Constitutive Receptor-independent Low Density Lipoprotein Uptake and Cholesterol Accumulation by Macrophages Differentiated from Human Monocytes with Macrophage-Colony-stimulating Factor (M-CSF)*

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Recently, we have shown that macrophage uptake of low density lipoprotein (LDL) and cholesterol accumulation can occur by non-receptor mediated fluid-phase macropinocytosis when macrophages are differentiated from human monocytes in human serum and the macropinophases are activated by stimulation of protein kinase C (Kruth, H. S., Jones, N. L., Huang, W., Zhao, B., Ishii, I., Chang, J., Combs, C. A., Malide, D., and Zhang, W. Y. (2005) J. Biol. Chem. 280, 2352–2360). Differentiation of human monocytes in human serum produces a distinct macrophage phenotype. In this study, we examined the effect on LDL uptake of an alternative macrophage differentiation phenotype. Differentiation of macrophages from human monocytes in fetal bovine serum with macrophage-colony-stimulating factor (M-CSF) produced a macrophage phenotype demonstrating constitutive fluid-phase uptake of native LDL leading to macrophage cholesterol accumulation. Fluid-phase endocytosis of LDL by M-CSF human macrophages showed non-saturable uptake of LDL that did not down-regulate over 48 h. LDL uptake was mediated by continuous actin-dependent macropinocytosis of LDL by these M-CSF-differentiated macrophages. M-CSF is a cytokine present within atherosclerotic lesions. Thus, macropinocytosis of LDL by macrophages differentiated from monocytes under the influence of M-CSF is a plausible mechanism to account for macrophage foam cell formation in atherosclerotic lesions. This mechanism of macrophage foam cell formation does not depend on LDL modification or macrophage receptors.

Macrophage cholesterol accumulation is considered a critical process in the development of atherosclerotic plaques, the cause of most heart attacks and strokes. LDL, the main carrier of blood cholesterol, enters the blood vessel wall and by some mechanism, monocyte-derived macrophages take up the LDL in the vessel wall. Macrophage cholesterol accumulation converts the macrophages into so-called foam cells and stimulates the macrophages to secrete proteases and tissue factor that contribute to plaque rupture and thrombosis, respectively (1–3).

Because incubation of macrophages with native LDL did not produce substantial macrophage cholesterol accumulation in past studies, it was proposed that LDL modifications that promote recognition and uptake of large amounts of the modified LDL by macrophage scavenger receptors is required to generate macrophage foam cells (4). LDL oxidation has been the most commonly studied LDL modification that stimulates LDL uptake (5).

Recently, we have shown that treatment of human monocyte-derived macrophages with protein kinase C activators such as PMA stimulates fluid-phase macropinocytosis of native (unmodified) LDL (6, 7). This results in uptake of LDL in amounts directly proportional to its concentration in the medium. As a result, incubation of human monocyte-derived macrophages with LDL at concentrations similar to those that exist within the normal vessel wall (0.7–2.7 mg/ml) (8–10) leads to macrophage foam cell formation.

Human monocytes can differentiate into macrophage phenotypes with different morphology and function depending on the culture conditions and differentiation factors included in the culture medium (11). In the studies discussed above, we differentiated human monocytes into macrophages in the presence of human serum, producing the macrophage phenotype that resembles a “fried egg.” In the present study, we show that differentiation of human monocytes into macrophages in the presence of fetal bovine serum with added M-CSF produces a macrophage phenotype having an elongated shape and showing high levels of constitutive macropinocytosis not dependent on activation of the macrophage with PMA. The constitutive macropinocytosis in these macrophages mediated fluid-phase uptake of LDL causing high levels of macrophage cholesterol accumulation. Thus, our findings demonstrate a macrophage model system showing constitutive cholesterol accumulation without the need for either LDL modification or LDL uptake mediated by receptors.

