Macrophage Foam Cell Formation with Native Low Density Lipoprotein

Received for publication, May 22, 2002, and in revised form, July 11, 2002
Published, JBC Papers in Press, July 12, 2002, DOI 10.1074/jbc.M205059200

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This investigation has elucidated a mechanism for development of macrophage foam cells when macrophages are incubated with native low density lipoprotein (LDL). LDL is believed to be the main source of cholesterol that accumulates in monocyte-derived macrophages within atherosclerotic plaques, but native LDL has not previously been shown to cause substantial cholesterol accumulation when incubated with macrophages. We have found that activation of human monocyte-derived macrophages with phorbol 12-myristate 13-acetate (PMA) stimulates LDL uptake and degradation and acyl-CoA:cholesterol acyltransferase-mediated esterification of LDL-derived cholesterol, resulting in massive macrophage cholesterol accumulation that could exceed 400 nmol/mg of cell protein. Cholesterol accumulation showed a biphasic linear LDL concentration dependence with LDL levels as high as 4 mg/ml similar to LDL levels in artery intima. Protein kinase C mediated the PMA-stimulated macrophage uptake of LDL because the protein kinase C inhibitors, Go6983 and GF109203X, inhibited cholesterol accumulation. LDL receptors did not mediate macrophage cholesterol accumulation because accumulation occurred with reductively methylated LDL and in the presence of an anti-LDL receptor-blocking monoclonal antibody. LDL-induced cholesterol accumulation was not inhibited by antioxidants, was not accompanied by increased LDL binding to macrophages, did not depend on the apoB component of LDL, and was not down-regulated by prior cholesterol enrichment of macrophages. We have shown that the mechanism of LDL uptake by macrophages was PMA-stimulated endocytosis of LDL taken up as part of the bulk phase fluid (i.e. fluid phase endocytosis). The amount of LDL taken up with the bulk phase fluid was measured with [3H]sucrose and accounted for a minimum of 83% of the LDL cholesterol delivery and accumulation in PMA-activated macrophages. This novel mechanism of macrophage cholesterol accumulation shows that modification of LDL is not necessary for foam cell formation to occur. In addition, the findings direct attention to macrophage fluid phase endocytosis as a relevant pathway to target for modulating macrophage cholesterol accumulation in atherosclerosis.

Engorgement of macrophages with cholesterol is the defining pathological characteristic of atherosclerotic plaques, the cause of most heart attacks and strokes. Cholesterol accumulation in macrophages not only contributes to cholesterol retention within the vessel wall, but also alters macrophage biology. Cholesterol-loaded macrophages secrete plaque-disrupting matrix metalloproteinases, and produce tissue factor that promotes thrombosis when plaques rupture (1–3). Thus, how macrophages accumulate cholesterol and become foam cells has been the subject of intense investigation.

Low density lipoprotein (LDL), the main carrier of plasma cholesterol, enters the vessel wall and then by some mechanism enters macrophages. Previously, native LDL could not be shown to cause foam cell formation because the cellular receptor that binds LDL is poorly expressed on differentiated macrophages and down-regulates during cholesterol uptake, limiting total cholesterol accumulation (4–7). Moreover, the LDL receptor is not expressed in human atherosclerotic plaques (8). Thus, most previous studies of macrophage foam cell formation have focused on modifying LDL in some way that increases its binding to macrophages. Increased macrophage binding of LDL has been achieved with chemical modifications to the apoB component of the LDL, aggregation of LDL induced by either vortexing or treatment of LDL with lipases, and complexing of LDL with other molecules, for example, glycosaminoglycans or antibodies, which bind macrophages and promote LDL uptake by piggyback endocytosis (9). Macrophages take up modified LDL by receptor-mediated endocytosis in pinocytic vesicles, phagocytic vacuoles, or patocytic surface-connected compartments (9).

One popular hypothesis of foam cell formation involves LDL oxidation. LDL oxidation promotes macrophage LDL uptake that is mediated by various macrophage scavenger receptors (10). Although oxidation of LDL has important biological effects that could influence atherosclerotic plaque development (11), oxidation of LDL does not readily explain foam cell formation. Incubation of human monocyte-derived macrophages with oxidized LDL, even strongly oxidized with artificial chemical systems, produces little macrophage cholesterol accumulation (12, 13). Also, oxidized LDL is poorly metabolized within lysosomes of macrophages because of partial inactivation by oxidized LDL of the lysosomal enzymes that degrade LDL (14–16). This limits the capacity of oxidized LDL to induce acyl-CoA:cholesterol acyltransferase (ACAT)-mediated cholesterol esterification and cholesteryl ester lipid droplet formation, the hallmark of foam cell formation.

