregation of oxidized LDL as assessed by electrophoresis correlated with the proportion of oxidized LDL degradation that was unaffected by unlabeled acetyl LDL. This result suggests that aggregation of oxidized LDL might account for the failure of acetyl LDL to compete fully for oxidized LDL degradation in mouse peritoneal macrophages.

Khoo and colleagues (55) reported that aggregated native LDL was internalized by LDL receptor-facilitated phagocytosis and that this was inhibited by cytochalasin D. To determine if an LDL receptor-independent but otherwise analogous phagocytic pathway was involved in the uptake of aggregates of oxidized LDL, we assessed the effect of cytochalasin D (an inhibitor of phagocytosis) on the degradation of oxidized LDL. Ten $\mu\text{g}/\text{ml}$ acetyl ^{125}I -LDL, very extensively oxidized ^{125}I -LDL (23% aggregates, electrophoretic mobility 1.04), ^{125}I -LDL oxidized at high LDL concentration (36% aggregates, electrophoretic mobility 0.89), or ^{125}I -LDL vortexed for 20 s (absorbance increase at 680 nm of 1 mg/ml solution = 0.9) were incubated with macrophages for 5 h in the absence or in the presence of 0.04 $\mu\text{g}/\text{ml}$ cytochalasin D. Cytochalasin D (0.04 $\mu\text{g}/\text{ml}$) inhibited the degradation of vortex aggregated LDL by $79 \pm 3\%$, but did not affect the degradation of acetyl LDL or of either oxidized LDL preparation. It should be noted that the turbidity (reflecting mean particle diameter) of aggregated LDL was substantially greater than that of either oxidized LDL preparation. This suggests that although oxidized LDL contains some aggregates, these are smaller than those in vortex-aggregated LDL and are not handled by the same phagocytic mechanism involved in uptake of vortex-aggregated LDL.

The preceding results indicate that extent of aggregation of oxidized LDL correlates with the proportion of its uptake by mouse peritoneal macrophages that is resistant to competition by acetyl LDL, but they do not prove that aggregation *per se* is the cause of this. We hypothesized that aggregates of modified LDL would have a higher apparent affinity for the scavenger receptor than monomeric lipoproteins because of their potential to interact with numerous receptor molecules, and that this might explain why acetyl LDL was unable to compete effectively for aggregates of oxidized LDL. Inspection of the curves shown in Fig. 2 suggests that if this hypothesis is true, then the

TABLE I
Estimation of extent of aggregation in different oxidized ^{125}I -LDL preparations

Oxidized LDL was prepared in the standard manner by incubating 200 $\mu\text{g}/\text{ml}$ LDL with 5 μM Cu^{2+} for 20 h at 37 $^{\circ}\text{C}$. Vortexed oxidized LDL was obtained by vortexing this standard preparation in a benchtop mixer for 15 s. "Very extensively" oxidized LDL was prepared by incubating 200 $\mu\text{g}/\text{ml}$ LDL with 5 μM Cu^{2+} for 30 h at 37 $^{\circ}\text{C}$. "High conc." oxidized LDL was prepared by incubating 2 mg/ml LDL with 20 μM Cu^{2+} for 40 h at 37 $^{\circ}\text{C}$. For each preparation, aggregation was measured as the proportion of total radioactivity that migrated between the origin and the edge of the major LDL band on electrophoresis, and as the proportion that sedimented on centrifugation for 10 min at $10,000 \times g$.

Fig. 4 samples	Electrophoretic mobility (relative to albumin)	% aggregates (by electrophoresis)	% aggregated ($10,000 \times g$ pellet)
"Standard" ox LDL	0.86	11	3
Very extensively ox LDL	1.01	22	4
High conc. ox LDL	0.92	36	5
Vortexed ox LDL	0.88	36	24

