ACAT1 Deficiency Disrupts Cholesterol Efflux and Alters Cellular Morphology in Macrophages

Dwayne E. Dove, Yan Ru Su, Wenwu Zhang, W. Gray Jerome, Larry L. Swift, MacRae F. Linton, Sergio Fazio

Objective—Acyl-coenzyme A: cholesterol acyltransferase (ACAT) converts intracellular free cholesterol (FC) into cholesteryl esters (CE) for storage in lipid droplets. Recent studies in our laboratory have shown that the deletion of the macrophage ACAT1 gene results in apoptosis and increased atherosclerotic lesion area in the aortas of hyperlipidemic mice. The objective of the current study was to elucidate the mechanism of the increased atherosclerosis.

Methods and Results—CE storage and FC efflux were studied in ACAT1(−/−) peritoneal macrophages that were treated with acetylated low-density lipoprotein (acLDL). Our results show that efflux of cellular cholesterol was reduced by 25% in ACAT1-deficient cells compared with wild-type controls. This decrease occurred despite the upregulated expression of ABCA1, an important mediator of cholesterol efflux. In contrast, ACAT1 deficiency increased efflux of the cholesterol derived from acLDL by 32%. ACAT1-deficient macrophages also showed a 26% increase in the accumulation of FC derived from acLDL, which was associated with a 75% increase in the number of intracellular vesicles.

Conclusions—Together, these data show that macrophage ACAT1 influences the efflux of both cellular and lipoprotein-derived cholesterol and propose a pathway for the pro-atherogenic transformation of ACAT1(−/−) macrophages.

Key Words: ACAT1 ▪ ATP-binding cassette A1 ▪ atherosclerosis ▪ macrophages ▪ cholesterol efflux

The development of atherosclerosis is influenced by abnormalities in cellular cholesterol homeostasis. Acyl-coenzyme A (CoA): cholesterol acyltransferase (ACAT) is responsible for the storage of cholesteryl ester (CE) within neutral lipid droplets in macrophage foam cells.1 The ACAT1 isoform is found at high levels in macrophages and steroidogenic tissues. The ACAT2 isoform is expressed only in the liver and the small intestine and is involved in the assembly of lipoproteins. Inhibition of cholesterol esterification in macrophages is expected to slow down foam cell formation and decrease lesion size by blocking the storage of cholesterol and facilitating cholesterol efflux. However, recent studies in our laboratory have shown that the deletion of the ACAT1 gene in macrophages results in an increase in atherosclerotic lesion area in the aortas of hyperlipidemic mice.2

Apolipoproteins (apo) E and AI, and the ATP-binding cassette (ABC) A1 are the main modulators of cholesterol balance in macrophages. Apolipoproteins (apo) E and AI, and the ATP-binding cassette (ABC) A1 are the main modulators of cholesterol efflux in macrophages.3–5 ABCA1 is responsible for the movement of phospholipids and cholesterol to apoAI, which results in the formation of nascent high-density lipoprotein.6 Systemically, ABCA1 is a critical part of the reverse cholesterol transport system. Tangier disease, caused by mutations in the ABCA1 gene, results in severely decreased high-density lipoprotein and apoAI levels, and accelerates atherosclerosis.5,7

Both CE storage and FC efflux are important physiological markers of cholesterol balance in macrophages. Pharmacological inhibition of ACAT has been shown to increase cholesterol efflux in many studies.8–10 However, there are conflicting reports of increased and decreased atherosclerosis in animal models after the administration of ACAT inhibitors.1 In our previous study, the worsening effect of ACAT1 gene deletion in hyperlipidemic mice may have been related to increased macrophage apoptosis induced by FC accumulation.2,9,11 In the current studies, we labeled cellular cholesterol to measure the effects of ACAT1 deficiency on CE formation and FC efflux from macrophages. The mild cholesterol loading conditions used in these studies are capable of perturbing cholesterol homeostasis without inducing toxicity. The goal of this investigation was to determine whether ACAT1 deficiency in macrophages was associated with changes in cholesterol efflux, cholesterol storage, or cellular morphology that would be consistent with increased suscep-
tibility to atherosclerosis. Our results suggest that ACAT1 deficiency disrupts the efflux of cholesterol and affects cholesterol homeostasis before any toxic cellular effects.

**Methods**

**Lipoprotein Preparation, ^3^H-Cholesteryl Olate Association, and Chemical Modification**

Low-density lipoproteins (LDL) (density = 1.019 g/mL to 1.063 g/mL) were isolated from human plasma by sequential ultracentrifugation as previously described. Incorporation of ^3^H-cholesteryl oleate into LDL was performed by incubating LDL with ^3^H-cholesteryl oleate in a dimethylsulfoxide solution. Acetyl-LDL (acLDL) was prepared by repeated addition of acetic anhydride to LDL. Lipoprotein species were confirmed by electrophoretic mobility.

