Lipoprotein aggregation protects human monocyte-derived macrophages from OxLDL-induced cytotoxicity

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Abstract **Oxidative modifications render low density lipoprotein cytotoxic and enhance its propensity to aggregate and fuse into particles similar to those found in atherosclerotic lesions. We showed previously that aggregation of oxidized LDL (OxLDL) promotes the transformation of human macrophages into lipid-laden foam cells (Asmis, R., and J. Jelk. 2000. Large variations in human foam cell formation in individuals. A fully autologous in vitro assay based on the quantitative analysis of cellular neutral lipids. *Atherosclerosis.* 148: 243–253). Here, we tested the hypothesis that aggregation of OxLDL enhances its clearance by human macrophages and thus may protect macrophages from OxLDL-induced cytotoxicity. We found that increased aggregation of OxLDL correlated with decreased macrophage injury. Using ^{3}H-labeled and Alexa546-labeled OxLDL, we found that aggregation enhanced OxLDL uptake and increased cholesteryl ester accumulation but did not alter free cholesterol levels in macrophages. Acetylated LDL was a potent competitor of aggregated oxidized LDL (AggOxLDL) uptake, suggesting that scavenger receptor A plays an important role in the clearance of AggOxLDL. Inhibitors of actin polymerization, cytochalasin B, cytochalasin D, and latrunculin A, also prevented AggOxLDL uptake and restored OxLDL-induced cytotoxicity. This suggests that OxLDL-induced macrophage injury does not require OxLDL uptake and may occur on the cell surface. Our data demonstrate that aggregation of cytotoxic OxLDL enhances its clearance by macrophages without damage to the cells, thus allowing macrophages to avoid OxLDL-induced cell injury.**—Asmis, R., J. G. Begley, J. Jelk, and W. V. Everson. **Lipoprotein aggregation protects human monocyte-derived macrophages from OxLDL-induced cytotoxicity. *J. Lipid Res.* 2005.** 46: **1124–1132.**

Supplementary key words cell death • atherosclerosis • oxidized low density lipoprotein • foam cells

Cell death and the appearance of a necrotic core are hallmarks of advanced atherosclerotic plaques (1). Emerging evidence suggests that the demise of macrophages and macrophage-derived foam cells plays a major role in the formation of the necrotic core. Several studies have demonstrated that macrophage death in advanced atherosclerotic lesions occurs predominantly in the proximity of the lipid-laden core of the atheroma (2–6). Furthermore, the necrotic core was shown to contain debris of dead macrophages, indicating that macrophage injury and subsequent cell lysis contribute to necrotic core formation (5, 7). Importantly, increased macrophage death in atherosclerotic lesions is likely to enhance the risk of clinically relevant events, because plaque ruptures associated with myocardial infarction are more likely to occur in lesions with large necrotic cores and a high concentration of macrophage-derived foam cells (8, 9).

The mechanisms involved in macrophage injury and subsequent cell death in human atherosclerotic plaques remain unclear, but immunohistological evidence together with a number of studies in human macrophages have implicated oxidatively modified LDL (OxLDL) (10–16). We showed that in human macrophages, OxLDL-induced cell injury occurs in a caspase-independent manner and results in cell lysis (14). Furthermore, we found that the transformation of human macrophages into lipid-laden foam cells increases their susceptibility to cell injury induced by OxLDL (14), supporting the hypothesis that OxLDL-induced macrophage injury and the concomitant uncontrolled release of the noxious cell contents may play an important role in promoting both vascular inflammation (17–19) and necrotic core formation and plaque destabilization (19).

Oxidation of LDL particles modifies their surface structure, resulting in loss of particle stability, particle aggregation, and subsequent fusion (20). LDL isolated from lesions has many physical and biological characteristics of in vitro-oxidized LDL, suggesting that oxidative modifications of LDL may play an important role in the formation of LDL aggregates and extracellular cholesteryl ester-rich

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Abbreviations: AcLDL, acetylated low density lipoprotein; AggOxLDL, aggregated oxidized low density lipoprotein; OxLDL, oxidized low density lipoprotein.

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lipid droplets found in lesions (10, 20). In vitro, lipoprotein aggregation is required for LDL and OxLDL to promote cholesteryl ester accumulation in human monocyte-derived macrophages (21, 22), and both aggregated LDL and aggregated OxLDL (AggOxLDL) are potent inducers of foam cell formation (20–24).

Oxidative modifications of LDL that render the lipoprotein cytotoxic also promote lipoprotein aggregation and foam cell formation. However, the effect of aggregation on OxLDL toxicity and macrophage death has not been investigated in any detail. Here, we show that aggregation enhances OxLDL uptake and metabolism by human macrophages but decreases OxLDL-induced cell injury. Although increased aggregation of OxLDL will promote foam cell formation, our results suggest that the enhanced clearance by macrophages of cytotoxic extracellular OxLDL after it has become aggregated is likely to reduce vascular cell injury and necrotic core formation.