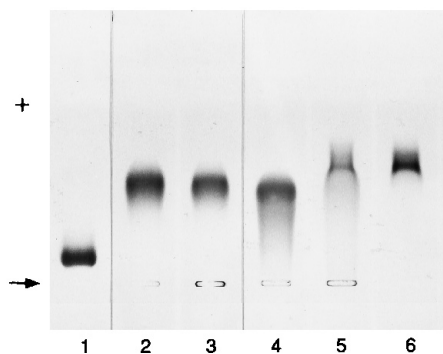


FIG. 3. **Agarose gel electrophoresis.** Oil red O-stained gel showing native LDL (lane 1), "standard" oxidized LDL (lane 2), vortexed oxidized LDL (lane 3), LDL oxidized by incubating a high concentration of LDL (2 mg/ml) with 20 μ M Cu^{2+} for 40 h at 37 $^{\circ}\text{C}$ (lane 4), LDL very extensively oxidized by incubating 200 $\mu\text{g}/\text{ml}$ LDL with 5 μ M Cu^{2+} for 30 h (lane 5), and acetyl LDL (lane 6). The arrow indicates the origin.

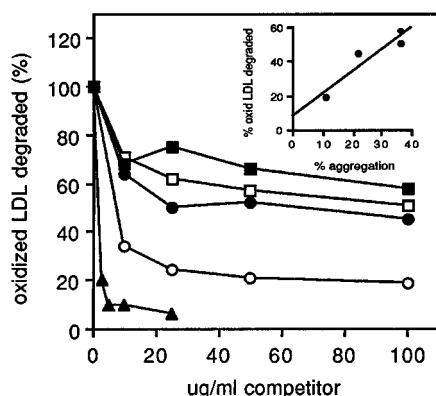


FIG. 4. **Effect of varying the extent of aggregation of oxidized ^{125}I -LDL on the ability of acetyl LDL to compete for its degradation.** "Standard" oxidized ^{125}I -LDL (\circ) or oxidized ^{125}I -LDL with aggregation induced by vortexing (\blacksquare), very extensive oxidation (\bullet , \blacktriangle), or oxidation at high LDL concentrations (\square) was incubated with macrophages for 5 h in the presence of the indicated concentration of acetyl LDL (\circ , \bullet , \square , \blacksquare) or polyinosinic acid (\blacktriangle). Characterization of these oxidized LDL preparations is shown in Table I. Oxidized LDL degradation is expressed as a percentage of that in the absence of competitor. Values are means of duplicate incubations that varied by less than 10%. The results shown are representative of three experiments. *Inset*, values for oxidized LDL degradation at 100 $\mu\text{g}/\text{ml}$ acetyl LDL competitor are plotted as a function of the percent aggregation of each oxidized preparation ($R^2 = 0.88$).

apparent affinity of aggregated oxidized LDL must be orders of magnitude greater than that of monomeric oxidized LDL. To test this, we generated ^{125}I -LDL preparations with varying degrees of oxidation, separated these by brief ultracentrifugation into aggregated and nonaggregated fractions, and tested the ability of acetyl LDL to compete for the uptake of each fraction. Results in Fig. 5 show that extensively oxidized LDL was relatively resistant to competition by acetyl LDL, but that this was the same for aggregated and nonaggregated fractions, and hence could not be attributed to aggregation alone. The notion that aggregates of modified LDL would have a higher apparent affinity for the scavenger receptor than monomeric lipoproteins because of their potential to interact with numerous receptor molecules was also tested by comparing the ability of vortex-aggregated acetyl LDL to compete for the degradation of oxidized LDL. Mouse peritoneal macrophages were incubated with 5 $\mu\text{g}/\text{ml}$ "standard" oxidized ^{125}I -LDL (electrophoretic mobility 0.83, 21% aggregates by electrophoresis), extensively oxidized ^{125}I -LDL (mobility 1.06, 64% aggregates), vortexed "standard" oxidized ^{125}I -LDL (mobility 0.83, 36% ag-

