The Pathogenesis of Foam Cell Formation
Modified LDL Stimulates Uptake of Co-Incubated LDL
Via Macropinocytosis

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Abstract—Previously, modified LDLs were shown to stimulate macropinocytosis in pigeon macrophages. Simultaneous intracellular trafficking of LDL and AcLDL, differentially labeled with colloidal gold, was done to determine whether uptake of LDL, which does not cause foam cell formation, was internalized via a separate route from AcLDL, which stimulates foam cell formation. AcLDL and LDL were followed at either low (12 μg/mL) concentrations near the saturation of high affinity binding sites or high (50 to 150 μg/mL) lipoprotein concentrations used to induce foam cell formation. The colloidal gold distribution and percentage of co-labeling as observed by transmission electron microscopy were determined for organelles involved with coated-pit endocytosis or macropinocytosis. LDL simultaneously incubated with AcLDL on macrophages at the low concentration was predominately internalized via coated-pit endocytosis. AcLDL was internalized via both coated-pit endocytosis and macropinocytosis at low concentration. At higher lipoprotein concentrations (50 to 150 μg/mL), AcLDL continued to be internalized via macropinocytosis. Interestingly, a significant portion of the co-incubated LDL, at high concentrations, also trafficked via macropinocytosis. LDL internalized by macropinosomes at high lipoprotein concentrations suggests that AcLDL-stimulated macropinocytosis might increase uptake of co-incubated lipoproteins. When 125I-LDL was incubated with cold AcLDL, LDL degradation at 37°C doubled, without a corresponding increase in cell association or total binding of LDL at 4°C. These studies suggest that modified LDL-stimulated macropinocytosis is a mechanism for increased degradation of co-incubated LDL potentially leading to foam cell formation. (Arterioscler Thromb Vasc Biol. 2000;20:773-781.)

Key Words: lipoproteins ■ endocytosis ■ macropinocytosis ■ foam cell formation

There is a generally accepted scenario for macrophage foam cell formation. Initially, lipoproteins are internalized by coated-pit endocytosis. These internalized lipoproteins use receptors that are not down-regulated by increases in cellular cholesterol concentrations. One such unregulated receptor is the scavenger receptor(s).1,2 The receptor-mediated internalized lipoproteins are then delivered to lysosomes where the cholesteryl ester is hydrolyzed to free cholesterol by the action of acid cholesterol esterase.3 Free cholesterol, perhaps transported to the plasma membrane,4 is re-esterified by acyl coenzyme A: cholesterol acyltransferase (ACAT) and stored as cholesterol ester in intracellular lipid droplets. The cholesterol ester can be hydrolyzed by neutral cholesterol esterases, and the free cholesterol can be released from the cell if an acceptor is available. An imbalance in the amount of cholesterol retained within the cell and the amount released to extracellular acceptors is believed to cause foam cell formation.

Uptake of native LDL alone does not cause in vitro foam cell formation.5 In short-term experiments, macrophages internalize ample LDL, but the cholesterol is unable to stimulate esterification.6 Uptake of LDL, and subsequently internalized cholesterol, leads to down-regulation of LDL receptors. An apparent quandary with the foam cell formation literature is that LDL, a reliable risk factor for predicting heart disease, does not cause macrophage foam cell formation in vitro. The presence of modified lipoproteins within the artery, and in vitro macrophage foam cell formation by uptake of these modified lipoproteins, has been suggested to explain this disparity.7

Pigeon blood monocytes have been cultured in vitro as a model of monocyte-derived foam cells. Like human macrophages,3 they can be stimulated by endocytosis of either βVLDL, AcLDL, or OxLDL to accumulate cholesterol esters resulting in a foam cell-like appearance,8-10 whereas native LDL does not cause cholesterol ester accumulation. Pigeon AcLDL is internalized in part by coated-pit endocytosis. However, AcLDL also stimulates macropinocytosis and is internalized in part by macropinocytosis.11,12 This stimulation of macropinocytosis by modified LDLs suggests an intriguing hypothesis for foam cell formation; the presence of some modified LDL may affect the internalization or intracellular trafficking of co-incubated LDL.
normally internalized by coated-pit endocytosis. The present study used simultaneous labeling methodology to determine whether LDL co-incubation with AcLDL was internalized via macropinocytosis.