METHODS

Isolation and culture of human monocyte-derived macrophages

Human mononuclear cells were isolated from buffy coats by density gradient centrifugation, purified, and cultured for 2 weeks in Teflon bags, as described elsewhere (25). Whole blood samples were obtained through the Central Kentucky Blood Center in Lexington and collected from apparently healthy male and female volunteers. Mature macrophages were plated on Aclar (22 mm diameter, transparent fluorinated-chlorinated thermoplastic film; ProPlastics) on 12-well plates at a density of 0.15×10^6 cells/well for all other experiments. The cell culture medium, referred to here simply as culture medium, consisted of RPMI (Gibco BRL) supplemented with 2 mM L-alanyl-L-glutamine (Glutamax-1; Gibco BRL), 1% (v/v) nonessential amino acids (Gibco BRL), 1 mM sodium pyruvate (Gibco BRL), penicillin G/streptomycin (100 U/l and 100 μg/ml, respectively; Gibco BRL), and 10 mM HEPES (Fluka). All solutions were routinely tested for endotoxin. Endotoxin levels of the culture medium and all buffers were less than 0.03 endotoxin units/ml. Cells were plated in culture medium supplemented with 5% human AB serum (Nabi). After 2 h, nonadherent cells were removed by washing with culture medium. Macrophages were incubated in culture medium containing 5% human AB serum for 48 h before the experiments. We observed no significant difference between macrophages cultured in 5% autologous serum and cells incubated in medium containing 5% human AB serum.

Isolation of CD36-negative macrophages

CD36-negative macrophages were isolated by negative selection using magnetic beads after the macrophages were differentiated for 2 weeks in Teflon bags (see above) as we described previously (15). Briefly, 200 μl streptavidin-coupled magnetic beads (6.7×10^8/ml, 2.8 μm; Dynal) were washed and coated with biotinylated anti-CD36 antibodies (SMO; Ancell, Bayport, MN) according to the manufacturer's recommendations. Cells were resuspended at a concentration of 9×10^6 mononuclear cells/ml in culture medium supplemented with 2% autologous human serum and cooled on ice. The cell suspension (6 ml) was added to the magnetic beads and incubated on ice for 30 min with mild shaking. Cell separation was achieved with a commercially available magnet (MPC-1; Dynal) and was performed at 10°C to avoid phagocytosis of the beads. The remaining cells were subjected to a second separation step to remove any residual bead-bound cells. Cells were plated at a concentration of 50,000 cells/well onto 12-well dishes containing Aclar and incubated at 37°C. After 2 h, the Aclar were transferred into 1 ml of fresh culture medium supplemented with 5% autologous human serum. Experiments were performed after 48 h of incubation. The absence of CD36-positive macrophages was confirmed by indirect immunofluorescence staining. Macrophages from the same blood sample that were not subjected to a selection process were plated as controls. Between 55% and 80% of these macrophages stained for CD36 (25).

Lipoproteins

Plasma obtained from six male donors was pooled, divided into aliquots, and kept at −30°C. Human LDL was prepared freshly for each experiment and was isolated by discontinuous gradient density and flotation ultracentrifugation, as described previously (26). LDL was concentrated by ultrafiltration in Centricon-100 concentrators (Amicon) and was further purified by gel filtration chromatography on Excellulose GF-5 columns (Pierce). To ensure reproducible oxidation conditions and to generate OxLDL preparations with consistent cytotoxic properties, LDL was diluted in PBS at a concentration of 3 mg/ml and was oxidized for 24 h at 37°C with 25 μM $CuSO_4$, as described by Esterbauer et al. (27). To remove low molecular weight contaminants and lipoprotein aggregates, OxLDL, prepared freshly for each experiment, was purified by ultrafiltration and gel filtration chromatography, as described above. The concentration of the remaining Cu^{2+} in the OxLDL preparations was less than 1 μM for 100 μg OxLDL/ml or less than five molecules of Cu^{2+} per molecule of OxLDL, as determined by atom absorption spectroscopy (13). OxLDL cytotoxicity was not attributable to residual Cu^{2+} ions because *a*) exposure of macrophages to 1 μM Cu^{2+} alone for 48 h did not induce macrophage lysis, and *b*) LDL oxidized for 24 h with the organic peroxyl radical generator 2,2′-azobis(2-amidinopropane) induced a similar dose-dependent cytotoxicity (13).

Acetylated LDL (AcLDL) was prepared by a modification of the method described by Basu et al. (28). AcLDL was purified by gel filtration and sterile-filtered, as described above. Protein concentrations of all LDL solutions were determined with bicinchoninic acid (Pierce) using BSA as a standard. OxLDL was aggregated by vortexing, as described previously (22). OxLDL (3 mg) was diluted to a concentration of 1.5 mg/ml and fluorescently labeled with 0.5 mg of Alexa546 carboxylic acid succimidyl ester (Molecular Probes, Eugene, OR) for 60 min in 0.1 M $NaHCO_3$ buffer (pH 8.3). Alexa546-labeled OxLDL was then purified by ultrafiltration and gel filtration chromatography, as described above.

Determination of lipoprotein uptake

[^{3}H]LDL was isolated from human plasma loaded with [^{3}H] cholesteryl oleoyl ether as described elsewhere (26). [^{3}H]LDL was oxidized and aggregated as described above. Uptake of ^{3}H-labeled AggOxLDL was measured in macrophages preincubated with culture medium for 1 h at 37°C in the absence or presence of inhibitors. Subsequently, cells were incubated with 20 μg/ml ^{3}H-labeled AggOxLDL for 4 h at 37°C, washed twice with culture medium containing 1% BSA, washed once with PBS, and harvested by scraping with a rubber policeman. Nonspecific binding was measured in the presence of a 50-fold excess (1 mg/ml) of unlabeled AggOxLDL. To determine cell number, DNA measurements were performed using the PicoGreen DNA Quantitation Kit (Molecular Probes). Fluorescence was measured with a FUSION plate reader (Packard) set to an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Cell-associated radiolabel was measured in lipid extracts by scintillation counting.