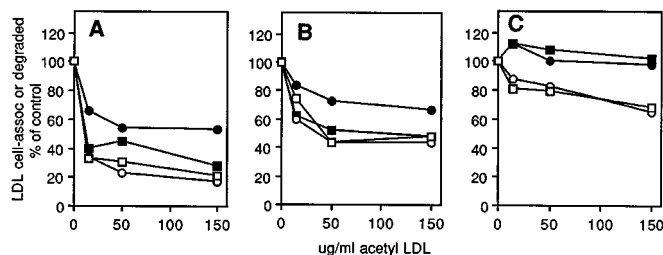


FIG. 5. **Aggregation accounts for only part of the uptake of oxidized LDL by alternate (non-scavenger receptor type I/II) pathways in mouse peritoneal macrophages.** Three preparations of oxidized ^{125}I -LDL that differed in their degree of oxidation were separated by ultracentrifugation into aggregated (solid symbols) and nonaggregated (open symbols) fractions. Mouse peritoneal macrophages were incubated with 5 $\mu\text{g}/\text{ml}$ labeled oxidized LDL together with the indicated concentration of unlabeled acetyl LDL. After 5 h, the amount of cell-associated LDL (squares) and degraded LDL (circles) was measured. A, oxidized LDL with electrophoretic mobility 0.6 relative to albumin (nonaggregated oxidized LDL); B, oxidized LDL with mobility 0.94; C, oxidized LDL with mobility 1.04. Respective 100% values for degradation and cell association of nonaggregated oxidized LDL in panel A were: 2.9 and 3.3 $\mu\text{g}/\text{mg}$, and of aggregated oxidized LDL, 1.3 and 4.3 $\mu\text{g}/\text{mg}$. Values for degradation and cell association of nonaggregated oxidized LDL in panel B were: 7.3 and 9.7 $\mu\text{g}/\text{mg}$, and of aggregated oxidized LDL, 3.8 and 13.1 $\mu\text{g}/\text{mg}$. Values for degradation and cell association of nonaggregated oxidized LDL in panel C were: 7.3 and 23.3 $\mu\text{g}/\text{mg}$, and of aggregated oxidized LDL, 6.5 and 30.5 $\mu\text{g}/\text{mg}$.

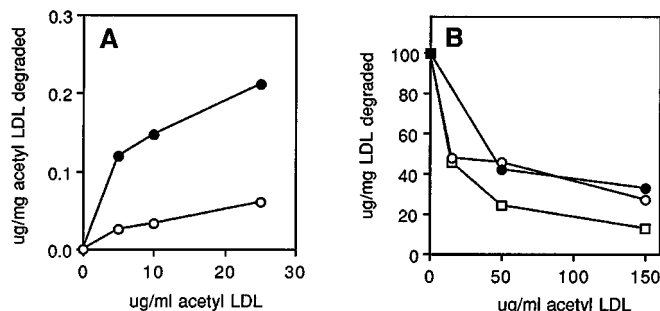


FIG. 6. **Aggregation does not account for incomplete competition for uptake and degradation of oxidized LDL by acetyl LDL in CHO cells stably transfected with human scavenger receptor type I.** CHO cells were transfected with plasmid pRC-CMV containing human scavenger receptor cDNA, and G418-resistant clones were selected for ability to internalize diI-labeled acetyl LDL. Panel A, varying concentrations of acetyl ^{125}I -LDL were incubated for 5 h with transfected cells (solid symbols) or control CHO cells (open symbols), and then degradation products in the media were measured. Panel B, 5 $\mu\text{g}/\text{ml}$ acetyl ^{125}I -LDL (\square), nonaggregated oxidized ^{125}I -LDL (\circ), or aggregated oxidized ^{125}I -LDL (\bullet) were incubated with the indicated concentration of unlabeled acetyl LDL for 5 h, and then LDL degradation products were assayed.

