Cholesterol Metabolism and Efflux in Human THP-1 Macrophages

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Abstract—This study has investigated in detail factors regulating accumulation, esterification, and mobilization of cholesterol in human THP-1 macrophages. Human THP-1 monocytes were differentiated into macrophages and then cholesterol enriched by exposure to acetylated LDL (AcLDL), together with \[^{3}H\]free cholesterol (FC). Although THP-1 macrophages accumulated FC and esterified cholesterol (EC), assessed by both mass and radioactivity, cellular EC always demonstrated a much lower specific activity (cpm/μg) than did cellular FC, and several potential causes of this finding were investigated. Inhibition of acyl-CoA:cholesterol acyltransferase (ACAT) during loading decreased cellular \[^{3}H\]EC by 95±1.4% but decreased cell EC mass by only 66.0±4.0%, indicating that some intracellular undegraded AcLDL-derived EC was present in these cells. Esterification of \[^{3}H\]oleate to EC in THP-1 cells loaded with AcLDL was 2.0 mmol · mg⁻¹ · h⁻¹, consistent with previous literature. However, EC, triglyceride, and phospholipid fractions respectively contained 1.0±0.07%, 80.0±0.5%, and 18.9±0.3% of cell \[^{3}H\]oleate, indicating triglycerides were much more metabolically active than EC. In addition, the mass of triglyceride in THP-1 macrophages exceeded that of EC both before and after exposure to AcLDL. Esterification of nonlipoprotein-derived cholesterol was compared in THP-1 cells and nonhuman Fu5AH, CHO, and RAW macrophage cells. Whereas the nonhuman cell lines all esterified over 30% of 2-hydroxypropyl-β-cyclodextrin (hp-β-CD)–delivered cholesterol within 6 hours, THP-1 cells esterified <8.0% of incorporated cholesterol. Kinetics of cholesterol efflux from AcLDL-loaded THP-1 cells were first investigated after loading with only FC, and interactions between efflux and EC hydrolysis were further assessed after loading cells with both EC and FC. Over 24 hours, human apolipoprotein (apo) A-I, apoHDL reconstituted with phosphatidylcholine, and HDL₃ respectively removed 46.6±3.7%, 61.3±3.4%, and 76.4±10.1% of \[^{3}H\]FC from FC-enriched THP-1 cells. Cholesterol efflux to apoA-I was saturated by 24 hours and was enhanced by using apoA-I–phospholipid instead of pure apoA-I. Kinetic modeling identified that 97% of effluxed FC derived from a slow pool, with a T_{1/2} ranging from 27.7 hours for HDL to 69.3 hours for apoA-I. Although efflux enhanced net clearance of EC, hydrolysis of EC during concurrent inhibition of ACAT was unaffected by cholesterol efflux. Supplementation of THP-1 cultures with cAMP to stimulate hormone-sensitive lipase did not significantly enhance net hydrolysis of EC or cholesterol efflux. In conclusion, human THP-1 macrophages contain a large and metabolically active pool of triglyceride and a relatively inactive pool of EC. The low specific activity of EC relative to FC is contributed to by reduced esterification of FC, slow hydrolysis of EC, and accumulated lipoprotein EC. The relative inactivity of the EC pool may further contribute to already impaired cholesterol efflux from these cells. Net cholesterol efflux from human macrophages is achieved by pure apoA-I and is substantially further enhanced by the presence of phospholipid in acceptor particles. (Arterioscler Thromb Vasc Biol. 1998;18:1589-1599.)

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previously available human monocyte lines. THP-1 cells, like mature human primary macrophages, synthesize and secrete M-CSF and apolipoprotein (apo) E, and IL-8 secretion has been described in THP-1 cells. THP-1 cells have been particularly useful for studying the induction of ScR expression during their differentiation from monocytes to macrophages during exposure to phorbol myristate acetate (PMA). Previous studies have confirmed both ScR expression and increased cholesterol esterification after exposure to acetylated LDL (AcLDL) in THP-1 cells, although the relative quantitative and metabolic activities of FC, EC, and triglyceride (TG) pools in THP-1 cells are as yet undefined. Given that ex vivo human foam cell macrophages and primary cultures of human monocyte-derived macrophages are described as containing significant amounts of TG, the size and metabolic activity of TG may be an important component of lipid metabolism of human macrophages in general and THP-1 cells in particular. Previous literature has suggested that there is deficient EC hydrolysis in THP-1 cells and that this factor was limiting for cholesterol efflux. Westman et al suggested that cholesterol efflux does not occur from THP-1 cells to phospholipid-deficient apoA-I, an observation which, if confirmed, would imply that THP-1 cells differed markedly from other macrophage species (see References 19 and 20). It would also imply that apolipoprotein-mediated efflux of cholesterol may be unimportant in human macrophages. The kinetics of cholesterol efflux per se, independent of EC hydrolysis, have not been established in THP-1 cells, nor is it known if cholesterol efflux from these macrophages is derived from multiple kinetic pools.

In these studies, we have investigated the accumulation and efflux of cholesterol and the relative size and metabolic activity of the EC and TG pools in human THP-1 macrophages. THP-1 cells contained large quantities of metabolically active TG and had a low specific activity in their EC pool compared with that of the FC pool. This finding was attributable to the combination of accumulated undegraded lipoprotein EC, slow esterification of exogenous FC, and sluggish EC hydrolysis. In addition, slow efflux of FC from THP-1 cells was observed, independent of impaired EC hydrolysis. Cholesterol efflux followed a 2-pool kinetic model, with important differences between cholesterol acceptors according to their phospholipid composition; however, definite efflux to pure apoA-I was observed.