Determination of membrane integrity

Membrane integrity was assessed with the [^{3}H]adenine release method described by Reid and Mitchinson (29). Loss of membrane integrity was measured as the percentage of radioactivity released into the supernatant. Intracellular radioactivity was measured after cell lysis with 1% (by volume) Triton X-100. Radioactivity was determined by scintillation counting. Total radioactivity per well was always proportional to cell number, as determined by DNA measurement as described above. Control experiments also confirmed that adenine loading did not interfere with any of our other assays.

Cellular cholesterol and cholesteryl ester determination

For lipid extraction, cells were washed with PBS for 10 min at 37°C. The supernatants were removed, and the cells were harvested with a rubber policeman. The cell suspensions were divided into aliquots for lipid extraction and DNA measurements. Lipids were extracted according to a modified technique (30) originally described by Bligh and Dyer (31). In brief, the volume of the cell suspension was adjusted to 800 μl with water, and 3 ml of methanol-dichloromethane with 0.001% 2,6-di-tert-butyl *p*-cresol was added. The sample was vortexed. Phase separation was obtained by adding 1 ml of dichloromethane and 0.5 ml of water. The aqueous phase was reextracted twice with 1 ml of dichloromethane. The combined organic phase was evaporated under nitrogen at 40°C and stored at −20°C.

TLC was performed as described previously (30). Spots were visualized by molybdatophosphoric acid staining and heating of TLC plates at 120°C for 4 min. The optical density of the plates was analyzed with a Personal Laser Densitometer (Molecular Dynamics, Sunnyvale, CA). Spot integration was performed with Image Quant®. Standard curves were fitted with the curve-fit program supplied with the SigmaPlot graphics program (SPSS, San Rafael, CA), which uses the Marquart-Levenberg algorithm, as described previously (30).

Confocal fluorescence microscopy

Confocal laser scanning microscopy was carried out using a Leica TCS SP confocal microscope equipped with argon, krypton, and helium neon lasers for excitation and four spectrophotometers interfaced through acousto-optic tunable filters. For each sample set, the wavelength range for each channel was adjusted to eliminate any overlap. For each image, a complete series of confocal sections was acquired along the *z* axis of the field of cells. Images were acquired using Leica TCS SP software, and images were processed using Adobe Photoshop and Adobe Illustrator.

Statistics

All experiments were performed in triplicate and repeated at least three times. All data are presented as means ± SD. Data were statistically analyzed using ANOVA. Multiple group comparisons were performed using the Tukey test. Results were considered significant at $P < 0.05$.

RESULTS

Aggregation of OxLDL reduces OxLDL cytotoxicity

Previously, we demonstrated that OxLDL promotes plasma membrane lysis in human monocyte-derived macrophages and induces cell death by a caspase-independent mechanism (13). Because OxLDL is predisposed to aggregation, we investigated whether OxLDL aggregation alters its cytotoxicity. Vortexing of freshly prepared and purified OxLDL induced lipoprotein aggregation, as demonstrated by a decrease in the soluble fraction of OxLDL (**Fig. 1**). Increased aggregation of OxLDL correlated with a decrease in OxLDL toxicity in macrophages (Fig. 1). Vortexing for 1 min induced partial aggregation and decreased the soluble fraction of OxLDL by 47.6% to 52.4 μg/ml. The cytotoxicity of the OxLDL preparation was reduced by 37.4%. Vortexing for 2 or 4 min decreased the concentration of soluble OxLDL by 77.3% or 90.2% and concomitantly decreased the cytotoxicity by 93.6% or 95.1%, respectively. Thus, aggregation of OxLDL decreased its cytotoxicity. Macrophages incubated with native LDL showed no increase in cytotoxicity (data not shown).

Aggregation of OxLDL enhances lipoprotein uptake and induces foam cell formation

Aggregation of native LDL enhances lipoprotein uptake by human macrophages (22, 23). To determine whether a similar effect was observed with OxLDL, we added OxLDL labeled with [^{3}H]cholesteryl ether to human macrophages for 4 h and measured OxLDL uptake as the amount of cell-associated radiolabel (**Fig. 2**). This procedure measures total cell-associated OxLDL (i.e., bound plus internalized lipoprotein). However, confocal microscopy studies revealed that in macrophages treated with fluorescently labeled OxLDL, the majority of cell-associated OxLDL and AggOxLDL was intracellular (**Fig. 3E**, **H**). It should be noted that to visualize the localization of the quantitatively low levels of OxLDL taken up by these cells, the sensitivity of the detector was increased by 2 to 3 orders of magnitude greater than the level needed to visualize intracellular AggOxLDL. This is consistent with the quantitative uptake data shown in Fig. 2. Less than 1.2% of the total OxLDL added was found to be cell-associated. Vortexing of ^{3}H-labeled OxLDL for 1, 2, or 4 min increased OxLDL uptake by 2.9-, 7.3-, and 9.7-fold, respectively, demonstrating that aggregation of OxLDL significantly increases lipoprotein uptake by human macrophages.