gregates), ^{125}I -LDL oxidized at high LDL concentration (mobility 0.75, 48% aggregates), or acetyl ^{125}I -LDL (mobility 1.06) in the presence or absence of up to 150 $\mu\text{g}/\text{ml}$ unlabeled acetyl LDL or acetyl LDL that had been aggregated by brief vortexing, which increased turbidity (absorbance at 680 nm) of a 1 mg/ml solution by 0.36. The competition profiles for the various oxidized LDL preparations were very similar to those shown in Fig. 2, and were essentially identical for untreated and vortexed acetyl LDL. A final test of the hypothesis that aggregation affects the apparent affinity of oxidized LDL for the scavenger receptor type I was carried out on CHO cells stably transfected with an expression vector containing human scavenger receptor type I cDNA. Results in Fig. 6 show that acetyl LDL was unable to compete fully for oxidized LDL degradation in transfected cells, and that there was no difference between its ability to compete for aggregated and non-aggregated oxidized LDL. Taken together, these findings lead one to reject the

postulate that an aggregation-related increase in affinity of oxidized LDL for the scavenger receptor type I is responsible for the failure of acetyl LDL to compete for its uptake, and supports the hypothesis that there is a second pathway.

CD36 is a cell surface adhesion molecule expressed in human macrophages and endothelial cells that has been proposed as a potential "receptor" for oxidized LDL (42). Unfortunately, neither an antibody to the murine analog of CD36 nor a CD36 knockout mouse is presently available, and so it was not possible to test directly if CD36 accounted for the component of oxidized LDL uptake that could not be competed for by acetyl LDL in mouse macrophages. To address this in human macrophages, we first carried out cross-competition experiments between oxidized LDL and acetyl LDL in human monocyte-derived macrophages and in THP-1 cells that had been induced to differentiate to a macrophage-like phenotype with phorbol ester. It was found that, as with mouse peritoneal macrophages, acetyl LDL competed for part but not all of the degradation of oxidized LDL (Fig. 7). However, in contrast to results with murine cells, in human macrophages oxidized LDL was a rather poor competitor for the degradation of acetyl LDL. It

was not ascertained if this was due to a species difference in affinity of the scavenger receptor type I/II for oxidized LDL or to other factors such as a second receptor specific for acetyl LDL (37). In human monocyte-derived macrophages, antibody to CD36 inhibited the uptake and degradation of "standard" oxidized LDL by about 25% (Fig. 8). The same concentration of antibody inhibited the binding of ^{125}I -thrombospondin, a putative ligand of CD36, to THP-1 cells by more than 50% (not shown). This is consistent with the notion that part of the uptake of oxidized LDL might be mediated by CD36. However, the anti-CD36 antibody inhibited the uptake and degradation of acetyl LDL to the same extent as very extensively and/or aggregated oxidized LDL. Therefore, it is difficult to implicate CD36 as being responsible for the failure of acetyl LDL to compete for the uptake of these forms of oxidized LDL.

DISCUSSION

In the present studies, we have shown that the apparent affinity of oxidized LDL as a competitor for acetyl LDL uptake in macrophages is dependent on the extent of oxidation of LDL, even though all of the oxidized LDL preparations had electrophoretic mobility at least 3-fold greater than that of native plasma LDL and were capable of interacting with macrophage scavenger receptors. This effect of the degree of oxidation has not generally been appreciated, as many investigators simply assess their oxidized LDL preparations to verify that oxidation has occurred, and do not determine precisely how extensive the modification is. With the standard protocol for generating copper-oxidized LDL used in this report (200 $\mu\text{g}/\text{ml}$ LDL incubated at 37 °C with 5 μM Cu^{2+} in PBS), incubation periods between 18 and 24 h have generally been used for generating oxidized LDL. The rate of LDL oxidation is directly correlated with copper concentration, and inversely correlated with LDL concentration, and the concentration of metal ion-binding substances, and some of these factors could lead to variability between laboratories, or even between LDL preparations in the same laboratory. In the present study, we found a large difference in the apparent uptake pathways between preparations oxidized by the standard protocol for 18 h compared to those oxidized for 30 h. Hence, it is perhaps not surprising that some groups found oxidized LDL to compete for 80% or more of acetyl LDL degradation in peritoneal macrophages (36, 38), while others reported only about 40% competition (34, 37, 41).