MATERIALS AND METHODS

Culture of Human Monocyte-derived Macrophages—Human monocytes were purified with counterflow centrifugal elutriation of mononuclear cells obtained by monocytepheresis of normal human donors. M-CSF monocyte-derived macrophage cultures were begun with the elutriated human monocytes suspended in RPMI 1640 medium (MediaTech) with 10% fetal bovine serum (FBS) (Invitrogen) seeded in 6-well plates (MatTek and Corning) at a density of 4 × 105 monocytes/cm2. After 2-h incubation in a cell culture incubator with 5% CO2/95% air at 37 °C, the attached monocytes were rinsed three times with RPMI 1640 and then incubated with RPMI 1640 containing 10% FBS, 50 ng/ml M-CSF (PeproTech), and 25 ng/ml interleukin-10 (IL-10) (PeproTech) (12). The medium was
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exchanged with fresh medium on day 6, and monocyte-derived macrophages were used for experiments on day 8. Elutriated human monocytes differentiated in human serum were cultured in 12-well plates as described previously (6).

For experiments, monocyte-derived macrophage cultures (about 0.25 mg of protein/well and 0.15 mg of protein/well for human serum and FBS+M-CSF+IL-10 cultured macrophages, respectively) were first rinsed three times with serum-free RPMI 1640. Cultures were incubated in RPMI 1640 without serum with the indicated concentration of LDL and the reagents specified in each experiment. For monocytes differentiated in FBS+M-CSF+IL-10, experimental incubations with these monocyte-derived macrophages were carried out with M-CSF and IL-10 added to RPMI 1640 medium.

Preparation of Lipoproteins for Use in Experiments—Before use, human lipoproteins, LDL, oxidized LDL, and HDL (Intactrel) were dialyzed against 1 liter of 0.15 M sodium chloride and 0.3 mM EDTA (pH 7.4) for 12 h at 4 °C, then against RPMI 1640 medium (2 changes, 1 liter/each change) for 24 h. LDL was incubated 24 h at 37 °C with 5 M CuSO4 to prepare oxidized LDL as described previously (13, 14). Human 125I-LDL (Biomedical Technologies) was dialyzed against 0.15 M sodium chloride and 0.3 mM EDTA (pH 7.4) over 36 h at 4 °C (three changes, 1 liter/each change). All dialysis was carried out with Pierce Slide-A-Lyzer cassettes (10,000 molecular weight cut-off). After dialysis, lipoproteins were sterilized by passage through a 0.45-μm pore size low protein-binding filter (Gelman Acrodisc). 125I-LDL specific activity was adjusted to 2.25 × 10−7 μCi/ng by adding unlabeled LDL. LDL concentration is expressed in terms of protein.

Assays of 125I-LDL Cell Association and Degradation—Macrophage cell association and degradation of 125I-LDL were determined according to the methods of Goldstein et al. (15). Lipoprotein degradation was quantified by measurement of trichloroacetic acid-soluble organic iodide radioactivity in supernatants of culture media samples that were centrifuged at 15,000 × g for 10 min. Values obtained in the absence of cells were <5% of those values obtained with cells.

Cell-associated 125I-LDL was determined by rinsing macrophages three times with Dulbecco’s phosphate-buffered saline (DPBS) plus Ca2+, Mg2+, and 0.2% bovine serum albumin (BSA), followed by 3 times with DPBS plus Ca2+ and Mg2+ all at 4 °C. Then, macrophages were dissolved overnight in 0.1 N NaOH at 37 °C. Aliquots of cell samples were assayed for 125I radioactivity with a γ counter. Values were subtracted for 125I radioactivity determined for wells incubated with 125I-LDL but without macrophages. These values were <1% of the cell-associated 125I-LDL.

Determination of Fluid-phase Endocytosis—125I-BSA (MP Biomedicals) cell association and degradation by macrophages was determined similar to 125I-LDL described above and served as a means to determine fluid-phase uptake by the macrophages. Before incubations, 125I-BSA specific activity was adjusted to 2.25 × 10−7 μCi/ng by adding unlabeled BSA. Macrophages were incubated 24 h with varying concentrations of 125I-BSA (31, 62, 125, and 250 μg/ml) to show non-saturable uptake of 125I-BSA (MP Biomedicals) cell-associated plus degraded 125I-BSA) by the concentration of 125I-BSA added to the medium (i.e. 250 μg/ml).