Materials
BSA (essentially fatty acid free), heat-inactivated fetal bovine serum (FBS), cholesteryl methyl ether, gentamicin, penicillin/streptomycin, cAMP, and FC were purchased from Sigma Chemical Company. Radioisotopes [1,2-3H]cholesterol (46.5 Ci/mmol), [4-14C]cholesterol (51.3 mCi/mmol), and [1,2-3H]oleate (10.0 Ci/mmol) were obtained from New England Nuclear. Organic solvents were obtained from Fisher Scientific. Tissue-culture flasks and plates were from Falcon and Corning. RPMI culture medium was purchased from Sandoz-58035 (S-58035) and Pfizer CP-113 818 (CP-113 818) were gifts from Sandoz (Dr J. Heider) and Pfizer (Dr M. Bamberger) Pharmaceuticals, respectively. 1-Palmityl-2-oleyl phosphatidylincholine (POPC) and egg PC were purchased from AVANTI polar lipids. 2-Hydroxypropyl-β-cyclodextrin (hp-β-CD) was purchased from Cyclodextrin Technologies Development Inc (Lot No. E8309) or was a gift from Cerestar, Hammond, Indiana.

Lipoprotein Preparation
Human LDL (1.019<d<1.063 g/mL) and HDL (1.21<d<1.25 g/mL) were isolated by sequential ultracentrifugation, dialyzed against 0.15 mol/L NaCl, and sterilized by 0.45 μm filter. LDL was acetylated with acetic anhydride. Dispersions containing FC and egg PC and preparations of pure human apoA-I were prepared as described. ApoHDL protein reconstituted with POPC (rHDL-PC) and apoA-I–PC were prepared by sonication of delipidated HDL apoprotein, or apoA-I, in 0.9% NaCl solution with POPC at 4°C under nitrogen, achieving a phospholipid-to-protein mass ratio of 2.5:1.

Culture and Loading of Cells
THP-1 monocytes (American Type Tissue Culture Collection, Camden, NJ) were grown in suspension at 37°C in 5% CO2 in bicarbonate-buffered RPMI containing 10% FBS (vol/vol), 50 μmol/L β-mercaptoethanol, and 50 μg/mL gentamicin, at a cell density of 0.2 to 1.0 × 10^6/mL. Cells were plated at a density of 1.2×10^6/cm^2 dish or 2.4×10^6/cm^2 dish in RPMI with 10% FBS, 50 μmol/L β-mercaptoethanol, 50 μg/mL gentamicin, and 50 ng/mL PMA for 3 to 4 days to become fully differentiated macrophages before use in experiments. Cells were used between passages 5 and 20.

Differentiated THP-1 macrophages were washed extensively with serum-free RPMI before exposure to RPMI containing AcLDL (150 μg protein per milliliter), 1% FBS, 50 ng/mL PMA, [3H]cholesterol (2 μCi/mL), and mercaptoethanol and gentamicin as above, for 24 to 96 hours. In the majority of experiments, cells were enriched with cholesterol by exposing them to AcLDL-containing medium for 24 hours. In some experiments, maximizing the size of the EC pool was desirable, which was achieved by increasing the duration of cholesterol enrichment to 48 or even 96 hours. (In initial experiments, cholesterol-phospholipid dispersions were included in the loading medium [150 μg cholesterol per milliliter medium], but these experiments were subsequently omitted, as they did not significantly affect accumulation of FC or EC mass [3H]cholesterol in THP-1 macrophages.) In typical experiments investigating cholesterol efflux, cells were loaded for 24 to 48 hours, then washed and incubated overnight in RPMI equilibration medium containing 2 mg/mL BSA and gentamicin, PMA, and mercaptoethanol as above. Cells were washed before efflux incubations with RPMI containing various cholesterol acceptors.

We systematically investigated the effects of withdrawal of PMA after differentiation of THP-1 monocytes into macrophages. Although previous literature indicated that PMA was an essential requirement for differentiation of THP-1 monocytes and maximizing ScR expression, in our studies, withdrawal of PMA after differentiation decreased cell viability over subsequent stages of long experiments. Our observations also agree with those by several other investigators. To exclude alterations to cell lipid metabolism caused by PMA directly or by its effects on protein kinase activity (see References 27 through 29), the effect of continuing or withdrawing PMA after differentiation was systematically investigated, and some of these comparative data are presented in “Results.” In general, there were only minor differences in lipid metabolism between groups with and without ongoing PMA, and PMA was routinely continued throughout experiments to maximize cell viability.

Resident mouse peritoneal macrophages were isolated by lavage from unstimulated QS mice and plated in 35-mm-diameter tissue-culture wells (Costar) at 5 to 6 × 10^6 cells per well as described. Cultures were incubated at 37°C for 1 to 2 hours to establish adherence, then washed 3 times with prewarmed PBS before incubation with Dulbecco’s modified Eagle’s medium containing lipoprotein-deficient serum (10% vol/vol, equivalent to final protein
concentration of 2.5 mg/mL), penicillin G, and streptomycin, plus AcLDL as previously described.20

Human monocytes were isolated from white cell concentrates using centrifugal elutriation as previously described.13 Purified monocytes (>95% purity by nonspecific esterase staining) were differentiated by plating at a density of 1.5 × 10⁶ cells per 22-mm diameter culture dish (Costar) in RPMI 1640 containing antibiotics and glucoseamine as above, and 10% (vol/vol) heat inactivated whole human serum, for 9 days, with fresh medium changes every 2 to 3 days. After differentiation, the cells were washed in warm PBS and incubated with RPMI 1640 containing lipoprotein-deficient serum (10%, vol/vol) and acetylated LDL (50 μg protein per milliliter) to achieve cellular enrichment with cholesterol.