Figure 1. AcLDL and LDL uptake at low concentrations. AcLDL 18 nm gold conjugate (small gold) and LDL 48 nm gold conjugated (large gold) were each incubated at 12 μg/mL for 2 hours at 4°C. A, B, and E, Macrophages were incubated for 10 minutes at 37°C 10 minutes or for 60 minutes (C, D, and F). A, The plasma membrane is highly ruffled at 10 minutes with both AcLDL (small gold) and LDL (large gold) present on ruffled portions of the membrane. AcLDL was present in a vesicular profile (arrow) and present with LDL in a macropinosome (MP). B, AcLDL and LDL were present in a single coated pit (arrow). E, The plasma membrane is highly ruffled at 10 minutes with both AcLDL (small gold) and LDL (large gold) present on ruffled portions of the membrane (large arrow). LDL was present in a vesicle and early endosomes (small arrows), whereas a large portion of AcLDL was in a macropinosome (MP). F, At 60 minutes, some lipoprotein was still on the plasma membrane with some AcLDL in or near coated pits (small arrows). More lipoprotein was present in a macropinosome (MP), spherical endosomes (SE), and lysosomes (L). Some lysosomes from this micrograph are further enlarged in C and D. C, These lysosomes only contain AcLDL (small gold), whereas others have both AcLDL and LDL present with in a single lysosome. Bars=0.5 μm. B, C, and D are the same magnification.

Figure 2. AcLDL and LDL uptake at high concentrations. A, AcLDL 37 nm gold conjugate (large) and LDL 14 nm gold conjugated (small) were each at 50 μg/mL for 2 hours at 4°C. B, C, and D, Macrophages were washed and incubated with AcLDL 17 and LDL 32 nm gold conjugate. B and C, Macrophages were incubated at 37°C for 5 minutes and D, 120 minutes. A, At time <1 minute, the plasma membrane was highly ruffled. Large macropinosomes were seen with both LDL or AcLDL present. An organelle designated by diameter as an early endosomes (EE) more likely was a macropinosomes formed directly under the membrane ruffled region. B, This macrophage had lipoprotein at 5 minutes in macropinosomes, coated vesicle (arrows), and early endosomes (EE). Note the membrane ruffle had both AcLDL and LDL present and macropinosomes were present in the membrane ruffle (arrowhead). C, A region from B was enlarged showing a large macropinosome with both AcLDL and LDL was present neighboring a coated pit containing LDL (arrow). D, A highly condensed lysosome is present. However, note that at 120 minutes some lipoprotein is on the plasma membrane associated with a membrane ruffle. Several large macropinosomes are also present at 120 minutes. This data are consistent with the time lapse video microscopy experiments in which AcLDL stimulated macropinosome formation continued throughout the first 2 hours of incubation with AcLDL. Bars=0.5 μm.

Methods

Macrophage Culture and Lipoproteins

White Carnea pigeon monocyte derived macrophages and adherent THP-1 cells were cultured for 7 days, as previously described. Hypercholesterolemic LDL (d≤1.006 and d≤1.080) was isolated.
from White Carneau pigeons and acetylated by the method of Basu et al. Lipoproteins (20 to 50 µg/mL) were conjugated to colloidal gold (small, 14 or 17 nm; large, 32 or 37 nm) and prepared as described in detail in the online publication (http://atvb.ahajournals.org/cgi/content/full/20/3/773/DC1).

Simultaneous Trafficking of Lipoproteins

Macrophages were incubated at either low (12.5 µg/mL) or high (50 to 150 µg/mL) equal protein concentrations of AcLDL and LDL. Cells were washed twice with ice-cold phosphate buffered saline (PBS) and then incubated with labeled lipoproteins for 2 hours at 4°C. The cells were then washed twice with PBS at 4°C. Media prewarmed to 37°C was added, and the cells were incubated at 37°C for 1, 5, 10, 30, 60, and 120 minutes. Cells were fixed with 2.5% glutaraldehyde, removed from the plates by scraping, and embedded in Spurr’s for standard, transmission electron microscopy. Silver thin sections (60 to 80 nm) were visualized at 80 keV in a Philips EM-400 transmission electron microscopy. Three experiments were done for the low (12.5 µg/mL) concentrations of lipoproteins, and 3 were done for the high (50 to 150 µg/mL) concentrations.

The colloidal gold distribution was determined for the following organelles of macrophages: plasma membrane, clathrin-coated pits and vesicular profiles, early endosomes, macropinosomes, spherical endosomes, late endosomal-prelysosomal tubuloreticular compartment (LEPT), and lysosomes. The time course is plotted for (A) LDL at low concentrations, (B) AcLDL at low concentrations, (C) LDL at high concentrations, and (D) AcLDL at high concentrations. Data for the plasma membrane and pits and vesicular profiles are shown separately in E and F, respectively. The percentage gold distribution for AcLDL and LDL was averaged for [Low] (solid line) and [High] (dashed line).