Assay of Cholesterol and Protein Contents of Macrophages—After incubations, macrophages were rinsed three times each with DPBS plus Mg2+, Ca2+, and 0.2% BSA and then DPBS plus Mg2+ and Ca2+. Macrophages were harvested from wells by scraping into 1 ml distilled water and then processed as described previously (16). Lipids were extracted from an aliquot of cell suspension using the Folch method (17). The cholesterol content of macrophages was determined according to the fluorometric method of Gamble et al. (18). Macrophage protein content was determined on another aliquot of cell suspension by the method of Lowry et al. (19) using BSA as a standard.

Time-lapse Microscopy—Time-lapse phase-contrast video microscopy was carried out by observing macrophages grown in a T-25 flask with a 10× long working distance planfluor objective lens (0.3 N.A.) mounted on an Olympus L70 inverted microscope. 5% CO2/95% hydrated air and 37 °C were maintained in an enclosed chamber during recording from the macrophages. Analogue video images were recorded using a Dage-MTI CD100 camera and JVC BR9000-U VHS video recorder. Video images were captured and digitized using MGI VideoWave software. When viewed at standard rates (i.e. 30 frames/s), the movie prepared from the time-lapse phase-contrast video is 180× real time.

Electron Microscopic Analysis of Macrophages—For immunolabeling of LDL, macrophages were seeded in 6-well culture plates. After incubation with LDL, macrophages were fixed, embedded in LR White resin, and prepared for immunogold labeling as described previously (16) except that 1% dry skim milk instead of 1% BSA was used to block nonspecific staining. LDL was labeled by incubating thin sections with 10 μg/ml affinity-purified rabbit anti-human LDL antibody (catalog number BT-905, Biomedical Technologies, Inc.) and then with a 1:10 dilution of 10 nm gold-conjugated goat F(ab)2 anti-rabbit IgG (BBInternational). For controls, the rabbit anti-human LDL antibody was substituted with the same concentration of purified rabbit IgG. This control showed no labeling.

Detection of Cell Death—Following a 1-day incubation with 2 mg/ml LDL or 125 μg/ml oxidized LDL, macrophages were rinsed once with serum-free culture medium, then the macrophages were incubated 30 min at 37 °C with 1 ml of 50 mM Tris-HCl (pH 7.5) containing 100 mM NaCl, 1% BSA, 100 ng/ml annexin V FITC conjugate (Sigma, catalog number A9210) (to detect apoptosis), and 1 μg/ml propidium iodine (to detect cell membrane permeability). The cells were rinsed once with PBS immediately. The annexin V FITC conjugate and propidium iodide bound to the macrophages were visualized by fluorescence microscopy (excitation at 460–490 nm and emission at 515 nm for FITC; excitation at 520–550 nm and emission at 580 nm for propidium iodide). Five predetermined microscopic fields for each of three culture wells were photographed by phase and fluorescence microscope, and macrophage staining was assessed. Averages of 735 ± 181, 672 ± 173, and 339 ± 66 macrophages were counted in each photographed field for control, LDL, and oxidized LDL-incubated cultures, respectively.

Statistical Analysis—All data are presented as the means ± S.E. of the mean. The means were determined from three culture wells for each data point. Standard error bars are not shown where the error range is smaller than the symbol size. Statistical comparisons of means were made using the Student’s t statistic (unpaired). A p value ≤ 0.05 was considered significant.