In the present study, we have investigated how modification of macrophages rather than modification of LDL affects macrophage metabolism of the LDL. We show that macrophage foam cell formation can occur with native LDL when macrophages are activated with PMA. Activation of cultured human mono-

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1 The abbreviations used are: LDL, low density lipoprotein; ACAT, acyl-CoA:cholesterol acyltransferase; PMA, phorbol 12-myristate 13-acetate; BSA, bovine serum albumin; DPBS, Dulbecco’s phosphate-buffered saline.
cyte-derived macrophages stimulated uptake and degradation of native LDL. LDL uptake did not depend on macrophage oxidation of LDL or increased macrophage binding of LDL. Also, uptake did not depend on LDL apoB protein that could be removed by protease digestion without affecting LDL uptake. Rather, activated macrophages showed greatly stimulated uptake of LDL as part of the bulk phase fluid that the macrophages took up (i.e. fluid phase endocytosis). This produced cholesterol accumulation in macrophages to levels characteristic of macrophage foam cells in plaques.

EXPERIMENTAL PROCEDURES

Reagents—Human LDL and human lipoprotein-deficient serum were obtained from Intracel. Human 125I-LDL was from Biomedical Technologies. [3H]Sucrose was from American Radiolabeled Chemicals. PMA was from Calbiochem. Papain was from Roche Molecular Biochemicals. Mannitol, EDTA, superoxide dismutase, catalase, ketoconazole, metapyrone, lipoid acid, N-acetylcysteine, aminoguanidine, glutathione, pro-bucal, butylated hydroxytoluene, Nε-methyl-l-arginine, and BSA were from Sigma. RPMI 1640 medium was from Cellgro. Heat-inactivated pooled human AB serum was from Pel-Freez. Congenital mouse control monoclonal IgG2b antibody, HEPES, and liquid scintillation cocktails (EcoLume) were from ICN. Dulbecco’s phosphate-buffered saline (DPBS), penicillin, and streptomycin were from Invitrogen. G66983 and GF109203X were from Calbiochem. SS6-035 was a gift from Sandoz.

Culture of Human Monocyte-derived Macrophages—Human monocytes were purified with counterflow centrifugal elutriation of mononuclear cells obtained by monocytopheresis of normal human donors. The monocytes were cultured as described previously (17). Macrophage and LDL protein contents were determined by the fluorometric method of Gamble et al. (21). Macrophages were harvested from wells by scraping 1 ml of distilled water and then processed as described previously (17).

Preparation of Lipoproteins—Reductively methylated LDL and acetylated LDL were prepared from LDL as described previously (18, 19). Before use, lipoproteins were dialyzed against a 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 (two changes, 1 liter/each change) for 24 h. All dialysis was carried out with Pierce Slide-A-Lyzer cassettes (10,000 molecular weight cut-off). After dialysis, lipoproteins were sterilized by passing them through a 0.45-μm ( pore size) Gelman Acrodisc filter. All lipoprotein concentrations are expressed in terms of protein.

Assays of 125I-LDL Cell Association and Degradation—Before adding to macrophages, 125I-LDL was dialyzed against 0.15 M sodium chloride and 0.3 mM EDTA (pH 7.4) for 36 h at 4 °C at 4 °C (three changes, 1 liter/each change). After dialysis, 125I-LDL was sterilized by passing through a 0.45-μm ( pore size) Gelman Acrodisc filter. All lipoprotein concentrations are expressed in terms of protein. LDL association and degradation of 125I-LDL were determined according to the methods of Goldstein et al. (20). Lipoprotein degradation was quantified by measurement of trichloroacetic acid-soluble iodide radioactivity in supernatants of media samples that were centrifuged at 15,000 × g for 10 min. Values obtained in the absence of cells were <3% of those obtained with cells. These control values were subtracted.

Cell-associated 125I-LDL was determined by rinsing macrophages five times (three quick rinses and two 10-min incubations all on ice) with DPBS plus Ca2+, Mg2+, and 0.2% BSA. After a final rinse with DPBS plus Ca2+ and Mg2+, macrophages were dissolved overnight in 0.1 N NaOH at 37 °C or scraped into 1 ml of distilled water if cholesterol content was also to be measured. Aliquots of cell samples were assayed for 125I radioactivity with a gamma 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.

Assay of Cholesterol and Protein Contents of Macrophages and LDL—Unesterified and esterified cholesterol contents of macrophages and LDL were determined according to the fluorometric method of Gamble et al. (21). Macrophages were harvested from wells by scraping 1 ml of distilled water and then processed as described previously (17). Macrophage and LDL protein contents were determined by the method of Lowry et al. using BSA as a standard (22). Protein contents of cultures generally ranged between 0.2 and 0.3 mg/well.