Analysis of Co-Labeling

The co-labeling of all organelle types, except the plasma membrane, was analyzed by the percentage of the labeled lipoprotein in the co-labeled organelles. The lipoprotein in the co-labeled organelles was divided by the total lipoprotein in each organelle type. The percentage of lipoprotein in the co-labeled organelles did not differ with time. Therefore, data were averaged for each organelle despite time. The nonparametric Wilcoxon paired t test was used to analyze significant differences between the lipoprotein pairs for each organelle. To determine whether the co-labeling of AcLDL or LDL was different between the following 2 organelle types, LEPTs and lysosomes, data were analyzed by a nonparametric unpaired t test (Instat Version 3.00, GraphPad Software Inc). Nonparametric multiple comparisons were made by the Kruskal-Wallis analysis to determine whether the co-labeling of organelles was different among the organelle types. When the null hypothesis was rejected, significant differences at the P<0.05 level between organelles were determined using a nonparametric Tukey-type comparison.

AcLDL Affect on Binding, Internalization, and Degradation of LDL

Macrophages were prechilled for 30 minutes at 4°C, before binding at 4°C for 2 hours. To determine whether AcLDL would affect the binding and degradation of LDL, a constant concentration of 125I-LDL (50 µg/mL) was added in the presence of either no other lipoprotein (control), AcLDL (12 µg/mL), or LDL (12 µg/mL). A 50-fold excess of unlabeled LDL (2.5 mg/mL) was added to determine nonspecific binding. After washing the macrophages 3 times with ice-cold PBS, the binding at 4°C was determined by
AcLDL and LDL Trafficking: Coated-Pit Endocytosis Versus Macropinocytosis

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<td>AcLDL</td>
<td>0.7±0.1*</td>
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<td>AcLDL</td>
<td>0.5±0.3†</td>
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The percent distribution was compiled for the <1, 5, and 10 minute time points to determine whether there were differences in the distribution of AcLDL versus LDL in organelles of coated-pit endocytosis: pits and vesicular profiles (P&amp;V), endosomes (EE), and spherical endosomes (SP) and the organelle of macropinocytosis: macropinosomes (MP). The distributions were averaged for <1, 5, and 10 minutes. The distribution was determined for trafficking at [Low] (12 μg/mL) and [High] (50 to 100 μg/mL) lipoprotein concentrations. Significant differences in the percent distribution of AcLDL versus LDL at the <0.05 level are indicated by an asterisk. At high concentration, the difference between AcLDL and LDL was approaching statistical significance (p = 0.09) as indicated by †.

measuring the cell-associated radioactivity after digestion of the cells with 1 N NaOH. The protein concentration was determined by the method of Lowry.

For degradation and internalization studies, macrophages were incubated with 125I-LDL (100 μg/mL) with either OxLDL, LDL, or AcLDL (20 μg/mL) for 2 or 5 hours to determine whether AcLDL could alter the uptake and degradation of co-incubated LDL. Macrophages were preincubated with Ringer’s Buffer (155 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2, 2 mmol/L Na2HPO4, 10 mmol/L glucose, 10 mmol/L Hepes, pH 7.2, 0.5 mg/mL bovine serum albumin) for 30 minutes at 37°C. Degradation was determined by measuring the trichloroacetic acid–soluble, non-iodide radioactivity in the media. The cell layer was washed extensively and digested with 1 N NaOH overnight. An aliquot was counted in a Beckman gamma counter to determine cell-associated 125I-radioactivity, and another aliquot was used to determine protein concentration. Counts were adjusted by subtracting degradation of lipoproteins on plates with no cells. The results were expressed as amount bound, cell-associated, and degraded per μg cell protein.

Results

Low Concentration Trafficking of AcLDL+LDL

LDL is internalized by coated-pit endocytosis, whereas AcLDL is internalized by both coated-pit endocytosis and macropinocytosis in pigeon macrophages.12 We hypothesize that ligands internalized by macropinocytosis will merge with ligands internalized by coated-pit endocytosis at the LEPT (please see Figure 1, published online at http://atvb.ahajournals.org/cgi/content/full/20/3/773/DC1). In the present study, a time course of simultaneous trafficking was done for LDL and AcLDL conjugated to different sized colloidal gold particles. Macrophages were incubated with lipoproteins at 12.5 μg/mL to examine the high affinity uptake of lipoproteins at <1, 5, 10, 30, 60, and 120 minutes (Methods).