RESULTS

We first determined whether human monocytes differentiated with M-CSF and IL-10 (hereafter referred to as M-CSF macrophages) metabolized LDL differently from monocytes differentiated in human serum. Unexpectedly, M-CSF-differentiated macrophages showed an extremely high level of 125I-LDL uptake (35 times greater) compared with human serum differentiated macrophages (Fig. 1). Greater than 90% percent of the 125I-LDL that was taken up by the M-CSF macrophages was degraded. Degradation of the 125I-LDL was not due to extra
cellular proteolysis of the \(^{125}\)I-LDL, as incubation of M-CSF macrophage conditioned media with \(^{125}\)I-LDL did not cause degradation of \(^{125}\)I-LDL. Furthermore, \(^{125}\)I-LDL degradation was inhibited 83% when the lysosomal inhibitor, bafilomycin A1, was added to incubations of macrophages with \(^{125}\)I-LDL. Macrophage uptake of \(^{125}\)I-LDL was linear over a 48-h incubation (Fig. 2).

Macrophage uptake of LDL led to macrophage accumulation of cholesterol. Incubation of M-CSF macrophages with concentrations of LDL up to 4 mg/ml did not show saturation of cholesterol accumulation (Fig. 3). This resulted in a tripling of macrophage cholesterol content to a level that exceeded 300 nmol/mg of cell protein with almost 50% of the cholesterol esterified. When macrophages were incubated 24 h with HDL and LDL at similar total cholesterol concentrations (i.e. 7928 nmol/ml equivalent to 9.5 mg/ml for HDL and 2 mg of protein/ml for LDL), the macrophages accumulated 252 and 485 nmol of cholesterol/mg of cell protein, respectively, showing that cholesterol accumulation was not specific for LDL.

Monocytes were routinely differentiated in the presence of M-CSF plus IL-10 because addition of IL-10 has been shown to produce a more homogeneous macrophage culture assessed by morphology and to augment the growth and differentiation of human monocytes stimulated with M-CSF (12). Monocyte-derived macrophages differentiated with M-CSF alone also showed cholesterol accumulation during incubation with LDL. However, these macrophages accumulated 29% less cholesterol than macrophages differentiated from monocytes with M-CSF and IL-10. Monocytes maintained in FBS without M-CSF did not survive as reported previously (11).

Macrophage uptake of \(^{125}\)I-LDL was not inhibited by addition of C7 antibody that blocks binding of LDL to the LDL receptor (20) (Table 1). Methylation of LDL also blocks its binding to the LDL receptor (21). Further evidence that LDL uptake was not mediated by the LDL receptor was shown by the finding that methylation of LDL did not decrease LDL-induced cholesterol accumulation (data not shown).

To learn more about the mechanism of macrophage LDL uptake and cholesterol accumulation, we examined the concentration dependence of \(^{125}\)I-LDL uptake by the M-CSF macrophages. We observed that uptake of \(^{125}\)I-LDL was linear not showing any evidence of saturation with \(^{125}\)I-LDL concentrations up to as high as 250 \(\mu\)g/ml (Fig. 4). Thus, \(^{125}\)I-LDL uptake was directly proportional to its concentration in the medium. These results suggested that the mechanism of LDL uptake was fluid-phase rather than receptor-mediated endocytosis.

Next, we determined the amount of macrophage fluid uptake to learn whether macrophage uptake of LDL in the fluid phase could account for \(^{125}\)I-LDL uptake by macrophages. There is no known receptor-medi-
**TABLE 1**

**Effect of competitive inhibitors on $^{125}$I-LDL uptake by macrophages**

Monocytes were differentiated 7 days in FBS containing M-CSF and IL-10, then the differentiated monocyte-derived macrophages were incubated 24 h with 100 µg/ml $^{125}$I-LDL with the indicated competitor. Following incubations, total uptake of the $^{125}$I-LDL was determined as the sum of cell-associated and degraded $^{125}$I-LDL and is expressed as µg/ml $^{125}$I-LDL protein.

| Competitor                  | Total $^{125}$I-LDL uptake (µg of protein/mg of cell protein) |
|-----------------------------|---------------------------------------------------------------|
| Anti-LDL receptor C7 monoclonal antibody (0.9 mg/ml) | 32 ± 2                                                        |
| Control IgG (0.9 mg/ml)     | 31 ± 1                                                        |
| LDL (2 mg/ml)               | 39 ± 3                                                        |
| HDL (2 mg/ml)               | 31 ± 2                                                        |

**FIGURE 4.** Effect of $^{125}$I-LDL concentration on $^{125}$I-LDL uptake. Monocytes were differentiated 7 days in FBS containing M-CSF and IL-10. Then, the differentiated monocyte-derived macrophages were incubated 24 h in serum-free medium, M-CSF, and IL-10 with the indicated concentrations of $^{125}$I-LDL. Total uptake of the $^{125}$I-LDL was determined as the sum of cell-associated and degraded $^{125}$I-LDL.