To investigate whether increased uptake of OxLDL induced by aggregation also enhanced lipoprotein degradation, we incubated human macrophages for 4 days with OxLDL vortexed for 0, 1, 2, or 4 min and quantified cellular cholesterol and cholesteryl ester mass levels. As we reported previously (22), without prior aggregation, OxLDL did not induce a significant increase in cholesteryl ester levels in human macrophages (0.05 ± 0.01 μg/μg DNA) compared with control cells (0.04 ± 0.01 μg/μg DNA; $P > 0.58$). In contrast, vortexing of OxLDL for 1 or 2 min increased cellular cholesteryl ester content by 34- or 53-fold, respectively (**Fig. 4A**). Vortexing for 4 min further increased cholesteryl ester accumulation, but the additional increase was not statistically significant. We observed a close correlation between the cell association of AggOxLDL and cholesteryl ester accumulation induced by AggOxLDL (correlation coefficient = 0.931, $P = 0.021$). Approximately 25% of the total cholesterol in human LDL is unesterified (32). If binding and uptake of AggOxLDL would occur without degradation and cholesteryl esterification, LDL-derived free cholesterol, ~34 μg/100 μg LDL, would

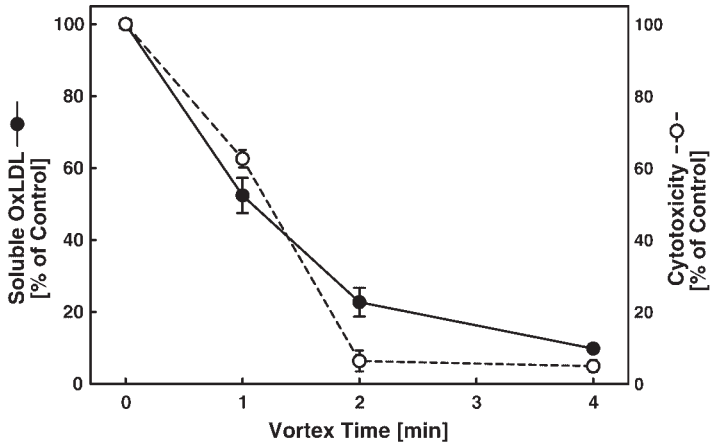

Fig. 1. Effect of vortexing on oxidized LDL (OxLDL) aggregation and OxLDL cytotoxicity. OxLDL (1.5 mg/ml) was aggregated by vortexing for the times indicated. To determine the extent of lipoprotein aggregation, the OxLDL preparations were centrifuged and the concentration of soluble, nonaggregated OxLDL was determined by protein assay (closed circles). [^{3}H]adenine-loaded macrophages were stimulated for 48 h with aliquots from each OxLDL preparation; the final OxLDL concentration was 100 μg/ml. Cytotoxicity (open circles) was determined as described in Methods and is expressed as the percentage of total cellular radioactivity released into the supernatant. Total cellular radioactivity was measured after cell lysis in the presence of 1% Triton X-100. Basal release of [^{3}H]adenine in the absence of OxLDL was 6.8 ± 0.5%. After subtraction of background, cytotoxicity was normalized to the cell lysis measured in the presence of 100 μg/ml nonaggregated OxLDL (100% of control = 95.5 ± 1.0% release of radiolabel). Data shown are means ± SD.

result in a significant increase in cellular free cholesterol levels. However, AggOxLDL induced no significant changes in cellular free cholesterol levels (Fig. 4B), indicating that the majority of AggOxLDL associated with macrophages was both internalized and metabolized. Our results demonstrate that aggregation of OxLDL accelerates both its uptake and its degradation.

Uptake of AggOxLDL is blocked by AcLDL but not OxLDL

To determine the role of scavenger receptors in AggOxLDL uptake, we first measured the cell association of ^{3}H-labeled AggOxLDL in both the absence and presence of a 10-fold molar excess of unlabeled OxLDL. Surprisingly, OxLDL inhibited the cell association of [^{3}H]AggOxLDL by only 23%, indicating that either the uptake of OxLDL and AggOxLDL is mediated by different receptors or aggregation of OxLDL increases its avidity toward the receptors involved in its uptake.

Between 55% and 80% of cultured human macrophages express CD36 on the cell surface (25) .To determine whether CD36 contributes to the uptake of AggOxLDL, we eliminated CD36-positive cells from the cell suspension before cell plating using anti-CD36-coupled magnetic beads. We observed no difference in the cell association of [^{3}H]AggOxLDL between unselected macrophages and the CD36-negative macrophage subset, suggesting that CD36 is not required for [^{3}H]AggOxLDL uptake by human macrophages.