**Metabolic Labeling**

THP-1 macrophages exposed to AcLDL for 22 hours were pulsed with [1H]oleate complexed with BSA (molar ratio oleic acid to BSA, 5:1; specific activity of oleic acid, 5.8 × 10⁶ cpm/μg) for 2 hours.31,32 To quantify lipid fractions isolated by thin-layer chromatography (TLC), [1H]cholesterol was used as an internal standard and was added to culture dishes at the time of addition of isopropanol.

To label cells with [1H]cholesterol during cholesterol enrichment, AcLDL and [1H]cholesterol in ethanol were coinubated with FBS and 10% of the calculated final volume of RPMI overnight at 37°C, then diluted to final concentrations of 150 μg protein per milliliter for AcLDL, 1% FBS (vol/vol), and 2 μCi/μL [1H]cholesterol in RPMI, as previously described.33,34 The final concentration of ethanol in RPMI was 0.2% (vol/vol). After enrichment with [1H]cholesterol, TLC of AcLDL in FBS-RPMI revealed that in excess of 98% of [1H]cholesterol was unesterified and that the specific activity of [1H]cholesterol was unchanged by 0.45 μmol/L filtration or by passage through Sepharose PD10 columns (columns supplied by Pharmacia; data not shown). To selectively load cells with [1H]fGluc, THP-1 macrophages were loaded in the presence of 1 to 10 μg/mL ACAT inhibitor (5-8035 or CP-113 818; stock solutions in DMSO with final volume ≤0.1%, vol/vol culture medium). In preliminary experiments, almost complete (>95%) maximal inhibition of cholesterol esterification in THP-1 cells was demonstrated by both ACAT inhibitors over this concentration range. To investigate cAMP-mediated stimulation of neutral cholesteryl ester hydrolase (nCEH) activity, stock solutions of cAMP were prepared in DMSO, frozen, and aliquots thawed immediately before use. A final concentration of 100 μmol/L cAMP (DMSO ≤0.1%, vol/vol culture medium) was added to cholesterol-enriched cells after overnight equilibration, as previously described.33,36

To label cells with nonlipoprotein-derived cholesterol, a cyclodextrin solution saturated with respect to cholesterol was prepared in RPMI containing 25 mmol/L Hp-BD (lot No. E8309), 0.625 mmol/L cholesterol, and 4.0 μCi/mL [1H]cholesterol (specific activity of cholesterol–hp-BD solution of 17 224 cpm/μg cholesterol). As described in detail previously,19 [1H]cholesterol and unlabeled cholesterol were mixed in chloroform, evaporated under nitrogen, and dissolved in toluene from which an aliquot was removed for scintillation counting. The toluene solution was re-evaporated and the film of dry cholesterol/[1H]cholesterol was incubated in RPMI containing hp-BD solution overnight at 37°C before filter sterilization (0.45 μmol/L) and incubation with THP-1 cells. Cholesterol–hp-BD solution thus prepared was added without addition of PMA or serum to culture medium for up to 8 hours, after which cell cultures were extracted in isopropanol and analyzed as described below. In preliminary experiments, coinubation of PMA with cholesterol–hp-BD solution had no effect on cholesterol accumulation or cell viability over short 6- to 8-hour incubations. To investigate the possibility that exposure to 10% FBS during differentiation subsequently modified the cellular response to nonlipoprotein-derived cholesterol, differentiated THP-1 cells were washed and preincubated for 24 hours with RPMI or RPMI containing 10% FBS (vol/vol) before incubation with cholesterol–hp-BD solution in each experiment.

**Cellular Lipid Analysis**

Monolayers were washed with PBS, allowed to dry, then incubated in 2 mL of isopropanol and cholesteryl methyl ether internal standard overnight. Isopropanol extracts were recovered and dried under nitrogen, and FCS and total cholesterol mass were respectively determined before and after saponification38 by gas-liquid chromatography (GC), and mass of cholesterol in EC was derived by difference.39 Dried isopropanol extracts were resuspended in toluene, and total [1H]cholesterol was determined from aliquots analyzed by scintillation counting. Residual tolune extracts were dried and redissolved in chloroform/methanol (1:1, vol/vol), and the proportion of label in [1H]FRC (R, 0.24) and [1H]FEC (R, 0.97) fractions was determined after separation by instant TLC (petroleum ether/ethyl acetate/acidic acid, 85:15:1, vol/vol)20 using standards of FC and EC identified by iodine vapor. To separate EC from TG fractions, a mobile phase of petroleum ether/ethyl ether/acidic acid (100:10:1, vol/vol) was used on silica gel TLC plates (Rf values 0.28, 0.67, 0.93 for PC, triolein, and cholesteryl oleate standards, respectively). TG mass (equivalent to micrograms of triolein) was determined on isopropanol cell lipid extracts using a modification of a commercially available colorimetric enzymatic assay (glycerol phosphate oxidase, Catalog No. 339–20, Sigma), after confirmation of linearity of glycerol and triolein standards under conditions used in these experiments.