**FIGURE 5.** Effect of cytochalasin D on $^{125}$I-LDL uptake and cholesterol accumulation by M-CSF-differentiated macrophages. Monocytes were differentiated 7 days in FBS containing M-CSF and IL-10, then the differentiated monocyte-derived macrophages were incubated 24 h in serum-free medium, M-CSF, and IL-10 with either 500 µg/ml $^{125}$I-LDL ± 2 µg/ml cytochalasin D (A) or no additions and 500 µg/ml LDL ± 2 µg/ml cytochalasin D (B). Following incubations, total uptake of the $^{125}$I-LDL was determined as the sum of cell-associated and degraded $^{125}$I-LDL in A, and total cholesterol content was determined in B.

actin-independent and macropinocytosis is actin-dependent, we examined the effect of cytochalasin D, an agent that interferes with actin function, on macrophage LDL uptake and cholesterol accumulation. Cytochalasin D blocked by greater than 95% both macrophage $^{125}$I-LDL uptake and cholesterol accumulation (Fig. 5) suggesting that macrophage LDL uptake and cholesterol accumulation were mediated by fluid-phase macropinocytosis of LDL. Time-lapse phase microscopic analysis of living M-CSF macrophages showed that macropinosomes were constitutively generated in these macrophages, and as a result, the macropinosomes contained numerous macropinosomes (Fig. 6A and movie in the supplemental material). We next confirmed that LDL was being internalized within the macropinosomes by examining with the electron microscope macrophages incubated with 2 mg/ml LDL for 20 min and then immunogold labeled for LDL. Macrophages incubated with LDL showed anti-LDL immunogold labeling of LDL scattered throughout the macropinosomes (Fig. 6B). Control macrophages incubated without LDL showed no anti-LDL immunogold labeling (Fig. 6C).

Previously, we reported that monocytes differentiated to macrophages in human serum showed PMA-stimulated macropinocytosis of LDL that was protein kinase C-dependent and could be inhibited with the protein kinase C inhibitor, GF109203X. However, GF109203X (4 µM) did not inhibit M-CSF macrophage uptake of $^{125}$I-LDL (500 µg/ml) during a 24-h incubation. This showed that the constitutive macropinocytosis of LDL by M-CSF macrophages was not protein kinase C-dependent (data not shown).

Finally, we examined whether macrophage cholesterol accumulation mediated by macropinocytosis was cytotoxic. Although incubation of macrophages for 24 h with 2 mg/ml LDL enriched macrophages with both unesterified and esterified cholesterol (net accumulation of 115 and 116 nmol/mg of cell protein, respectively), cholesterol accumulation did not induce apoptosis or necrosis of the macrophages. Only 1.2 ± 0.8% of macrophages showed staining with annexin V FITC conjugate (to detect apoptosis) following incubation with LDL. This was not
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HDL also produced cholesterol accumulation. The amount of cholesterol accumulation that occurred with HDL was less than what accumulated with a similar amount of LDL cholesterol, presumably because HDL is known to stimulate cholesterol efflux from macrophages. In contrast to LDL, it is unlikely that HDL can contribute substantially to macrophase cholesterol accumulation in the vessel wall. This is because while LDL concentration in the vessel wall can exceed 1 mg protein/ml, the vessel wall concentration of HDL is only about 12% of the LDL concentration (8-10, 25, 26).