Measurement of Fluid Phase Endocytosis—Fluid phase endocytosis was determined by incubating macrophages with 0.8 nmol/ml [3H]sucrose (specific activity of 12.3 Ci/mmol). After the incubations, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol contents as described under “Experimental Procedures.” Error bars are not shown where the error range is smaller than the symbol.

FIG. 1. Time course of PMA-stimulated macrophage cholesterol accumulation. Two-week-old macrophage cultures were incubated for varying times with 2 mg/ml LDL without or with 1 μg/ml PMA. After the incubations, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol contents as described under “Experimental Procedures.” Error bars are not shown where the error range is smaller than the symbol.

FIG. 2. Effect of LDL concentration on PMA-stimulated macrophage cholesterol accumulation. Two-week-old macrophage cultures were incubated for 2 days with varying concentrations of LDL and 1 μg/ml PMA. After the incubations, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol contents.
buffered with 10 mM HEPES (pH 7.4). After a 2-h incubation, macrophages were rinsed three times with ice-cold Tris-saline buffer (50 mM Tris-HCl, 0.15 M NaCl, at pH 7.4) containing 2 mg/ml BSA, then incubated twice for 10 min on ice with the buffer. This was followed by one rapid rinse with the Tris-saline buffer without BSA. Next, the macrophages were dissolved in 0.1N NaOH overnight at 37 °C. Aliquots of the dissolved macrophages were assayed for 125I radioactivity and protein concentration. Nonspecific binding was measured in parallel incubations in the presence of a 20-fold excess of unlabeled LDL. Specific binding was calculated by subtracting nonspecific binding from the total binding.

Preparation of ApoB-free LDL—ApoB-free LDL was prepared by incubating 10 mg of LDL (5 mg/ml) with 5 mg of papain (10 mg/ml, 40 units/mg) and 0.4 ml of penicillin-streptomycin (penicillin at 10,000 units/ml, streptomycin at 10,000 μg/ml) in 0.6 ml of Tris-saline buffer (50 mM Tris-HCl, 0.15 mM EDTA, 0.15 M NaCl, at pH 7.2) for 1 day at 37 °C. Then, 5 mg of fresh papain was added to the mixture, which was incubated another 1 day. After this incubation, the papain-treated LDL was purified by gel filtration chromatography through a 1.6 × 70-cm column of Bio-Gel A-50m-agarose gel eluted with DPBS containing 2 mM EDTA. This and all subsequent procedures were carried out at 4 °C.

The papain-treated LDL particles eluted in the void volume fractions. These fractions were pooled and concentrated with an Amicon stirred cell with a 10,000 molecular weight cut-off cellulose filter. Before use, the papain-treated LDL particles were dialyzed against RPMI 1640 medium as described above for LDL and then passed through a 0.45-μm filter. Standard SDS-gel electrophoresis was carried out to confirm that papain had digested the apoB component of LDL.

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 Student’s t test (unpaired). A p value ≤ 0.05 was considered significant.

RESULTS

PMA-stimulated Macrophage Uptake of LDL and Cholesterol Accumulation—PMA-activated human monocyte-derived macrophages showed a biphasic linear progressive increase in macrophage cholesterol content (from 71 ± 10 to 394 ± 19 nmol/mg of cell protein) during a 2-day incubation with 2 mg/ml LDL, similar to the LDL concentration in artery intima (23–25) (Fig. 1). The rate of increase in cholesterol content was greater during the initial 3 h of incubation compared with the later hours of incubation. In contrast to macrophages incubated with LDL plus PMA, macrophages incubated for the same time with 2 mg/ml LDL without PMA showed only a slight increase in cholesterol content (to 117 ± 8 nmol/mg of cell protein).

Incubation of macrophages with PMA and increasing LDL concentrations up to 4 mg/ml for 2 days showed a curvilinear nonsaturating increase in macrophage cholesterol content which reached a level greater than 400 nmol/mg of cell protein (Fig. 2). This resulted in macrophages loaded with phase-refractile lipid (Fig. 3) similar in appearance to foam cells isolated from atherosclerotic lesions (26).

Cholesterol accumulation in PMA-stimulated macrophages incubated for 2 days with 2 mg/ml LDL reached levels of greater than 700 nmol of cholesterol/mg of cell protein in some experiments.