Lipoprotein phospholipids were determined by the method of Sokoloff and Rothblat41 after Bligh and Dyer extraction32 to remove aqueous phosphate. Cell and lipoprotein protein values were determined by a modification of the Lowry assay43 by Markwell et al.44

**Cholesterol Efflux**

After equilibration, cells were washed and incubated in RPMI (containing β-mercaptoethanol and gentamicin as above) with or without pure apoA-I (25 μg protein per milliliter), HDL₃ (200 μg phospholipid per milliliter), rHDL-PC (200 μg phospholipid per milliliter or 25 μg protein per milliliter, the latter equivalent to 62.5 μg phospholipid per milliliter), or apoA-I–PC (25 μg protein per milliliter). These concentrations of apoA-I, HDL₃, and rHDL-PC have previously been found to be optimal for maximal cholesterol efflux from macrophages.38,39

Efflux incubations were performed for up to 24 hours in 22-mm (4.6-cm²) dishes, each containing 2 mL of medium. Aliquots (120 μL) were removed at various times, filtered through 0.45-μmol/L multiscreen filtration plates (Millipore), and 100-μL aliquots were analyzed by scintillation counter to quantify efflux of [1H]cholesterol from cells.45 In other experiments, 1-mL aliquots of media were removed and spun at 14 000 rpm in an Eppendorf microfuge for 15 minutes to pellet any floating cells, and the supernatant was removed and counted. Media samples analyzed using both methods demonstrated identical efflux data were obtained with the filtration plate or the Eppendorf microfuge methods (data not shown).

**Data Analysis**

Percent cholesterol efflux was calculated by dividing total [1H]cholesterol in the medium after efflux by that present in cells before efflux (t₀) for kinetic studies with multiple time points or by dividing cholesterol in the medium by the sum of cholesterol in medium and in cells at the end of 24 hours’ efflux when both cells and media were extracted. Efflux values derived by either method gave almost identical results. In addition, depletion of cellular cholesterol or EC during efflux is presented as a percentage of that in cells at t₀. In figures and tables, all data points represent the mean±SD of 3 cell cultures from a single representative experiment. Computer modeling of cholesterol efflux kinetic data was analyzed by Cell Chol molecular modeling program48 to generate the data described in the Table and Figure 8. Data were analyzed to fit either a single kinetic pool or a 2–kinetic pool model and compared for minimization of error as described in detail previously.46
Cholesterol Metabolism in THP-1 Macrophages

**Results**

**Accumulation of EC Mass and[^3]H]EC in THP-1 Cells**

Before exposure to AcLDL, THP-1 cells differentiated for 3 days in 10% FBS with PMA typically contained 10 to 30 μg FC per milligram cell protein and undetectable or very small quantities of EC. After 24 hours’ incubation with AcLDL, the cell FC mass doubled and EC mass increased severalfold, but after 24 hours’ loading, cell EC mass was always less than that of FC. Continued exposure of cells to PMA during loading with AcLDL appeared to increase FC content of THP-1 cells in some experiments, but any such effects were modest and varied between experiments (Figure 1A).

During 24-hour incubations with AcLDL and[^3]H]FC, cells accumulated[^3]H]EC, and this accumulation was largely unaffected by the continued presence or withdrawal of PMA during incubation with AcLDL (Figure 1B). The relative sizes of the cellular[^3]H]FC and[^3]H]EC pools were consistently even more discrepant than were the respective masses of FC and EC, as indicated by the specific activity (cpm/μg cholesterol; Figure 1C). The low specific activity of EC compared with FC indicated that these 2 pools had not reached equilibrium by the end of 24 hours’ loading (without additional equilibration).

Even more prolonged cholesterol loading (48 hours), followed by a prolonged equilibration incubation (24 hours) did not achieve equal specific activities in[^3]H]FC and[^3]H]EC pools (Figure 2A through 2C). In comparison with cells loaded for 24 hours, cells loaded for 48 hours contained more EC mass than FC; however, the specific activity of FC and EC pools remained unequal even after attempted equilibration. It was concluded that mass data and radioactivity could not be simply correlated when using THP-1 macrophages, and data for each was always calculated independently by GC and scintillation counting, respectively, in all experiments.

We hypothesized that cellular accumulation of undegraded, lipoprotein-derived (hence unlabeled) EC contributed to the low EC specific activity. To test this, THP-1 cells were loaded with unlabeled AcLDL and[^3]H]cholesterol for 24 hours or 48 hours in the presence or absence of ACAT inhibitor (CP-113 818), and FC and EC mass and radioactivity were measured (Figure 3). ACAT inhibition during cholesterol enrichment decreased cell[^3]H]EC to <5% of that present without ACAT inhibition, whereas EC mass was decreased to 35% of that present without ACAT inhibition. Even though EC mass in THP-1 cells was clearly greater after 48-hour than after 24-hour loading, the proportion of cell EC that persisted in the presence of ACAT inhibitor was approximately 30% in the 2 conditions. The discrepancy between substantial residual EC mass and trivial residual[^3]H]EC implied that immediately after loading, approximately 30% of EC mass in THP-1 cells was derived from undegraded AcLDL-derived EC and may contribute to the relatively low specific activity of[^3]H]EC.