Earlier studies showed non-saturable LDL uptake by cultured human fibroblasts that was attributed to fluid-phase endocytosis in these cells (22, 27). Because fibroblasts are not known to carry out macropinocytosis, the fluid-phase endocytosis in those cells was most likely mediated by micropinocytosis. However, LDL taken up by fluid-phase endocytosis in fibroblasts does not cause cholesterol accumulation because the cholesterol delivered by LDL is excreted by the fibroblasts (27). On the other hand, we have shown that LDL entering macrophages by fluid-phase endocytosis mediated by macropinocytosis does cause cholesterol accumulation. It remains to be determined whether this difference in cholesterol trafficking is because of a difference in processing of cholesterol delivered by macropinocytosis and micropinocytosis or because of a difference in fibroblast and macrophase processing of cholesterol delivered to these cells. Macrophage macropinocytosis delivered fluid (and as a result LDL cholesterol) at a rate some 18 times that shown by fibroblasts (calculated from Table 5 in Ref. 22), and this may be another factor contributing to macrophase accumulation of cholesterol delivered by fluid-phase endocytosis. Receptor-independent uptake of LDL not only occurs in cultured cells but occurs in animals and humans where it contributes substantially to LDL metabolism and tissue cholesterol balance (28, 29). The reticuloendothelial system in which macrophages are a prominent cell type functions in receptor-independent catabolism of LDL (30). Thus, it is possible that macrophase receptor-independent macropinocytotic uptake of LDL that we have demonstrated in vitro contributes to receptor-independent catabolism of LDL in vivo.

In previous work, we have shown that when monocytes are differentiated into macrophages using human serum, they do not show constitutive macropinocytosis. Rather, these macrophages show macropinocytosis of LDL causing foam cell formation when protein kinase C is stimulated in these macrophages (6, 7). Thus, human monocyte-derived macrophages can show two distinct phenotypes with respect to foam cell formation mediated by fluid-phase macropinocytosis of LDL: macropinocytosis can be either constitutive as in macrophages differentiated from monocytes in M-CSF and FBS or inducible as in macrophages differentiated from monocytes in human serum. The signaling that mediates macropinocytosis in these two macrophase phenotypes also appears to be different because macropinocytosis in the M-CSF-differentiated macrophages was not protein kinase C-dependent.

M-CSF acutely stimulates macropinocytosis when added to mouse bone marrow-derived macrophages (31). However, in the case of the human monocyte-derived macrophages differentiated in the presence of M-CSF, macropinocytosis did not depend on the continuous presence of exogenously supplied M-CSF. Macropinocytosis and cholesterol accumulation during incubation with LDL continued after removal of the exogenous M-CSF. This may be because the macrophages secrete M-CSF or because M-CSF functions differently in mouse and human macrophages, acutely stimulating macropinocytosis in the

3 B. Zhao and H. S. Kruth, unpublished data.
former but chronically stimulating macropinocytosis in the latter through a differentiation process.

Accumulation of unesterified cholesterol in macrophages has been associated with triggering of macrophage apoptosis (32, 33). However, macrophage cholesterol accumulation, which included a substantial increase in unesterified cholesterol content, did not induce apoptosis in our study. Recent work shows that other factors such as scavenger receptor engagement and location of accumulated unesterified cholesterol are important factors in triggering apoptosis (34, 35).

Differentiation of human monocytes with M-CSF added to FBS results in development of a macrophage phenotype that shows constitutive macropinocytosis-mediating uptake of large amounts of LDL without requiring LDL modification. M-CSF is an important factor necessary for atherosclerotic lesion development in mice (36, 37). Because M-CSF is present in atherosclerotic plaques (38, 39), it is conceivable that monocytes differentiate into macrophages capable of macropinocytosis in vivo similar to what we have shown here in vitro.

A recent report shows that scavenger receptors, which function in macrophage uptake of oxidized LDL, are not necessary for in vivo macrophage foam cell formation (40, 41). Our finding that macrophage foam cell formation can occur through receptor-independent fluid-phase macropinocytosis helps explain why scavenger receptor function is not necessary for in vivo foam cell formation. Our findings further suggest that receptor-independent uptake of LDL is an important pathway to study for possible discovery of new drugs targeting foam cell formation in atherosclerosis.

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