The difference in uptake between "standard" oxidized LDL (electrophoretic mobility about 0.85 relative to albumin) and very extensively oxidized LDL (electrophoretic mobility greater than 1.0) could reflect increased affinity of binding of monomeric very extensively oxidized LDL to scavenger receptors. Alternatively, it could be due to aggregates in preparations of very extensively oxidized LDL causing an apparent increase in affinity for the receptor because aggregated LDL particles could interact with a greater number of receptor molecules but

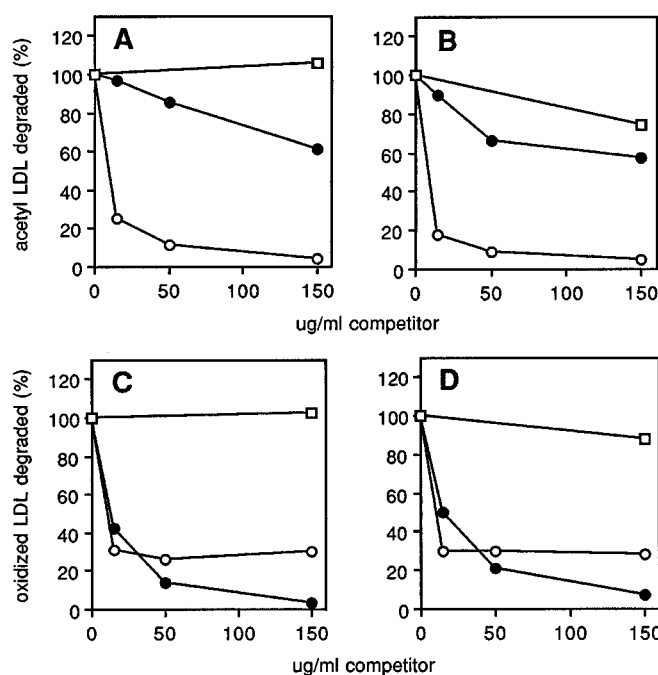
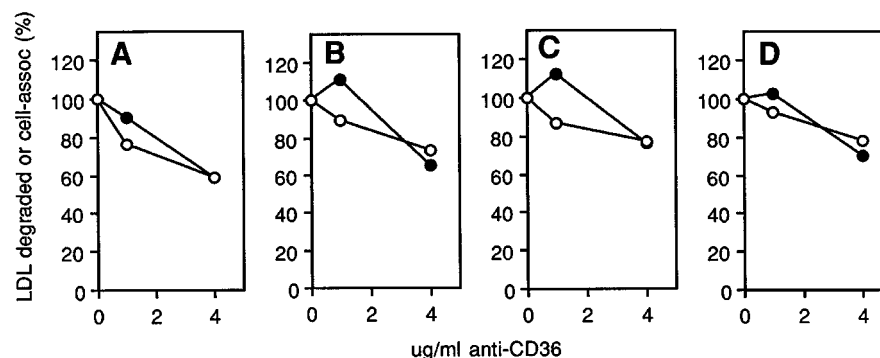


FIG. 7. **Incomplete cross-competition between oxidized LDL and acetyl LDL in human macrophages.** Cultured human THP-1 macrophages (panels A and C) or human monocyte-derived macrophages (panels B and D) were incubated for 5 h with 5 $\mu\text{g}/\text{ml}$ acetyl ^{125}I -LDL (panels A and B) or 5 $\mu\text{g}/\text{ml}$ oxidized ^{125}I -LDL (panels C and D) and the indicated concentration of unlabeled acetyl LDL (○), extensively oxidized LDL (●), or native LDL (□). Degradation products in the medium were then measured.

FIG. 8. **Inhibition of uptake of modified LDL by antibody to CD36.** Human monocyte-derived macrophages were incubated for 5 h with 5 $\mu\text{g}/\text{ml}$ "standard" oxidized ^{125}I -LDL (panel A), extensively oxidized ^{125}I -LDL (panel B), ^{125}I -LDL oxidized at high concentration (panel C), or acetyl ^{125}I -LDL (panel D) together with the indicated concentration of monoclonal antibody to human CD36. Cell-associated radioactivity (○) and LDL degradation products (●) were then measured. At 4 $\mu\text{g}/\text{ml}$, this antibody inhibited the binding of ^{125}I -thrombospondin to THP-1 cells by more than 50%.



the present studies provide no support for this possibility. Otnad and co-workers (56) also found a correlation between the extent of LDL oxidation and its ability to compete for the binding of acetyl LDL to liposome-reconstituted hepatic scavenger receptors, although in that report the amount of oxidation required for displacement of acetyl LDL from reconstituted receptors was much less than that required in the present study for competition for acetyl LDL degradation in cultured macrophages.