To establish that cholesterol esterification by THP-1 cells in our laboratory was consistent with that of previous literature, THP-1 cells were loaded with unlabeled AcLDL for 22 hours, then pulsed with[^3]H]oleate complexed to BSA.[^32] Inhibition
of ACAT effectively inhibited the formation of cholesteryl \(^3\)H\(\text{oleate}\) in THP-1 cells. THP-1 cells esterified \(\approx 2.0\) nmol of \(^3\)H\(\text{oleate}\) per milligram of cell protein per hour, and did so regardless of the presence or absence of PMA during exposure to AcLDL (Figure 4A). This rate of esterification is consistent with previous THP-1 literature ranging between 1.0 and 5.0 nmol \(\cdot \) mg \(^{-1}\) \(\cdot\) h \(^{-1}\), but is substantially less than rates of approximately 12 nmol \(\cdot\) mg \(^{-1}\) \(\cdot\) h \(^{-1}\) achieved with mouse peritoneal macrophages exposed to AcLDL.\(^{31}\)

Despite similar rates of \(^3\)H\(\text{oleate}\) esterification as in previous THP-1 literature, we found that esterification of oleate to EC represented a minute proportion of the total cell oleate separated by TLC (Figure 4B). Nineteen percent of cellular \(^3\)H\(\text{oleate}\) was within the phospholipid fraction, 80% was in the TG pool, and only 1% was present in the EC pool, indicating that differentiated THP-1 cells have a previously uncharacterized metabolically active TG pool and a relatively inactive EC pool.

To further investigate the quantitative importance of the TG pool in these cells, TG mass was measured and related to cholesterol mass in the same cell cultures (Figure 5A). As indicated above, THP-1 cells loaded for 24 hours with AcLDL accumulated both FC and EC. However, the mass of TG in THP-1 cells was greater than that of total cell cholesterol both before exposure to AcLDL and after 24
hours' incubation with AcLDL. Although incubation with AcLDL achieved a greater increment in EC than TG mass, TG mass was 3-fold greater than EC mass in THP-1 cells after loading with AcLDL. Systematic manipulations of culture conditions during the 24-hour loading phase, such as omission of FBS, varying the concentration of FBS from 1% to 10%, or incubating cells with BSA (to bind free fatty acids) did not significantly alter the mass of the TG pool in these cells (data not shown).

To evaluate the relative size of the TG pool in other commonly used macrophages, primary cultures of murine and human macrophages were incubated with AcLDL and analyzed (Figure 5B). Murine macrophages accumulated more EC than did human macrophages (or THP-1 cells) in response to incubation with AcLDL for 24 hours, but the mass of accumulated TG in murine cells was clearly less than the total cell cholesterol. Human macrophages accumulated less EC than THP-1 cells after exposure to AcLDL, as previously described, and, even more markedly than in THP-1 cells, TG mass in primary human macrophages exceeded total cell cholesterol even after exposure to AcLDL. Interestingly, all 3 types of macrophage accumulated some intracellular TG during incubation with AcLDL. The presence of an intracellular pool of TG that was large relative to the mass of accumulated EC was thus confirmed to be a property shared by both primary and THP-1 human macrophages, but not primary murine macrophages.

**Esterification of [3H]Cholesterol Delivered to Cells Through the Plasma Membrane via hp-β-CD**

From the above data, it appeared likely that THP-1 cells esterified cholesterol relatively sluggishly compared with other cell types. To address the issue of cholesterol esterification independently of lipoprotein binding and degradation, we investigated accumulation and esterification of nonlipoprotein-derived cholesterol delivered by hp-β-CD. Cyclodextrins have been used to stabilize sterols in solution and to enrich cells with cholesterol, and cellular loading and esterification of cholesterol stabilized in solution by cyclodextrins have recently been characterized in this laboratory in detail. Delivery of cholesterol to cells by cyclodextrins avoids potential cholesterol crystal formation in aqueous solution, while allowing direct assessment of cholesterol incorporation and esterification. THP-1 cells were differentiated as described in “Methods,” incubated for 24 hours in RPMI/10% FBS (10%, vol/vol), then incubated with cholesterol labeled with [3H]oleate and with AcLDL for 24 hours (THP-1 t0). Aliquots of isopropanol extracts of cell cultures were analyzed for TG, total cholesterol (TC), FC, and EC mass as described in “Methods.” B. Resident murine macrophages were isolated by peritoneal lavage and adhered to culture dishes as described in “Methods” and immediately extracted for cell lipids as above (M t0) or were washed and incubated with AcLDL for 24 hours (M t4) and then extracted. Similarly, primary human monocyte-derived macrophages were isolated by elutriation, differentiated as described in “Methods,” and extracted (H t0) or incubated with AcLDL for 24 hours (H t4) and then extracted. Note that EC is greater than TG only in murine macrophages incubated with AcLDL.
Efflux of Cholesterol From THP-1 Macrophages

Efflux of Unesterified Cholesterol

To assess cholesterol efflux independent of the rate of hydrolysis of EC, cholesterol efflux was evaluated after loading and equilibrating THP-1 macrophages in the presence of ACAT inhibitor (CP-113 818). More than 98% of cholesterol label (TLC) and mass (GC) in THP-1 cells so loaded were confirmed to be unesterified (data not shown). Because previous investigations indicated that pure apoA-I did not cause cholesterol efflux from THP-1 macrophages and to evaluate the relative contribution of selected structural components of HDL, in causing cholesterol efflux, we compared several cholesterol acceptors. Lipid-free apoA-I (25 μg protein per milliliter), reconstituted HDL-PC (rHDL-PC) and HDL3 (both at 200 μg phospholipid per milliliter), and rHDL-PC (25 μg protein per milliliter, equivalent to 62.5 μg phospholipid per milliliter) were compared with RPMI (control medium) for their capacity to induce efflux of [3H]cholesterol. (As identical kinetics were observed with or without PMA in the efflux medium, only data with PMA are presented.)