The plasma membrane surface was highly convoluted and ruffled at both low and high concentrations of lipoproteins (Figures 1 and 2). Approximately 70% of AcLDL and LDL was on the plasma membrane at time zero at low concentrations (Figure 3A). Correspondingly, at <1 minute, 30% of AcLDL and LDL was in early endocytic organelles such as clathrin-coated pits and vesicular profiles, early endosomes, and macropinosomes. From 5 to 10 minutes, the percent distribution of lipoproteins on the plasma membrane increased slightly (Figure 3A). This elevation of lipoprotein, AcLDL, and LDL on the plasma membrane at 10 minutes might reflect unruffling of the plasma membrane. With time, the percentage of AcLDL and LDL on the plasma membrane progressively decreased until only 18% remained on the plasma membrane at 120 minutes (Figure 3E).

AcLDL and LDL were present in coated-pits, endosomes, and macropinosomes at early times (Figure 1). AcLDL was more frequently seen in macropinosomes than LDL. Organelles consistent with only coated-pit endocytosis are clathrin-coated pits, early endosomes, and spherical endosomes. Organelles consistent only with macropinocytosis are macropinosomes (Figure 1 published online at http://atvb.ahajournals.org/cgi/content/full/20/3/773/DC1). The distribution of AcLDL and LDL were compared in these organelles to determine whether uptake was via coated-pit endocytosis or macropinocytosis. At <1 through 10 minutes, the distribution of LDL was consistently increased in pits and vesicular profiles, endosomes, and spherical endosomes compared with the distribution of AcLDL (Table). The distribution of LDL was 7-, 3-, and 3-fold increased versus AcLDL in pits, endosomes, and spherical endosomes, respectively. Conversely, the distribution of AcLDL in macropinosomes was 7-fold greater than for LDL (Table and Figure 3). At 5 and 10 minutes, the distribution of AcLDL was significantly greater than LDL in macropinosomes with 32% and 17% of AcLDL compared with 7% and 1% of LDL, respectively (Figure 3).

Time Course (Low Concentration)

Generally, LDL moved more rapidly through the endocytic organelles than AcLDL (Figure 3). The time course for maximal distribution of LDL was 0 to 10 minutes for coated pits, 0 to 10 minutes for early endosomes, 5 to 10 minutes for spherical endosomes, 30 to 120 minutes for LEPT, and 120 minutes for lysosomes. The distribution of LDL peaked in macropinosomes at 30 minutes. The time course for maximal distribution of AcLDL was 0 to 10 minutes for coated pits, 10 to 30 minutes for early endosomes, 10 to 60 minutes for spherical endosomes, 60 to 120 minutes for LEPT, and 120 minutes for lysosomes. AcLDL was present in macropinosomes through 30 minutes.

Co-Trafficking (Low Concentration)

The simultaneous incubation of lipoprotein pairs for this experiment was designed to detect if lipoproteins trafficked through the same endocytic organelles or whether these pathways remain separate. The quantitative data support the hypothesis that most of LDL trafficks via coated-pit endocytosis with little LDL present in macropinosomes (Figure 3A). A significant portion of AcLDL is present both in macropinosomes and endosomes, indicating that AcLDL trafficks in part by coated-pit endocytosis and in part by macropinocytosis (Figure 3B). The co-labeling data also support this conclusion. At low concentrations of LDL+AcLDL, trafficking of AcLDL was more segregated from LDL in the coated-pit endocytic pathway. AcLDL had a significantly lower extent of co-labeling than LDL in clathrin-coated pits, early endosomes, and spherical endosomes (Figure 4A). These data indicate that at low concentrations, 60% of AcLDL trafficks through separate early endosomes and...
Co-Trafficking of LDL + AcLDL

A. [Low] - High Affinity

B. [High] - Foam Cell

Figure 4. Co-Trafficking of LDL + AcLDL. The percentage of lipoprotein in co-labeled organelles was determined for A (Low) and B (High) concentration experiments (see Methods). Co-labeling was determined for the following organelles: clathrin-coated pits and vesicular profiles (PV), early endosome (EE), spherical late endosomes (SE), late endosomal-prenysosomal tubuloreticular compartment (LEPT), lysosomes (LY), and macropinosomes (MP). Asterisk designates a significant difference at the \( P < 0.05 \) level between AcLDL and LDL in PV, EE, MP, and SE at low concentrations. @ designates a significant difference \( P < 0.05 \) level in the percent of co-labeling in LEPT versus LY for LDL and AcLDL at high concentrations.