AcLDL is a scavenger receptor ligand with high selectivity for scavenger receptor A (33). Previously, we showed that AcLDL is a potent inhibitor of OxLDL uptake in human macrophages (15). In the presence of a 10-fold mo-

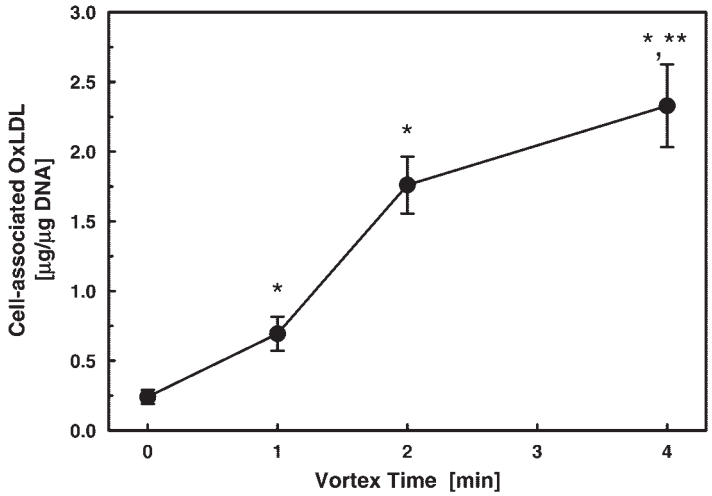

Fig. 2. Effect of aggregation on OxLDL uptake. [^{3}H]LDL was isolated from [^{3}H]cholesteryl oleoyl ether-loaded human plasma, oxidized, and aggregated by vortexing for the times indicated. Human macrophages were incubated with 20 μg/ml ^{3}H-labeled aggregated oxidized LDL (AggOxLDL) for 4 h at 37°C, washed extensively, and harvested by scraping with a rubber policeman. Nonspecific binding was measured in the presence of 1 mg of unlabeled AggOxLDL. Cell number was determined by DNA measurement. Lipoprotein uptake was determined as the amount of cell-associated radiolabel measured in lipid extracts by scintillation counting, as described in Methods. Data shown are means ± SD. * $P < 0.05$ versus nonaggregated OxLDL (0 min); ** $P > 0.05$ versus 2 min.

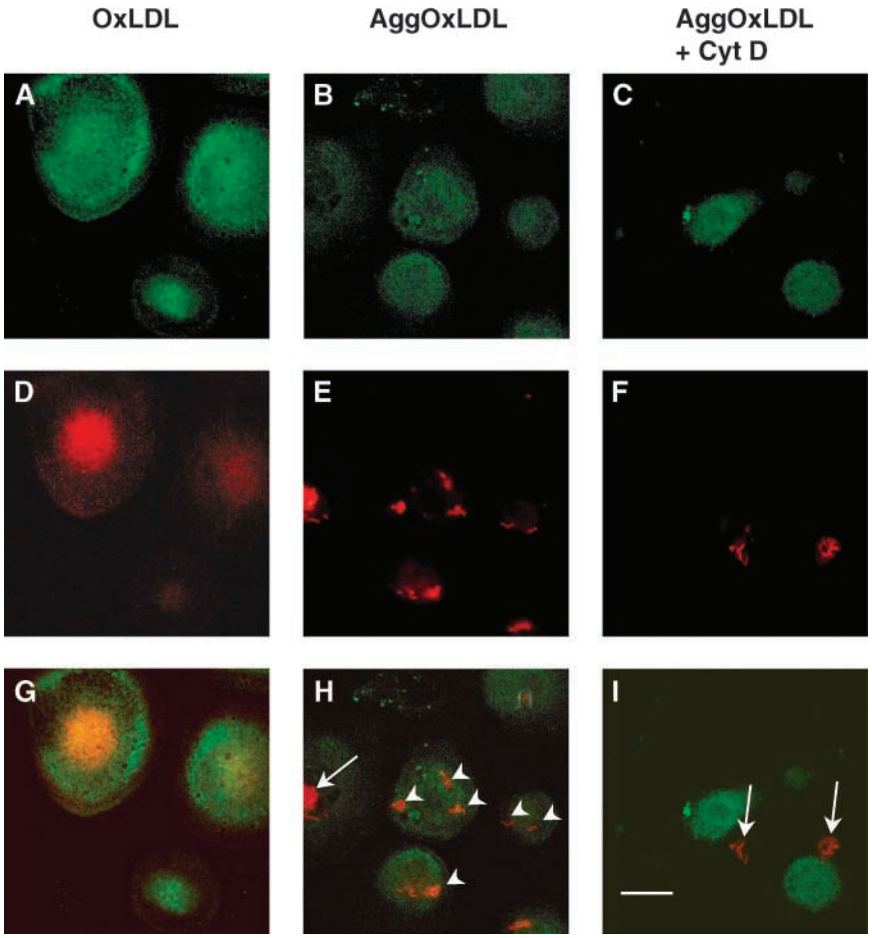

Fig. 3. Effect of cytochalasin D on AggOxLDL binding and internalization. Human macrophages were loaded for 1 h with Alexa488-dextran (500 μg/well; molecular weight 10,000), pretreated for 1 h with either vehicle (A, B, D, E, G, H) or 4 μM cytochalasin D (Cyt D; C, F, I), and then incubated for 4 h with either Alexa546-labeled OxLDL (20 μg/ml; A, D, G) or Alexa546-labeled AggOxLDL (20 μg/ml; B, C, E, F, H, I). Cells were washed, fixed, and mounted on cover slips. Images were acquired with a Leica TCS SP confocal microscope as described in Methods. A single representative slice through the midsection of the cells is shown. The signal from Alexa488-labeled dextran was pseudocolored green (A–C), and the signals from Alexa546-labeled OxLDL and AggOxLDL were pseudocolored red (D–F). Overlays are shown in G–I. Extracellular and intracellular AggOxLDLs are identified by arrows and arrowheads, respectively. The detector sensitivity and emission wavelength range were set independently for optimal special resolution of each fluorescence signal. For example, in D and G, detector sensitivity was set at 2–3 orders of magnitude greater than for E, F, H, and I to account for the difference in ligand uptake. Bar = 20 μm.

lar excess of unlabeled AcLDL, the cell association of ^{3}H-labeled AggOxLDL was blocked by 70% (**Fig. 5**), suggesting that scavenger receptor A plays an important role in the uptake of AggOxLDL by human macrophages.