Although our experiments typically were carried out without serum, the presence of serum did not affect macrophage cholesterol accumulation. Macrophages incubated with LDL and PMA in the absence or presence of 10% human lipoprotein-deficient serum showed similar amounts of cholesterol accumulation when compared with macrophages incubated without serum.

Effect of prior macrophage cholesterol enrichment on PMA-stimulated macrophage cholesterol accumulation

Two-week-old macrophage cultures were incubated for 2 days with RPMI 1640 medium without any addition or with 50 μg/ml acetylated LDL (AcLDL) to enrich the macrophages with cholesterol. Then, macrophages were rinsed and incubated for 2 additional days in RPMI 1640 medium either without any additions or with 2 mg/ml LDL and 1 μg/ml PMA. After the incubations, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol contents. The increments in cholesterol content caused by LDL + PMA were similar whether or not the macrophages initially had been cholesterol enriched by incubating with AcLDL (increments of 297 ± 20 and 301 ± 34 nmol cholesterol/mg of cell protein without and with initial cholesterol enrichment, respectively).

Effect of Prior Macrophage Enrichment on PMA-stimulated Macrophage Cholesterol Accumulation

TABLE I

Effect of prior macrophage cholesterol enrichment on PMA-stimulated macrophage cholesterol accumulation

Two-week-old macrophage cultures were incubated for 2 days with RPMI 1640 medium without any addition or with 50 μg/ml acetylated LDL (AcLDL) to enrich the macrophages with cholesterol. Then, macrophages were rinsed and incubated for 2 additional days in RPMI 1640 medium either without any additions or with 2 mg/ml LDL and 1 μg/ml PMA. After the incubations, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol contents. The increments in cholesterol content caused by LDL + PMA were similar whether or not the macrophages initially had been cholesterol enriched by incubating with AcLDL (increments of 297 ± 20 and 301 ± 34 nmol cholesterol/mg of cell protein without and with initial cholesterol enrichment, respectively).

| Condition                              | Macrophage cholesterol content (nmol/mg of cell protein) |
|----------------------------------------|----------------------------------------------------------|
| No addition 2 days/no addition 2 days | 85 ± 4                                                   |
| No addition 2 days/LDL + PMA 2 days    | 382 ± 38                                                 |
| AcLDL 2 days/no addition 2 days        | 178 ± 12                                                 |
| AcLDL 2 days/LDL + PMA 2 days          | 479 ± 70                                                 |
Thus, in both cases, macrophages degraded the majority of 125I-LDL: 73 and 82%, was degraded by untreated macrophages (Table II). The latter being the highest concentration we tested with radiolabeled LDL. The total 125I-LDL uptake without PMA showed saturation above 250 nmol of cholesterol/mg of cell protein, and cholesterol-enriched macrophages accumulated a similar amount, 301 ± 34 nmol of cholesterol/mg of cell protein.

The effect of PMA concentration on macrophage cholesterol accumulation showed that between 0.3 and 1 µg/ml PMA produced maximal levels of cholesterol accumulation (Fig. 4). Thus, 1 µg/ml PMA was used in experiments. The effect of PMA was mediated through its action as an activator of protein kinase C. This was shown by the findings that each of two protein kinase C inhibitors, GF109203X (4 g/ml), and Go6983 (4 µM), completely blocked PMA stimulation of macrophage cholesterol accumulation (Fig. 5A). In addition, another protein kinase C activator, bryostatin, stimulated macrophage cholesterol accumulation similar to that of PMA (Fig. 5B).

Next, we determined whether the PMA-stimulated increase in macrophage cholesterol content was accompanied by uptake and degradation of LDL. PMA stimulated a curvilinear nonsaturating increase in cell association and degradation of 125I-LDL incubated at concentrations between 50 and 500 µg/ml, the latter being the highest concentration we tested with radiolabeled 125I-LDL (Fig. 6, A and B). Without PMA, cell association of 125I-LDL also showed a curvilinear increase with increasing 125I-LDL concentration but reached a level that was only 38% of the level reached when macrophages were incubated with 125I-LDL plus PMA. Without PMA, degradation of 125I-LDL saturated above 250 µg/ml and was only 20% of the level reached when macrophages were incubated with 125I-LDL plus PMA. The net total 125I-LDL uptake stimulated by PMA (i.e., cell-associated + degraded 125I-LDL with PMA minus cell-associated + degraded 125I-LDL without PMA) was linear with increasing 125I-LDL concentration (Fig. 6C). On the other hand, the total 125I-LDL uptake without PMA showed saturation with increasing 125I-LDL concentration. At 500 µg/ml 125I-LDL, the total macrophage uptake of 125I-LDL was between five and six times greater in the presence of PMA compared with the absence of PMA (Table II). 78 and 83% of 125I-LDL taken up by PMA-treated macrophages for 1 and 2 days, respectively, was degraded, and a similar amount of 125I-LDL, 73 and 82%, was degraded by untreated macrophages (Table II). Thus, in both cases, macrophages degraded the majority of 125I-LDL taken up. In an analysis to be presented below, the amount of 125I-LDL taken up by PMA-stimulated macrophages could account for the increase in cholesterol content of the macrophages.