AcLDL was twice as likely to co-traffick with LDL via macropinocytosis (macropinosomes) than coated-pit endocytosis (coated-pits, early endosomes, and spherical endosomes). The greatest extent of co-labeling in any organelle occurred in macropinosomes; if LDL was present in macropinosomes, 82% of the time AcLDL was also present. Previously, the greatest extent of co-trafficking possible with this co-trafficking methodology at this lipoprotein concentration was 80% to 90%. Therefore, 100% of LDL co-trafficked with AcLDL. This suggests that for LDL to traffic in macropinosomes, AcLDL must be present.

High Concentration Trafficking of AcLDL + LDL

At high AcLDL concentrations, the hypothesis is that the pathway associated with foam cell formation, either coated-pit endocytosis or macropinocytosis, will have a greater distribution of AcLDL. LDL at high concentrations should be primarily internalized by coated-pit endocytosis, unless AcLDL-stimulated macropinocytosis influences LDL uptake. A time course of simultaneous trafficking was done for LDL + AcLDL at concentrations of lipoproteins that stimulate foam cell formation 50 to 150 \( \mu \)g/mL.

At <1 minute, the plasma membrane surface was highly convoluted and ruffled (Figure 2A). At high concentrations, 65% of AcLDL and LDL was on the plasma membrane at <1 minute (Figure 3). At 5 minutes, the distribution of both AcLDL and LDL on the plasma membrane dropped to 40%. At 10 minutes, the distribution of lipoproteins on the plasma membrane increased to 50% (Figure 3). This dramatic drop at 5 minutes and subsequent increase at 10 minutes probably reflects membrane ruffling activity. Some of AcLDL on membrane ruffles might have appeared internalized within the cell; when the membrane ruffles unfolded, an elevation of lipoproteins on the plasma membranes at 10 minutes resulted. With time, the distribution of AcLDL and LDL decreased on the plasma membrane until 30 minutes. After that, the distribution of lipoproteins on the plasma membrane remained stable or perhaps slightly increased with 35% of AcLDL and LDL remaining associated with the plasma membrane at 120 minutes (Figure 2). This elevation in the distribution of lipoproteins on the plasma membrane at these later times may reflect regurgitation from macropinosomes. Time-lapse phase contrast microscopy showed that during AcLDL incubation, both nonfusion of membrane ruffles and regurgitation of macropinosomes occurred.

AcLDL and LDL were present in coated-pits, endosomes, and macropinosomes at early times (Figure 2). The distribution of AcLDL and LDL was compared in organelles consistent with coated-pit endocytosis versus macropinocytosis to determine whether AcLDL and LDL were taken up by coated-pit endocytosis versus macropinocytosis (Table). LDL was 5- and 2-fold increased versus AcLDL in the percent distribution in pits and vesicles and spherical endosomes, respectively. LDL and AcLDL had the same distribution to early endosomes with 17% and 20%, respectively, in early endosomes at <1 to 10 minutes. This was a greater distribution of AcLDL and LDL to the early endosomes than at low concentrations.

At <1 to 10 minutes, the same proportion of AcLDL and LDL (24%) was present in macropinosomes (Table). Comparing trafficking of LDL at 5 and 10 minutes showed 28%
and 27% of LDL trafficked in macropinosomes at high concentrations compared with 7% and 1% of LDL at low concentration. The same distribution of AcLDL trafficked in macropinosomes at high and low concentrations. These data indicated that when AcLDL is present at high concentrations, a significant portion of LDL trafficks via macropinocytosis. At high concentrations of AcLDL, there was a shift toward more trafficking via macropinocytosis. The total volume of AcLDL present in macropinosomes over the entire time course was elevated, whereas the distribution of AcLDL in coated pits and vesicles and spherical endosomes were decreased (Figure 3).

Time Course (High Concentration)

The time course for maximal distribution of LDL at high concentrations was 0 to 5 minutes for pits and vesicular profiles, 0 to 10 minutes for early endosomes, 10 to 30 minutes for spherical endosomes, 10 to 60 minutes for LEPT, and 120 minutes for lysosomes. The time course for maximal distribution of AcLDL at high concentrations was 10 minutes for pits and vesicles, 0 to 10 minutes for early endosomes, 60 to 120 minutes for spherical endosomes, 10 to 60 minutes for LEPT, and 120 minutes for lysosomes (Figures 3). A greater proportion of the input lipoprotein (15%) reached the lysosomes at low versus high concentrations of lipoproteins. This difference in distribution to the lysosomes could be accounted for by the distribution of lipoproteins remaining associated with the plasma membrane at 120 minutes. Approximately 18% versus 35% of the input lipoprotein remained associated with the plasma membrane at low versus high concentrations. The time course of residence in macropinosomes doubled at high concentrations; AcLDL and LDL were present in macropinosomes for 60 minutes versus 30 minutes at low concentration.