All acceptors caused clear time-dependent efflux of [3H]cholesterol (Figure 7A), and extraction of cells at t₀ (onset of efflux incubation) and at 24 hours confirmed significant depletion of both cell [3H]cholesterol and cell cholesterol mass during efflux incubations (Figure 7B). The kinetic profiles of efflux of [3H]cholesterol into the medium (Figure 7A) indicated that lipid-free apoA-I was the only acceptor to clearly become saturated by 24 hours. These kinetic data suggested that phospholipids were important in maintaining the net gradient for cholesterol efflux to occur from human THP-1 cells. To exclude the possibility that apolipoprotein composition alone explained the difference between rHDL-PC (25 μg protein per milliliter) and apoA-I (25 μg protein per milliliter), THP-1 cells were incubated with apoA-I, apoA-I–phospholipid particles, and HDL3 (Figure 7C). These data confirmed that like macrophages from other species, lipid-free apoA-I can cause significant cholesterol efflux from these cells, but phospholipid itself enhanced apoA-I–mediated efflux.

In view of the complex kinetics observed, the experimental data derived from cells enriched exclusively with FC were subjected to detailed modeling using the Cell Chol model (Table). Data were first modeled to fit a single kinetic pool (1 pool) of cellular [3H]cholesterol. rHDL-PC was the most effective of the acceptors not containing cholesterol (unlike LDL), and thus, interpretation of efflux to this acceptor is not complicated by 2-way flux of lipoprotein-derived cholesterol and cell-derived cholesterol. The t½ for efflux to rHDL-PC at 200 μg phospholipid per milliliter was 18.2 hours when estimated using a single-pool model.

Modeling data to a fast and slow pool of cholesterol (2 pools) of unspecified cellular location, both of which were accessible for efflux and which could exchange cholesterol with each other, revealed a far superior fit of the data (Table). Error decreased 5-fold to 10-fold using a 2-pool model. During efflux to maximally effective concentrations of acceptors, approximately 97% of cholesterol derived from the initial 2 hours, whereas [3H]EC accumulation was approximately linear over 6 hours, consistent with esterification of 35.0 ± 1.5% (Table). Data were first modeled to fit a single kinetic pool (1 pool) of cellular [3H]cholesterol. rHDL-PC was the most effective of the acceptors not containing cholesterol (unlike LDL), and thus, interpretation of efflux to this acceptor is not complicated by 2-way flux of lipoprotein-derived cholesterol and cell-derived cholesterol. The t½ for efflux to rHDL-PC at 200 μg phospholipid per milliliter was 18.2 hours when estimated using a single-pool model.

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Slow pool. Efflux from the slow pool best discriminated between the different acceptors, as all were similarly effective at removing cholesterol from the fast pool. The concentration of phospholipid-containing particles clearly affected the rate of efflux from the slow pool. For example, $t_{1/2}$ increased from 33.0 hours to 63.0 hours as the total concentration of rHDL-PC phospholipid decreased from 200 mg to 62.5 mg phospholipid per milliliter. Phospholipid-containing particles also demonstrated less apparent influx of cholesterol back to the cells, which is discussed in detail in the Discussion section.

Hydrolysis of Cell Esterified Cholesterol and Cholesterol Efflux

The rate of hydrolysis of cytosolic (ACAT-generated) [3H]EC was measured in THP-1 cells loaded with widely differing EC masses (range 19.9 ± 2.0 to 128.7 ± 8.5 μg/mg) by incubating THP-1 cells with AcLDL and [3H]cholesterol for between 24 and 96 hours. Cells were then equilibrated overnight, washed, and incubated for a further 24 hours with an ACAT inhibitor S-58035 (1 μM) or both S-58035 and cAMP (100 μmol/L). Results were identical with and without PMA during efflux; thus, only +PMA results are included.

A. Aliquots of media were removed as described in Methods at specified intervals, filtered, and counted by scintillation. Cumulative efflux was calculated separately at each time point for each dish and percent efflux by dividing total counts in the medium per dish by counts in control cell extracts removed at t0. B. Percent efflux of [3H]cholesterol into medium after 24 hours' incubation in specified media. Each point represents the mean ± SD of 3 dishes, and efflux data in B are derived from the same cell cultures generating data in A.

Cells undergoing efflux of cholesterol cleared more EC than cells incubated in control medium such as BSA (Figure 8). However, the extent of clearance was entirely consistent of efflux from the slow pool. For example, $t_{1/2}$ increased from 33.0 hours to 63.0 hours as the total concentration of rHDL-PC phospholipid decreased from 200 μg to 62.5 μg phospholipid per milliliter. Phospholipid-containing particles also demonstrated less apparent influx of cholesterol back to the cells, which is discussed in detail in the Discussion section.