PMA-stimulated uptake of LDL led to ACAT-dependent cholesterol esterification. Incubating macrophages with LDL and PMA in the absence and presence of the ACAT inhibitor, SS5035, showed this. The ACAT inhibitor almost completely blocked accumulation of cholesteryl ester in the macrophages (Table III). Simultaneously, the macrophage unesterified cholesterol content increased. This is consistent with the known action of ACAT to re-esterify lipoprotein-derived unesterified cholesterol after lysosomal hydrolysis of lipoprotein-derived cholesteryl ester (27). The ACAT inhibitor decreased PMA-stimulated macrophage cholesterol accumulation by 30% compared with macropahes incubated without the ACAT inhibitor. The decrease in macrophage cholesterol accumulation was not the result of a decrease in LDL uptake because the ACAT inhibitor had no effect on PMA-stimulated 125I-LDL uptake (data not shown). The decrease is consistent with spontaneous efflux of unesterified cholesterol from the macrophages as we reported previously (28).

The LDL Receptor Did Not Mediate PMA-stimulated Uptake of LDL—Differentiated human monocyte-derived macrophages poorly express the classical LDL receptor (4). However, it was possible that PMA treatment of macrophages increased expression of this receptor. Reductive methylation of LDL apoB blocks its binding to the LDL receptor (29). This LDL modification decreased cholesterol accumulation by a small amount, 9 ± 1% in one experiment and 20 ± 2% in another experiment (the latter experiment is shown in Table IV). However, the LDL receptor was not responsible for the small decrease in cholesterol accumulation which occurred with reductively methylated LDL. The anti-LDL receptor-blocking monoclonal antibody, C7 (30), added at a 10-fold molar excess based on protein, showed no inhibition of PMA-stimulated cholesterol accumulation compared with an isotype-matched control antibody (Table IV). Moreover, we found that PMA treatment of macrophages did not increase 125I-LDL binding. Rather, PMA decreased total and specific 125I-LDL binding (i.e., inhibitable by a 20-fold excess of unlabeled LDL) and did not change nonspecific 125I-LDL binding (Fig. 7).

Role of ApoB in PMA-stimulated LDL Uptake—PMA-stimulated uptake of LDL did not depend on apoB, the major protein component of LDL. This was shown by treating LDL with papain to digest apoB completely and then purifying the papain-treated LDL with gel filtration chromatography. SDS-gel electrophoresis showed that papain completely digested the

**Fig. 5. Effect of protein kinase C inhibitors and activators on macrophage cholesterol accumulation.** Two-week-old macrophage cultures were incubated for 2 days with 2 mg/ml LDL and either 1 µg/ml PMA plus 4 µM protein kinase C inhibitor (A) or 0.04 µM bryostatin, a protein kinase C activator (B). After the incubations, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol contents.
shows the total 125I-LDL uptake (LDL in the absence and presence of PMA) minus total 125I-LDL uptake with PMA stimulation (i.e. total 125I-LDL uptake without PMA).

Fig. 6. LDL concentration dependence of macrophage 125I-LDL cell association and degradation. Two-week-old macrophage cultures were incubated for 1 day with varying concentrations of 125I-LDL in the absence and presence of 1 μg/ml PMA. After the incubations, the amount of cell-associated 125I-LDL (A) and the amount of trichloroacetic acid-soluble organic 125I in media (i.e. the degraded 125I-LDL) (B) were determined as described under “Experimental Procedures.” C shows the total 125I-LDL uptake (i.e. cell-associated plus degraded 125I-LDL) without PMA stimulation and the net total 125I-LDL uptake with PMA stimulation (i.e. total 125I-LDL uptake with PMA minus total 125I-LDL uptake without PMA).

Our results show how macrophage foam cells can form from native LDL. PMA activation of monocyte-derived macrophages stimulated an LDL concentration-dependent increase in macrophage cholesterol accumulation sufficient to reproduce the appearance and cholesterol levels of foam cells isolated from atherosclerotic plaques (9, 26). PMA activation of macrophages has been shown previously to stimulate macrophage fluid phase endocytosis as we observed here (34, 36). Cholesterol accumulation in our study resulted from PMA-stimulated macrophage cholesterol uptake.