Co-Trafficking (High Concentration)

The quantitative data suggest that at high concentrations, AcLDL continued to be internalized via coated-pit endocytosis and macropinocytosis. However, LDL was now internalized in part via macropinocytosis as well as coated-pit endocytosis. The co-labeling data suggest that at high lipoprotein concentrations, no segregation of AcLDL trafficking from LDL trafficking occurred (Figure 4B). At high concentrations, the extent of co-labeling was greater than at low concentrations in clathrin-coated pits with 30% to 50%, but greater co-labeling was seen in the rest of the organelles. The percentage distribution in coated pits, early endosomes, and spherical endosomes were 61% and 56% for AcLDL and 72% and 53% for LDL, respectively. Macropinosomes had the greatest extent of co-labeling with 75% and 85% for AcLDL and LDL, respectively. Interestingly, nearly all of the lipoproteins were mixed in the LEPT compartment, with 92% of AcLDL and 88% of LDL in co-labeled organelles. lysosome co-labeling was less than the LEPT compartment with 62% of AcLDL and 51% of LDL in co-labeled lysosomes. Previously, the LEPT compartment has been designated as an organelle where fusion and segregation into distinct lysosomes occurs.21 This suggests that these lipoproteins are sorted to different lysosomes.

Lipoprotein Binding Sites

Previously, AcLDL was shown to bind predominately to membrane ruffles and smooth portions of the membrane, whereas LDL bound to microvilli.15 At both low and high concentrations, AcLDL bound predominately to membrane ruffles and smooth areas of the plasma membrane. However, when LDL was incubated with AcLDL, at both low and high concentrations, LDL bound predominately to membrane ruffles (please see Figure II, published on-line at http://atvb.ahajournals.org/cgi/content/full/20/3/773/DC1). The shift of LDL binding to membrane ruffles probably reflects the stimulation of membrane ruffling stimulated by AcLDL.11,12

Enhanced Degradation of LDL Co-Incubated With AcLDL and OxLDL

Co-incubation of LDL with AcLDL caused a shift in the intracellular trafficking of LDL. Potentially, this could result in internalization of LDL via fluid-phase or adsorptive endocytosis during AcLDL stimulated macropinocytosis. To determine whether AcLDL affects the degradation of co-incubated LDL, 125I-LDL was incubated with cells in the presence of AcLDL. First, the binding was determined for 125I-LDL (50 μg/mL) alone and with co-incubated cold AcLDL or LDL (12 μg/mL). The total binding and specific binding of LDL were reduced with AcLDL and LDL present in pigeon macrophages (Figure 5) and THP-1 cells (data not shown). In some experiments, nonspecific binding of LDL increased, which may be due to trapping in AcLDL stimulated membrane ruffles (data not shown).

To determine whether AcLDL-stimulated macropinocytosis could increase the uptake and subsequent degradation of LDL, 125I-LDL was incubated with macrophages at 100 μg/mL, a concentration above saturation of high affinity receptors. Cold AcLDL (20 μg/mL) was added to stimulate macropinocytosis during incubation of 125I-LDL. A control condition was co-incubation with cold LDL (20 μg/mL). The 125I-LDL in the cell associated fraction (Figures 5B and 5C) was not increased with AcLDL or LDL in pigeon macrophages at 2 hours (data not shown) or 5 hours (Figure 5B). However, the degradation of 125I-LDL was increased when AcLDL was present (Figure 5B).

OxLDL also stimulates macropinocytosis in pigeon macrophages.12 OxLDL also increased the degradation of 125I-LDL, with no affect on the 125I-LDL in the cell associated fraction (Figure 5C). OxLDL also stimulated the degradation of 125I-LDL in THP-1 macrophages. In THP-1 cells, OxLDL decreased the cell association of 125I-LDL (Figure 5D). Incubation 125I-LDL with cold AcLDL also decreased the cell association but had no affect on the degradation of co-incubated 125I-LDL in THP-1 macrophages (data not shown).