Hydrolysis of Cell Esterified Cholesterol and Cholesterol Efflux

The rate of hydrolysis of cytosolic (ACAT-generated) [3H]EC was measured in THP-1 cells loaded with widely differing EC masses (range 19.9 ± 2.0 to 128.7 ± 8.5 μg/mg) by incubating THP-1 cells with AcLDL and [3H]cholesterol for between 24 and 96 hours. Cells were then equilibrated overnight, washed, and incubated for a further 24 hours with an ACAT inhibitor S-58035 (1 μM) or both S-58035 and cAMP (100 μmol/L). A, Aliquots of media were removed as described in Methods at specified intervals, filtered, and counted by scintillation. Cumulative efflux was calculated separately at each time point for each dish and percent efflux by dividing total counts in the medium per dish by counts in control cell extracts removed at t0. B. Percent efflux of [3H]cholesterol into medium after 24 hours' incubation in specified media. Each point represents the mean ± SD of 3 dishes, and efflux data in B are derived from the same cell cultures generating data in A.
with the hydrolysis observed during ACAT inhibition alone. Stimulation of efflux did not enhance hydrolysis of EC beyond that observed during ACAT inhibition, indicating that cholesterol efflux facilitated clearance of EC by preventing FC from participating further in ACAT-dependent reesterification. Identical results with regard to hydrolysis of EC were obtained with HDL₃ and apoA-I-mediated efflux (data not shown).

cAMP supplementation has been previously found to enhance EC hydrolysis and cholesterol efflux in murine macrophages. Via stimulation of nCEH (generally considered synonymous with hormone-sensitive lipase [HSL]). More recently, cAMP appeared to cause net accumulation of EC in human monocyte-derived macrophages rather than to promote hydrolysis, and this may be related to the absence of HSL in human macrophages. We tested cAMP-mediated stimulation of EC hydrolysis and cholesterol efflux by adding it to THP-1 cells in which EC synthesis was inhibited by coincubation with ACAT inhibitor (Figure 8). Because PMA stimulates protein kinase C and cAMP can enhance nCEH activity via a cAMP-dependent protein kinase, was omitted after AcLDL loading for this experiment. At 24 hours, there was no significant enhancement of EC hydrolysis or cholesterol efflux by cAMP. This may indicate similarity between THP-1 cells and human monocytes, although persisting stimulation of nCEH activity initiated by PMA during differentiation and loading of THP-1 cells cannot be excluded.

To ensure that the apparent net decrease in [³H]EC caused by efflux reflected changes occurring in cell EC mass and that native HDL₃ and reconstituted rHDL-PC caused similar mass depletion of EC, EC mass was measured before and after efflux. THP-1 cells were loaded with AcLDL for 48 hours to maximize EC accumulation, then incubated with RPMI (control) or HDL₃ or HDL-PC, both at 200 µg/mL phospholipid for 24 hours. FC and EC content (µg/mg cell protein) of cell cultures after incubation with each of the media were respectively RPMI (36.4±2.0 FC, 96.7±11.1 EC), rHDL-PC (27.9±5.3 FC, 65.8±7.5 EC), and HDL₃ (37.6±2.8 FC, 64.8±0.13 EC). Thus, rHDL-PC and HDL₃ respectively caused a 29.6±8.0% and 30.7±0.2% depletion in EC mass compared with control RPMI medium, consistent with the reduction in [³H]EC described above.

**Physical State of Lipid Droplets**

Previous literature indicates that the physical state of lipid droplets markedly affects the rate of hydrolysis of EC with isotropic droplets being hydrolyzed more rapidly than anisotropic droplets. We hypothesized that the physical state of lipid droplets in THP-1 cells may be anisotropic and that this may contribute to impaired EC hydrolysis. Cultures of THP-1 cells were loaded with AcLDL for 48 hours and inspected under oil immersion by polarized microscopy as previously described after 2, 24, and 48 hours' equilibration. Ubiquitous large lipid droplets were evident in loaded THP-1 cells under light microscopy, but almost all of these were isotropic, demonstrating no phase polarization. Rare clusters of small anisotropic droplets detected at 2 hours were undetectable at later time points. The isotropic nature of the lipid droplets is consistent with the presence of cellular TG but argues against the physical nature of the droplets as being a sufficient explanation for slow cytosolic [³H]EC hydrolysis in THP-1 cells.

**Discussion**

Foam cell macrophages are characterized by the presence of cytoplasmic lipid droplets. EC and TGs can both contribute to cytoplasmic droplets and cannot be distinguished by oil red O staining. Foam cell macrophages from human plaque contain large quantities of EC and phospholipid and lesser but significant quantities of TG. The interaction between TG and EC accumulation in human macrophages is poorly understood. It is clear from our studies that TGs are a major lipid component in human THP-1 macrophages and primary human monocyte-derived macrophages, both immediately after maturation and after loading with AcLDL, and generally exceed EC accumulation. Given that intracellular TG can affect the hydrolysis of EC droplets, the reasons for such TG accumulation in human macrophages require further investigation.

We have identified that THP-1 foam cell macrophages differ in their metabolism of cholesterol compared with other cell types. THP-1 cells are more resistant to the accumulation of EC than murine macrophages; after exposure to AcLDL for 24 hours, the mass of EC accumulated and the esterification oleate in THP-1 cells are less than are achieved with murine macrophages. They also demonstrate slow hydrolysis of cytosolic EC, ie, 37% hydrolysis of EC over 24 hours in THP-1 cells compared with 60% in 24 hours in murine macrophages. Even though cholesterol efflux caused net clearance of EC in THP-1 macrophages, the rate of EC hydrolysis, assessed during ACAT inhibition, was minimally affected by concurrent cholesterol efflux. Thus, apolipoprotein-mediated stimulation of EC hydrolysis described for various cell types does not appear to be quantitatively important in human THP-1 macrophages.