Role of Selective Cholesterol Uptake from LDL—Selective uptake of cholesteryl ester from LDL into cells mediated by the SR-B1 receptor has been described (33). With this mechanism of cholesterol accumulation, the amount of cholesterol delivered to cells exceeds the amount of lipoprotein taken up and degraded by the cells. This occurs because cholesteryl ester is selectively delivered into the cell compared with the protein components of the lipoprotein. However, selective uptake of LDL cholesteryl ester was not the mechanism of PMA-stimulated cholesterol accumulation by macrophages incubated with LDL. Experiments showed that the total amount of 125I-LDL uptake (cell association plus degraded 125I-LDL) could account for cholesterol accumulated by PMA-stimulated macrophages incubated with 125I-LDL (Table V). Thus, selective cholesteryl ester uptake did not occur.

Role of Fluid Phase (i.e. Bulk Phase) Endocytosis in Macrophage LDL Uptake—Because PMA did not stimulate increased binding of LDL to macrophages and PMA stimulates fluid phase endocytosis in macrophages (34, 35), we next examined whether fluid phase endocytosis could explain the PMA-stimulated uptake of LDL. Fluid phase endocytosis was measured using [3H]sucrose. We found that PMA treatment of macrophages increased macrophage uptake of medium during a 24-h incubation from 5.8 ± 0.4 to 25.5 ± 1.2 μl/mg of cell protein (n = 10 experiments). Macrophage uptake of [3H]sucrose showed a biphasic linear increase over 24 h (data not shown) consistent with previous data (36, 37). Measurement of [3H]sucrose uptake in the presence of 500 μg/ml LDL (the concentration of LDL used for determining 125I-LDL uptake) or a 20-fold excess of unlabeled sucrose did not affect [3H]sucrose uptake. Comparison of the amount of 125I-LDL uptake that occurred with the amount of 125I-LDL uptake predicted from fluid phase endocytosis of medium showed that an average of 83 ± 4% of 125I-LDL uptake could be accounted for from fluid phase endocytosis (Table VI).

DISCUSSION

Our results show how macrophage foam cells can form from native LDL. PMA activation of monocyte-derived macrophages stimulated an LDL concentration-dependent increase in macrophage cholesterol accumulation sufficient to reproduce the appearance and cholesterol levels of foam cells isolated from atherosclerotic plaques (9, 26). PMA activation of macrophages has been shown previously to stimulate macrophage fluid phase endocytosis as we observed here (34, 36). Cholesterol accumulation in our study resulted from PMA-stimulated uptake of LDL as part of the bulk phase fluid that the activated macrophages took up. Macrophage foam cell formation that occurs by uptake of bulk phase fluid (i.e. fluid phase endocytosis) does not require LDL modification or binding to macrophage receptors.
Macrophage Foam Cell Formation with Native LDL

Two-week-old macrophages were incubated with 500 μg/ml 125I-LDL for the indicated days without and with 1 μg/ml PMA. Then, media were collected, macrophages were rinsed, and cell-associated 125I-LDL was determined. Next, trichloroacetic acid-soluble organic 125I was degraded 125I-LDL determined in four separate experiments. Shown in parentheses is the percentage of the total 125I-LDL taken up which was degraded by the macrophages.

| Incubation time (days) | Total 125I-LDL uptake | +PMA | +PMA:–PMA ratio |
|------------------------|-----------------------|------|-----------------|
| 1                      | 2.8 ± 0.6 (73 ± 6)     | 16.6 ± 1.0 (78 ± 5) | 5.9 |
| 2                      | 8.3 ± 0.3 (82 ± 1)     | 42.8 ± 2.4 (83 ± 2) | 5.2 |

Table III

Effect of ACAT inhibition on cholesterol accumulation by PMA-stimulated macrophages

Two-week-old macrophage cultures were incubated for 2 days with RPMI 1640 and the indicated additions of 2 mg/ml LDL, 1 μg/ml PMA, and 4 μg/ml of the ACAT inhibitor, SS8–035. After the incubations, macrophages were rinsed, harvested, and analyzed for their protein and cholesterol contents. The decrease in the total cholesterol content of macrophages incubated with LDL + PMA + ACAT inhibitor compared with the total cholesterol content of macrophages incubated with LDL + PMA was statistically significant.