Discussion

The time course for AcLDL and LDL internalization at low concentrations via receptor-mediated endocytosis is similar to other examples of coated-pit endocytosis reported in the literature. Interaction of AcLDL and LDL with receptors leads to uptake via coated-pit endocytosis at the base of microvilli on the plasma membrane. The contents of multiple pits and their derivative endosome vesicles fuse with each other by <1 to 10 minutes.17–20 These early endosomes pass on their contents or mature to spherical endosomes by 5 to 10 minutes. Multiple spherical endosomes may fuse with the
LDL) (20 μg/mL), LDL or OxLDL (100 μg/mL) for 5 hours at 37°C with buffer (LDL) or AcLDL (+AcLDL) or LDL (+LDL) (20 μg/mL). Cell association (Cell Asoc) and degradation (degraded) was determined as described in Methods. Data are compiled from 4 experiments with triplicates in each experiment. Asterisk shows significant difference in total binding compared with LDL alone. B, Affect of LDL Degradation. Pigeon macrophages were incubated with 125I-LDL (100 μg/mL) for 5 hours at 37°C with buffer (LDL) or AcLDL (+AcLDL) or LDL (+LDL) (20 μg/mL). Cell association (Cell Asoc) and degradation (degraded) was determined as described in Methods. Data are compiled from 4 experiments with triplicates in each experiment. Asterisk shows significant difference in degradation (degraded) between LDL and AcLDL and LDL.

Figure 5. Affect of AcLDL on LDL binding, uptake, and degradation. A, LDL Binding. Pigeon macrophages were preincubated for 30 minutes at 4°C before binding at 4°C for 2 hours under the following conditions: LDL; 125I-LDL (50 μg/mL) plus 12 μg/mL of unlabeled LDL, LDL or AcLDL, LDL and 125I-LDL (50 μg/mL) plus 12 μg/mL unlabeled LDL. These binding studies were done in the absence (Total) and presence of (Nonspecific) of 50X excess of unlabeled LDL (2.5 mg/mL). Asterisk shows significant difference in total binding compared with LDL alone. B, Affect of AcLDL on LDL Degradation. Pigeon macrophages were incubated with 125I-LDL (100 μg/mL) for 5 hours at 37°C with buffer (LDL) or AcLDL (+AcLDL) or LDL (+LDL) (20 μg/mL). Cell association (Cell Asoc) and degradation (degraded) was determined as described in Methods. Data are compiled from 4 experiments with triplicates in each experiment. Asterisk indicates a significant difference in degradation (degraded) between LDL and +AcLDL.

At high concentrations, a similar time course is seen for coated-pit endocytosis of LDL and AcLDL. In contrast, the residence of both AcLDL and LDL in macropinosomes was doubled to 60 minutes at the higher concentration. Another difference between high and low concentrations was the increase in the distribution of both AcLDL and LDL on the plasma membrane at 120 minutes.

Most internalization studies use lipoproteins near the saturation of high affinity binding sites. However, foam cell formation requires higher concentrations of AcLDL. These studies speculated that at high lipoprotein concentrations, AcLDL would be segregated from LDL trafficking and more AcLDL would be internalized via macropinocytosis. At high concentrations, most AcLDL trafficked via macropinosomes. However, unexpectedly, LDL also trafficked through macropinosomes. If LDL is co-incubated with AcLDL at concentrations near the saturation of the high affinity receptor (low concentrations), LDL predominately trafficks through coated-pit endocytosis with little trafficking in macropinosomes. As shown here, at concentrations above the saturation of the high affinity receptor, a large portion of LDL trafficks through macropinosomes. Subsequent studies showed AcLDL has a similar influence on trafficking of Pigeon βVLDL. βVLDL co-incubated with AcLDL shifted from 5.8% to 8.4% of βVLDL in macropinosomes at low versus high lipoprotein concentrations. As a control, βVLDL and LDL were co-incubated at high or low concentrations. Both Pigeon βVLDL and LDL had only 1% to 2% traffic in macropinosomes (data not shown).

The method for entry into macropinosomes of co-incubated ligands is not known. Earlier studies indicated that modified LDLs stimulate fluid-phase uptake in pigeon macrophages.12 However, adsorptive endocytosis cannot be ruled out. The ultrastructural methodology with 4°C loading, washing, and subsequent incubation at 37°C would not indicate true fluid-phase uptake but rather adsorptive endocytosis. Recently glycosaminoglycans and proteoglycans have been shown to contribute to the catabolism of OxLDL.23,24 One study showed a cooperation between glycosaminoglycans and cell-surface scavenger receptors (SR-As). Digestion of heparan sulfate and chondroitin sulfate could reduce degradation by 40%.23 This effect was not seen at 10 μg/mL but only at concentrations >25 μg/mL. Perhaps some modified LDL is internalized by attachment to the plasma membrane via glycosaminoglycans, but stimulation of macropinocytosis by interaction with the scavenger receptor is necessary. Interestingly, pigeon AcLDL-specific binding is saturable, but total binding was linearly dependent on concentration, similar to fluid phase uptake.25
macropinocytosis results in an increase of fluid-phase uptake and membrane internalization. When other lipoproteins are co-incubated with modified LDLS, if receptors are available, these co-incubated ligands will still be predominately internalized via receptor-mediated endocytosis in coated pits. At ligand concentrations above the saturation of high affinity sites, significant portions of the co-incubated lipoproteins are internalized in macropinosomes, presumably by fluid-phase or adsorptively endocytosed via nonspecific binding, low affinity-high capacity binding, and/or proteoglycan bound ligands due to increased membrane internalization during membrane ruffling.