Accumulation of cholesterol in cells exposed to AcLDL will be expected to occur via both whole-particle uptake via the ScR and direct transfer of cholesterol from AcLDL to the plasma membrane. The low specific activity of [³H]EC compared with [³H]PC in THP-1 cells incubated with AcLDL is therefore likely due to a combination of several factors: (1) a cold pool of EC derived from undegraded lipoprotein but perhaps also contributed to by the esterification of undefined cold pools of cell cholesterol; (2) slow esterification of cholesterol, including that from the plasma membrane, compared with other cell types; (3) preferential incorporation of fatty acids into TG and phospholipid pools in preference to their esterification to cholesterol; (4) slow hydrolysis of cytosolic everted cholesterol, necessitating longer incubations if equilibrium is to be achieved. Selective utilization of synthesized cholesterol for incorporation into EC could contribute to the low specific activity of EC in these cells. However, previous studies in differentiated THP-1 cells indicate that cholesterol synthesis, as assessed by acetate incorporation, is markedly inhibited after cholesterol accumulation after exposure to AcLDL, making this possibility less likely. From our studies, it appears that at least several
days of equilibration would be required to achieve equivalent specific activity between FC and EC pools in THP-1 cells. Preliminary experiments in our laboratory showed that such very prolonged equilibration phases resulted in loss of cells and were not practicable. Consequently, variable specific activity must be anticipated between different cholesterol pools, and sterol metabolism must be interpreted cautiously using THP-1 cells.

Although we have not at this time fully characterized the rate of lysosomal hydrolysis of AcLDL-derived EC in THP-1 cells, it is clear that immediately at the end of loading, a substantial pool of undegraded EC is present. Previous literature does not adequately indicate how this finding differs from other cell types, because inhibition of ACAT and concurrent measurement of EC mass and [3H]EC have not been performed. Importantly, studies from Kruth et al56 indicate that human macrophages can sequester AcLDL and cholesterol crystals in compartments contiguous with the extracellular space. If this is also the case in human THP-1 macrophages, the hydrolysis of lipoprotein lipids in THP-1 cells would reflect not only lysosomal degradation but also delivery of lipids from the sequestered compartment to the lysosome. Studies in progress in our laboratories addressing the hydrolysis of lipoprotein-derived EC and the effect of lysosomotropic agents will help characterize more fully the accumulation of undegraded lipoprotein in these cells.

THP-1 cells demonstrated cholesterol efflux to phospholipid-free apoA-I, and in this respect THP-1 macrophages behave like murine macrophages.6,29,30 In addition, consistent with previous work, phospholipids played a major role in enhancing the rate of efflux of cholesterol from THP-1 cells.18 This was best demonstrated in the comparison of efflux mediated to 25 μg protein per milliliter apoA-I and 25 μg protein per milliliter rHDL-PC (Figure 7 and Table) and in the direct comparison of apoA-I with apoA-I–PC (Figure 7C). Kinetic modeling indicated that THP-1 cells, which had accumulated only [3H]FC, effuxed cholesterol from a large slow pool and a relatively small fast pool. Slow [3H]EC hydrolysis would be expected to further increase the size of the slow pool and further retard overall efflux.17

Net clearance of cellular cholesterol represents the balance of bidirectional cholesterol movement, i.e., of cholesterol efflux from and influx to the cell.26 In the absence of exogenously supplied cholesterol (as with apoA-I and rHDL-PC), influx represents reuptake by the cells of [3H]FC previously removed. Kinetic modeling in the Table indicated that t1/2 for influx from apoA-I to the cellular cholesterol pool was 3 times faster than that from phospholipid-containing acceptors, consistent with the apparent early saturation of apoA-I. This observation indicates that acceptor particle phospholipid prevented saturation and influx of cholesterol back to THP-1 cells, as well as accelerating the rate of efflux. The small size and slow hydrolysis of the EC pool probably underlies the failure of ACAT inhibition during efflux to significantly enhance cholesterol efflux. That CAMP did not enhance EC hydrolysis or cholesterol efflux may be due to various factors. First, if HSL is either not responsible for nCEH activity in human macrophages30 or present in very low amounts,57 human macrophages may use a different enzyme to hydrolyze cytosolic EC. Second, if the lack of effect of cAMP in THP-1 cells indicates that nCEH activity is already maximal because of prior PMA exposure, then alternative avenues for stimulating hydrolysis of EC in human cells must be established, as EC hydrolysis is clearly slow in these cells in spite of previous or ongoing PMA exposure.

In summary, these studies have established that although THP-1 cells accumulated both EC and FC in response to loading with AcLDL, they contain a relatively small and metabolically inactive pool of EC compared with the size and activity of the TG pool and demonstrate slow esterification of nonlipoprotein-derived cholesterol. In addition to slow hydrolysis of EC, efflux of FC is itself slow in THP-1 cells. Cholesterol efflux is substantially enhanced by the presence of phospholipid in acceptor particles, confirming the importance of phospholipid in establishing net efflux from human macrophages.

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