Cytochalasin B and D are potent inhibitors of actin polymerization and phagocytosis (34–36). We found that pretreating macrophages for 30 min with cytochalasin D reduced the cell association of [^{3}H]AggOxLDL by 73% (Fig. 5) and blocked the internalization but not the binding of fluorescently labeled AggOxLDL (Fig. 3I), confirming that the majority of cell-associated AggOxLDL was indeed internalized by human macrophages. Our data also suggest that AggOxLDL uptake involves an actin-dependent mechanism. Finally, AcLDL and cytochalasin D inhibited the cell association of [^{3}H]AggOxLDL to the same extent, indicating that AggOxLDL is phagocytosed by human macrophages in a scavenger receptor A-dependent manner.

Inhibition of lipoprotein uptake restores the cytotoxicity of AggOxLDL

To determine whether blocking the uptake of AggOxLDL restores OxLDL toxicity in human macrophages, we pretreated human macrophages with cytochalasin D and latrunculin A, an inhibitor of actin polymerization structurally unrelated to the cytochalasins. Cells were then incubated with either OxLDL or AggOxLDL (i.e., OxLDL vortexed for 2 min). As shown in **Fig. 6**, the protection from cell injury conferred by the aggregation of OxLDL was lost and cytotoxicity was restored in macrophages pretreated with either cytochalasin D or latrunculin A. A third inhibitor of actin polymerization, cytochalasin B (21 μM), also restored

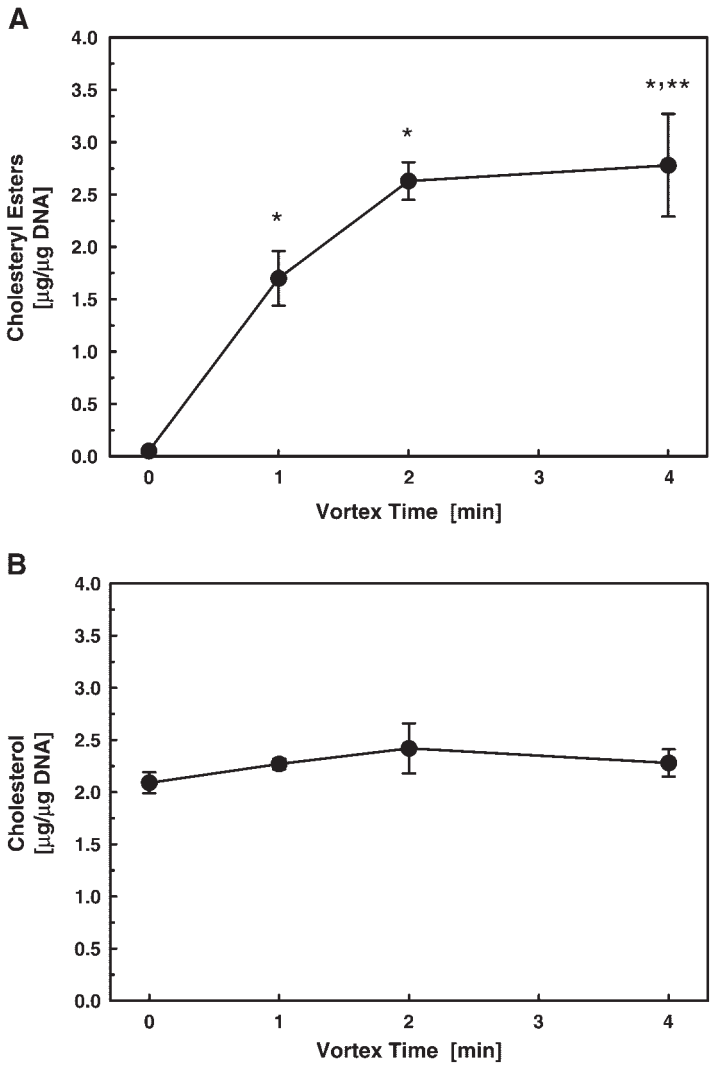

Fig. 4. Effect of aggregation on OxLDL degradation and foam cell formation. Human macrophages were incubated with OxLDL (100 μg/ml) and vortexed for the times indicated. After 4 days, cells were washed, harvested with a rubber policeman, and divided into aliquots for lipid extraction and DNA measurements. Lipids were separated by thin-layer chromatography, and cholesteryl esters (A) and cholesterol (B) were quantified as described in Methods. In macrophages incubated in the absence of OxLDL, cholesteryl ester and cholesterol levels were 0.04 ± 0.01 μg/μg DNA and 2.03 ± 0.20 μg/μg DNA, respectively. Data shown are means ± SD. * $P < 0.05$ versus nonaggregated OxLDL (0 min); ** $P > 0.05$ versus 2 min.

OxLDL cytotoxicity (data not shown). These data indicate that phagocytosis of AggOxLDL protects macrophages from cell death.