| Incubation condition | Total cholesterol content | Unesterified | Esterified | Esterified cholesterol |
|----------------------|---------------------------|--------------|------------|-----------------------|
| No addition          | 94 ± 1                    | 94 ± 2       | 0 ± 2      | 0 ± 2                 |
| LDL                  | 126 ± 4                   | 113 ± 2      | 13 ± 2     | 10 ± 2                |
| LDL + PMA            | 487 ± 23                  | 188 ± 5      | 299 ± 22   | 61 ± 2                |
| LDL + PMA + ACAT inhibitor | 379 ± 17             | 359 ± 14     | 20 ± 3     | 5 ± 1                 |

Table IV

Evaluation of LDL receptor function in cholesterol accumulation by PMA-stimulated macrophages

Our discovery of foam cell formation mediated by fluid phase endocytosis resulted from consideration of factors that could affect foam cell formation in atherosclerotic plaques. One factor is that LDL and cholesterol concentrations in atherosclerotic plaques are much higher than those concentrations that saturate most cell receptors. LDL concentrations of less than 100 μg/ml LDL are commonly used for in vitro studies of foam cell formation, whereas LDL concentrations in the artery intima where foam cells form have been shown to be much higher, ranging from about 0.7 to 2.7 mg/ml LDL (23–25). Another factor is that macrophages in atherosclerotic plaques are likely activated because of their exposure to inflammatory mediators released from other cells, which include lymphocytes within atherosclerotic plaques (38). By testing the combined effects of macrophage activation and LDL concentrations similar to levels that occur in atherosclerotic plaques, we discovered that macrophage foam cell formation occurs readily with native LDL.

In past studies of fibroblasts and inactivated macrophages, non-LDL receptor-mediated uptake of LDL did not produce cellular cholesterol accumulation (39–41). This was because the cholesterol released from the degraded LDL in those studies was excreted into the medium rather than remaining within the cells. Non-LDL receptor-mediated LDL uptake in these earlier studies was attributed to both nonspecific low affinity
binding processes and fluid phase endocytosis (6, 42). The fact that cholesterol accumulation occurred in our study but not in these previous studies can be attributed in part to PMA activation of the macrophages here which caused a substantial increase in LDL uptake by the macrophages. Also, PMA activation of macrophages has been reported to increase ACAT activity, which could increase cholesterol esterification (43). Previously, it was shown that reductive methylation of LDL decreases the saturable nonspecific low affinity binding uptake of LDL observed at high LDL concentrations (44). Possibly, uptake of LDL through this low affinity binding could have produced the small amount of non-LDL receptor-mediated cholesterol accumulation in our study which was not the result of fluid phase endocytosis.

PMA stimulates macroinocytosis in macrophages, a fluid phase endocytic pathway distinct from micropinocytosis which accounts for fluid phase uptake in fibroblasts and inactivated macrophages (34, 35). Micropinocytosis of extracellular fluid occurs during endocytosis mediated by small vesicles (<200 nm) that pinch off from the plasma membrane (45). During micropinocytosis, large vacuoles (>1 μm) form from plasma membrane extensions that envelop extracellular fluid. Jones et al. (46) have suggested that macroinocytosis can mediate uptake of LDL by adsorptive endocytosis of LDL bound to macropinosome membranes and LDL taken up with the bulk phase fluid of macropinosomes. PMA did not stimulate cholesterol accumulation when cultured fibroblasts were incubated with LDL, and PMA is not known to stimulate macroinocytosis in fibroblasts. Thus, PMA stimulation of macroinocytosis possibly contributes to the increased fluid phase endocytosis of LDL by PMA-activated macrophages. We are currently investigating to what extent macroinocytosis and other endocytic pathways contribute to macrophage fluid phase endocytosis of LDL.

It also will be important in future research to learn which naturally occurring macrophage activators can stimulate macrophage LDL uptake similarly to PMA. In this regard, the macrophage activator, microbial-derived lipopolysaccharide, also stimulates macrophage LDL uptake (47–51). Analysis of the effect of lipopolysaccharide on LDL uptake is complicated by the fact that lipopolysaccharide binds LDL (52). This interaction potentially produces LDL uptake through lipopolysaccharide rather than LDL binding to macrophages. In any case,

![Graph](image.png)

**FIG. 8.** Macrophage cholesterol accumulation during incubation with papain-treated LDL. LDL was treated for 48 h with papain to remove apoprotein from the LDL. Then, the papain-treated LDL was purified by gel filtration column chromatography. Next, 2-week-old macrophage cultures were incubated for 2 days with the papain-treated LDL (3.050 nmol of total cholesterol/ml) without or with 1 μg/ml PMA. After the incubations, macrophages were rinsed, harvested, and analyzed for their protein and total cholesterol contents.