A more important issue relates to the incomplete competition by acetyl LDL for oxidized LDL uptake and degradation in macrophages. Some investigators reported that acetyl LDL competed for only about 40% of oxidized LDL uptake and interpreted this as evidence for additional receptor(s) for oxidized LDL (36–38, 42). However, others have found essentially complete competition by acetyl LDL (23, 41) or acetoacetylated LDL (34). In the present report, we show that these differences can be explained at least in part by heterogeneity of oxidized LDL, in that very extensive oxidation and/or aggregation of oxidized LDL correlated with reduced ability of acetyl LDL to compete for its uptake. One possible explanation for the inability of acetyl LDL to compete for aggregates of oxidized LDL is that such aggregates might interact with many identical receptor molecules, thereby increasing the apparent affinity. However, we found no difference in the ability of acetyl LDL to compete for the uptake and degradation of the aggregated compared to nonaggregated fractions of oxidized ¹²⁵I-LDL in mouse peritoneal macrophages or CHO cells transfected with human scavenger receptors, and aggregation of acetyl LDL by vortexing did not augment its ability to compete for the degradation of oxidized LDL. Therefore, a second pathway is apparently required to account for the uptake of oxidized LDL, particularly of very extensively oxidized preparations.

Kho and colleagues characterized the uptake by macrophages of LDL that had been aggregated by vortexing (55), and found that the uptake involved a phagocytic mechanism that was dependent on LDL receptor binding. LDL receptor binding cannot be invoked in the present experiments because oxidation of LDL is associated with derivatization of lysine residues and fragmentation of apo B, and LDL receptor binding is abolished even after relatively modest degrees of oxidation, corresponding to electrophoretic mobility about 0.5 relative to albumin (23). None of the oxidized LDL preparations used in the present work would have been capable of interacting with the LDL receptor, but it was possible that LDL receptor-independent phagocytosis was involved. However, cytochalasin D (an inhibitor of phagocytosis) blocked the degradation of vortexed native LDL but not that of oxidized LDL or acetyl LDL.

Reference has been made above to reports that CD36 can interact with oxidized LDL and mediate its internalization (42–44). However, we found that although antibody to CD36 resulted in a slight inhibition of the uptake of oxidized LDL in human monocyte-derived macrophages, it affected the uptake of nonaggregated and aggregated forms of oxidized LDL and acetyl LDL to the same extent, and hence CD36 did not appear to explain the observed differences in uptake pathways for these modified LDLs. Acton and co-workers (57) cloned a new scavenger receptor termed SR-BI that is 30% homologous to CD36 and binds oxidized LDL. This receptor also binds acetyl LDL, and hence is unlikely to explain the acetyl LDL-independent component of oxidized LDL uptake that is the focus of the present work. Otnad and co-workers (58) have described a 94–97 kDa plasma membrane protein in macrophages that binds to oxidized LDL on ligand blots and have proposed that this may represent a receptor for oxidized LDL. As well, phosphatidylcholine/cholesterol vesicles containing small amounts

of acidic phospholipids are internalized and metabolized by macrophages via a pathway that is completely inhibitable by oxidized LDL but only partly by acetyl LDL (59), and this pathway does not involve scavenger receptors type I or II (60). Once the receptors involved in these pathways have been fully characterized, it should be possible to determine if they account for the scavenger receptor-independent component of the uptake of oxidized LDL by macrophages. In the meantime, the present results indicate that inferences based on competition studies between oxidized LDL and other scavenger receptor ligands need to be interpreted cautiously because the apparent mechanism of uptake of oxidized LDL depends on the extent of LDL oxidation.

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