DISCUSSION

A large number of studies implicate OxLDL in vascular cell injury (11). However, the well-documented cytotoxicity of OxLDL in cultured human macrophages and foam cells has been difficult to reconcile with the apparent coexistence in vivo of OxLDL and macrophage-derived foam cells in developing lesions. If OxLDL is as cytotoxic in vivo as it is in vitro, why do necrotic cores form only late in lesion development (i.e., in advanced plaque)? Here, we show that the enhanced uptake of cytotoxic OxLDL induced by lipoprotein aggregation allows macrophages to escape cell injury. Our data suggest that the propensity of oxidatively modified LDL to aggregate and fuse may facilitate the macrophage-mediated clearance of the noxious material and thus protect vascular cells from OxLDL cytotoxicity.

Our findings have several implications for the mechanisms of OxLDL cytotoxicity in macrophages. First, uptake of OxLDL does not appear to be required to induce macrophage injury. Under conditions that prevent AggOxLDL internalization, OxLDL cytotoxicity was increased, not decreased, and AggOxLDL was as cytotoxic as nonaggregated OxLDL. Our results in primary human macrophages, therefore, contrast with results obtained in studies performed in macrophage-like cells and other cell lines showing that OxLDL-induced cell injury involves lysoso-

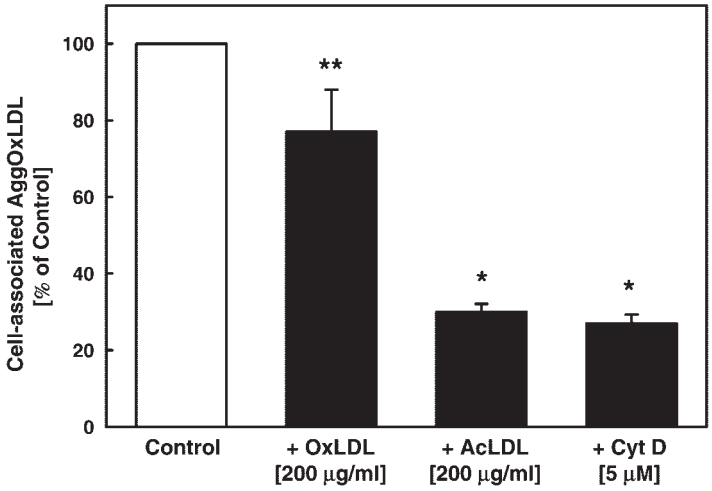

Fig. 5. Effect of cytochalasin D (Cyt D), acetylated LDL (AcLDL), and OxLDL on AggOxLDL uptake. [^{3}H]LDL was isolated from [^{3}H]cholesteryl oleoyl ether-loaded human plasma, oxidized, and aggregated by vortexing for 2 min. Macrophages were preincubated for 30 min with either vehicle or 8 μM cytochalasin D and incubated for 4 h at 37°C with 20 μg/ml ^{3}H-labeled AggOxLDL. Where indicated, a 10-fold excess (200 μg/ml) of OxLDL or AcLDL was added together with the ^{3}H-labeled AggOxLDL. Cells were washed extensively and harvested by scraping with a rubber policeman. Nonspecific binding was measured in the presence of 1 mg of unlabeled AggOxLDL. Cell number was determined by DNA measurement. Lipoprotein uptake was determined as the amount of cell-associated radiolabel measured in lipid extracts by scintillation counting, as described in Methods. Data shown are means ± SD; 100% = 1.1 ± 0.2 μg protein/μg DNA. * $P <$ 0.05 versus control; ** $P < 0.05$ versus AcLDL.

mal damage and therefore appears to require OxLDL uptake (37, 38). Although we cannot exclude the possibility that OxLDL uptake by nonphagocytic mechanisms (e.g., endocytosis) may contribute to OxLDL-induced cell injury, our results strongly suggest that the primary insult induced by OxLDL in macrophages (i.e., cell damage by OxLDL or transfer of OxLDL-derived cytotoxins) occurs extracellularly, possibly at the plasma membrane.

The mechanisms involved in macrophage death are not clear. LDL oxidation generates a large number of cytotoxins, such as reactive aldehydes, lysophospholipids, and oxysterols (for review, see 11). Although most OxLDL-derived cytotoxins appear to activate caspase-dependent apoptotic pathways, some oxysterols, including 7β-hydroperoxy cholesterol, induce cell lysis (39) and therefore may play a role in the caspase-independent cell death induced by OxLDL in primary human macrophages (13). Cytotoxins such as 7β-hydroperoxy cholesterol could enter the cell either by diffusing into the plasma membrane or by selective uptake mechanisms (e.g., via scavenger receptor class B type I) (40, 41). Alternatively, OxLDL, once in close proximity to the cell surface, may directly (per)oxidize plasma membrane lipids and hence induce the formation of potential cytotoxins on the cell surface. This mechanism is supported by our previous finding that peroxyl radical scavengers protect human macrophages from OxLDL cytotoxicity (13). It is also conceivable that OxLDL oxidizes membrane proteins. Various aldehydes formed during LDL oxidation, including 4-hydroxynonenal, can modify proteins by reacting with lysine residues to form Schiff bases (42, 43) or with cysteine residues to form thioether conjugates (42). Oxidation of cholesteryl esters in LDL was shown to promote the formation of cholesteryl ester core aldehydes and covalent adducts with apolipoprotein B-100 (44). A similar reaction may occur on the cell surface and result in the loss of membrane integrity. We found that 4-hydroxynonenal, like OxLDL, induces cell lysis in human macrophages and mimics several other aspects of OxLDL cytotoxicity, including the depletion of intracellular glutathione (R. Asmis, unpublished data), suggesting that lipid peroxide-derived alkenals may be involved in OxLDL toxicity in human macrophages. However, in contrast to OxLDL, 4-hydroxynonenal toxicity in macrophages was not blocked by Trolox, suggesting that 4-hydroxynonenal may