| Table V: Cholesterol accumulation predicted from 125I-LDL uptake by PMA-stimulated macrophages |
|---|
| **Experiment no.** | **Total 125I-LDL uptake (μg protein/mg of cell protein)** | **Cholesterol accumulation (nmol/mg of cell protein)** |
| | Predicted | Actual |
| 1 | 38 ± 1 | 153 ± 6 | 160 ± 22 |
| 2 | 43 ± 2 | 205 ± 10 | 184 ± 6 |
| 3 | 49 ± 1 | 195 ± 1 | 199 ± 5 |

**Table VI: 125I-LDL uptake predicted from fluid phase endocytosis in PMA-stimulated macrophages**

Two-week-old macrophage cultures were incubated for 1 day with 500 μg/ml 125I-LDL, 0.8 nmol/ml [3H]sucrose, and 1 μg/ml PMA. After the incubations, media were collected, macrophages were rinsed and harvested, and cell-associated 125I-LDL and [3H]sucrose were determined. Then, trichloroacetic acid-soluble organic 125I (i.e., degraded 125I-LDL) was determined in the media samples. The total 125I-LDL uptake was calculated as the sum of the cell-associated and degraded 125I-LDL. The predicted cholesterol accumulation was calculated by multiplying the LDL cholesterol content (nmol/mg of LDL protein) and the total 125I-LDL uptake (μg of LDL protein/mg of cell protein). The actual cholesterol accumulation was calculated by subtracting the macrophage cholesterol content at the beginning of the experiment from the macrophage cholesterol content after the 2 days of incubation. There were no statistical differences between the actual and predicted cholesterol accumulations for the three experiments, each carried out with macrophages from a different donor.

| Table VI: 125I-LDL uptake predicted from fluid phase endocytosis in PMA-stimulated macrophages |
|---|
| **Experiment no.** | **Fluid uptake μl/mg cell protein/24 h** | **Total 125I-LDL uptake (μg protein/mg of cell protein)** | **Cholesterol accumulation (nmol/mg of cell protein)** |
| | Predicted | Actual | Predicted:actual |
| 1 | 21.7 ± 3.2 | 10.9 ± 1.6 | 14.2 ± 0.2 | 77 |
| 2 | 25.1 ± 2.5 | 12.6 ± 1.2 | 16.3 ± 0.9 | 77 |
| 3 | 23.7 ± 2.6 | 14.6 ± 0.5 | 16.8 ± 0.6 | 87 |
| 4 | 34.7 ± 2.7 | 17.3 ± 1.4 | 19.0 ± 0.3 | 91 |

Average 83 ± 4
lipopolysaccharide-stimulated LDL uptake by macrophages is different from PMA-stimulated LDL uptake in many respects. Lipopolysaccharide-stimulated LDL uptake depends on LDL binding to macrophages, does not involve protein kinase C activation, and produces only modest levels of macrophage LDL uptake and cholesterol accumulation compared with the effects of PMA on LDL uptake shown here.

Because fluid phase endocytosis of LDL does not depend on recognition of the apoB component of LDL, this pathway could mediate macrophage uptake of lipid particles that comprise the lipid core of atherosclerotic plaques. These cholesteryl ester-rich lipid particles lack apoB and are believed to form from LDL that has lost its apoB component because of proteolysis within the plaque (53–55). This is believed because similar LDL-derived lipid particles form in vitro when LDL is treated with proteases that extensively degrade the apoB component of LDL (56). Loss of apoB from LDL causes the LDL lipid cores to fuse, forming lipid particles similar to those in plaques that are up to 10 times the size of native LDL (54, 57). Here we showed that PMA-activated macrophages also accumulated cholesterol when incubated with these LDL-derived lipid particles that lack apoB after treating LDL with protease.

In conclusion, we demonstrate a mechanism of foam cell formation in atherosclerotic plaques which does not require LDL modification as a trigger for macrophage LDL uptake. Rather, activated macrophages can take up native LDL and apoB-free lipid particles derived from LDL in the bulk phase fluid without their binding to the macrophage surface. Uptake of LDL by fluid phase endocytosis in activated macrophages produces massive storage of LDL-derived cholesterol in the macrophages. Cholesterol storage in macrophages alters cholesterol trafficking and macrophage function. Thus, altering the state of macrophage activation and fluid phase endocytosis in atherosclerotic plaques are factors that could influence atherosclerotic plaque cholesterol accumulation and progression.

Acknowledgments—We thank Janet Chang and Rani Rao for help in carrying out experiments and the Department of Transfusion Medicine, Clinical Center, National Institutes of Health, for providing elutriated monocytes.

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