The atherogenic potential of macropinocytosis remains to be determined. First, macropinocytosis may be atherogenic due to large capacity uptake via fluid-phase or a stimulation of membrane internalization of nonspecific and low affinity binding of ligand. This uptake would be dependent on the scavenger receptor(s) and independent of cholesterol content in the cell. However, a net increase in cell association of $^{125}$I-LDL with AcLDL and OxLDL did not occur. Some experiments at 1 and 2 hours showed an increased cell association of LDL with modified LDLS present. The inconsistency could be due to a transient cell association and subsequent regurgitation. Macropinocytosis may also allow differential trafficking of cholesterol, with perhaps increased access to ACAT. Macropinosomes, compared with clathrin-coated and noncoated pits, are long-lived (up to 30 to 60 minutes). Previously, retention of lipoproteins on the plasma membrane and in surface tubules for entry into macrophages was suggested to be associated with a stimulation of ACAT.26 The surface tubules for entry into macrophages was an environment in which partial degradation of VLDL occurred. Macropinocytosis may also stimulate modification of LDL by prolonged contact with the macrophage surface and/or regurgitation. Sequestration of lipoproteins within tightly closed membrane ruffles and regurgitation from macropinosomes may be environments that enhance modification of LDL by macrophages. Recently, it was shown that cyclic adenosine monophosphate could selectively promote regurgitation of macropinosomes.27 Conditions may exist in the arterial wall that would selectively promote uptake or regurgitation of lipoproteins. The increase in degradation without a consistent increase in cell association of $^{125}$I-LDL suggests either degradation in the membrane ruffled areas or regurgitation of macropinosomes.

An apparent quandary in the foam cell formation literature is that, although high LDL plasma concentration is a reliable risk factor for predicting heart disease, LDL uptake by macrophages in vitro does not cause macrophage foam cell formation. The data from this study suggest an intriguing hypothesis; the presence of some modified LDL can affect the intracellular trafficking and potentially the metabolism of unmodified LDL. This would suggest that not all of the arterial LDL would need to be modified for foam cell formation to occur with high concentrations of LDL. Modified LDLS stimulated an increase in the total degradation of LDL at 37°C, without increasing the total binding at 4°C. These new findings present a novel working hypothesis: first, macropinocytosis rather than coated-pit endocytosis is associated with foam cell formation; second, that nonreceptor mediated endocytosis and low affinity uptake is an important

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**Figure 6.** Foam cell formation model—modified LDL stimulated macropinocytosis. Modified LDLS stimulate membrane ruffling and subsequent macropinocytosis via interaction with the scavenger receptor(s). Lipoproteins, LDL, and AcLDL, at concentrations near the saturation of their high affinity binding site, will be internalized via coated-pit endocytosis via clathrin-coated pits. The early endosomes are sites of fusion of multiple vesicles. These early cisternal endosomes pass on their contents or mature to spherical endosomes by 5 to 10 minutes. Multiple spherical endosomes may fuse with the LEPTs from 10 to 60 minutes. Lysosomes are then matured from the LEPTs. Lipoproteins, AcLDL, and LDL incubated with the macrophages during modified LDL–stimulated macropinocytosis above the saturation of high affinity lipoprotein receptors will be internalized via fluid-phase and adsorptive endocytosis. AcLDL enters macropinosomes on average by 30 minutes, but macropinocytosis is stimulated by modified LDLS for greater than 60 minutes. Materials internalized by macropinosomes can also arrive at lysosomes. The atherogenic potential of modified LDL stimulated macropinocytosis results from uptake of lipoproteins both dependent on and independent of their receptors. Additionally, increase residence of lipoprotein on the plasma membrane, unfolding of membrane ruffles with ligands attached or trapped within membrane ruffles that did not form macropinosomes, and/or regurgitation from macropinosomes will result in an environment favoring further modification of lipoproteins.
pathway for foam cell formation; and third, that modified LDL stimulation of macropinocytosis causes increased degradation of co-internalized lipoproteins.

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