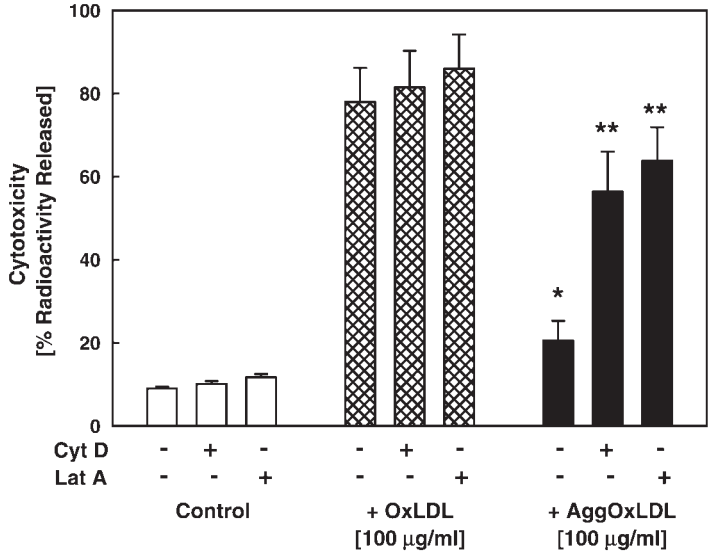

Fig. 6. Effect of cytochalasin D (Cyt D) or latrunculin A (Lat A) on OxLDL cytotoxicity. [^{3}H]adenine-loaded macrophages were preincubated with vehicle, cytochalasin D (4 μM), or latrunculin A (2 μM) for 1 h (open bars) and subsequently stimulated for 48 h with either OxLDL (hatched bars) or OxLDL vortexed for 2 min (AggOxLDL; closed bars). The final OxLDL concentration was 100 μg/ml. Cytotoxicity was determined as described in Methods and is expressed as the percentage of total cellular radioactivity released into the supernatant. Total cellular radioactivity was measured after cell lysis in the presence of 1% Triton X-100. Data shown are means ± SD. * $P >$ 0.05 versus control; ** $P < 0.05$ versus AggOxLDL.

not be the actual toxin in OxLDL. Finally, membrane Na^+/K^+ and Ca^{2+} pump ATPases are sensitive to oxidative inactivation, and failure of these ion pumps would increase membrane permeability and result in cell lysis. Further studies are needed to elucidate the mechanism involved in OxLDL-induced macrophage lysis.

Our results show that phagocytosis of cytotoxic OxLDL aggregates protects human macrophages from cell damage. Several receptors present on human macrophages bind OxLDL, including scavenger receptor A and CD36, suggesting that multiple pathways may be involved in AggOxLDL uptake and processing (33). However, our data suggest that scavenger receptor A is the primary scavenger receptor involved in the clearance of AggOxLDL by human macrophages.

Interestingly, peroxyl radical formation induced by OxLDL (13) was not decreased by aggregation (data not shown). Why phagosomes appear to be resistant to the lytic properties of OxLDL is not clear, because the limiting membrane of the phagosome immediately after formation still closely resembles the plasma membrane (45). However, shortly after sealing, the phagosome undergoes massive changes in its composition, and in the course of this maturation process the phagosome acquires its bactericidal ability. Compared with the rapid uptake of AggOxLDL, OxLDL-induced macrophage death is a slow process, lasting 24–48 h (13). Hence, it is conceivable that damage induced by OxLDL to the phagosomal membrane is repaired in the course of phagosome maturation. Analysis of the phagosome proteome reveals that the phagosome acquires a number of proteins that may participate in the degradation of AggOxLDL, such as cholesteryl ester hydrolase and a large number of proteases (46). More importantly, during maturation, the phagosome also recruits antioxidant enzymes, such as thioreductase peroxidase 2 and gamma-Interferon-inducible lysosomal thiol reductase, a thiol reductase, that may prevent further oxidative damage to the phagosome membrane induced by AggOxLDL-derived oxidants. The combined effect of AggOxLDL degradation, membrane repair, and the presence of antioxidant enzymes may explain the relative resistance of phagosomal membranes to OxLDL-induced damage compared with the plasma membrane.

In summary, our results show that aggregation of cytotoxic OxLDL enhances the uptake and metabolism of OxLDL by human macrophages but decreases OxLDL-induced cell injury. Even though aggregation of OxLDL would be expected to promote foam cell formation in atherosclerotic lesions, our data suggest that the enhanced clearance by macrophages of cytotoxic extracellular OxLDL is likely to reduce vascular cell injury and necrotic core formation and hence delay the progression of early atherosclerotic lesions into advanced unstable plaques prone to rupture.

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