**14C-Adenine Release Assay for Cellular Toxicity**

Cholesterol-induced toxicity in macrophages was assessed by measuring the leakage of ^14^C-adenine into media. Murine peritoneal macrophages were elicited with thioglycollate as previously described. Cells were labeled in DMEM with 0.4 μCi/mL ^14^C-adenine (Amersham) for 3 hours. Cells were rinsed and the release period was initiated by the addition of loading media with DMEM/1% fetal bovine serum (FBS) and 70 μg/mL acLDL. Loading media was removed after release periods of 24 or 48 hours and cell debris was removed by centrifugation. Remaining cellular ^14^C-adenine and protein mass were harvested by rinsing and lysing cells with 0.1N NaOH. ^14^C-adenine counts from media samples and lysate samples were detected by liquid scintillation. 14C-adenine release to the media removed after release periods of 24 or 48 hours and cell debris was removed by centrifugation. Remaining cellular ^14^C-adenine and protein mass were harvested by rinsing and lysing cells with 0.1N NaOH. ^14^C-adenine counts from media samples and lysate samples were detected by liquid scintillation. ^14^C-adenine release to the media was expressed as a percentage of the total counts (lysate plus media).

**Quantitation of Sterol Mass in Cultured Macrophages**

Cellular lipids were extracted by the Bligh–Dyer method and dried under nitrogen. Cholesterol mass was determined by gas chromatography as previously described.

**Efflux of Cholesterol Mass**

Macrophages were cultured for 36 hours in DMEM/1% serum with 70 μg/mL acLDL. Cells were rinsed and efflux was initiated by the addition of 10 μg/mL lipid-free human apoAI in DMEM. After 18 hours of efflux, cellular lipids were extracted with isopropanol and the total cholesterol mass was determined by gas chromatography as previously described. The cholesterol mass remaining after the efflux period was expressed as a percentage of the mass of parallel samples harvested before the efflux period.

**Efflux of Radiolabeled Cellular or Lipoprotein-Derived Cholesterol**

Macrophages were labeled by 2 different methods of sterol delivery, either to preferentially label cellular (membrane) cholesterol or to label lipoprotein-derived cholesterol. Cells were labeled in DMEM with 0.4 μCi/mL ^14^C-adenine (Amersham) for 3 hours. Cells were rinsed and the release period was initiated by the addition of loading media with DMEM/1% fetal bovine serum (FBS) and 70 μg/mL acLDL. Loading media was removed after release periods of 24 or 48 hours and cell debris was removed by centrifugation. Remaining cellular ^14^C-adenine and protein mass were harvested by rinsing and lysing cells with 0.1N NaOH. ^14^C-adenine counts from media samples and lysate samples were detected by liquid scintillation. ^14^C-adenine release to the media was expressed as a percentage of the total counts (lysate plus media).

**Separation of Free and Esterified Cholesterol by Thin Layer Chromatography**

The samples were extracted by the Bligh–Dyer method, dried, and spotted on silica G thin layer chromatography plates (Alltech). Plates were developed in 90:10:1 petroleum ether/ethyl ether/acetic acid and visualized with iodine vapor (Sigma). FC and CE bands were scraped and counted by liquid scintillation.

**Quantitation of ABCA1 mRNA and ABCA1 Protein**

Cells were cultured for 36 hours in DMEM/1% FBS media with 70 μg/mL acLDL. Total RNA was isolated using Trizol reagent (Invitrogen). The relative quantities of ABCA1 message were measured by real time reverse-transcription polymerase chain reaction. TaqMan one-step reverse-transcription polymerase chain reaction master mix reagent kit (ABI) was used for reverse-transcription polymerase chain reaction. Relative quantification of ABCA1 was normalized with 18S ribosomal RNA. For Western blot analysis of ABCA1 protein, cell extracts (30 μg total protein per lane) were separated by 3% to 8% NovPAGE Tris-Acetate gels (Novex) and transferred to nitrocellulose membranes. Murine ABCA1 was detected with a primary antibody (Novus Biological), visualized by a chemiluminescent ECL Plus (Amersham), and quantified by densitometric analysis.

**Transmission Electron Microscopy and Image Analysis**

Macrophages were treated with DMEM/1% FBS for 48 hours with 0 or 70 μg/mL acLDL. Cellular morphology was characterized by transmission electron microscopy as described. Sections (80 nm) of Spurr-embedded macrophages were stained with uranyl acetate and lead citrate and visualized by a Philips CM12 transmission electron microscope operated at 80 keV. Twenty macrophages per group were randomly selected. The percentages of total cellular volume occupied by vesicles were determined by using point counting stereologic techniques. Volume percentages were calculated from the number of points on vesicles as a percentage of total points on macrophages.

**Immunofluorescence Microscopy for Endosomes and Lysosomes**

Macrophages were treated with 0 or 70 μg/mL acLDL in DMEM/1% FBS for 36 hours. Macrophages were fixed, permeabilized, and treated with primary antibodies against the endosomal marker, EEA1 (Affinity BioReagents), or the lysosomal marker, LAMP1 (BioDesign). Cells were labeled with TRITC-conjugated secondary antibody (BioDesign). Fluorescence was visualized with a Zeiss Axioplan Imaging fluorescence microscope with digital camera and analyzed with MetaMorph 5.0, imaging software (Universal Imaging). The threshold was set to display the brightest 30% of positive pixels for EEA1 fluorescence and brightest 45% of positive pixels for LAMP1 fluorescence. The area of fluorescence from five random fields is expressed as pixels per cell.

**Results**

**Cholesterol Mass**

To confirm that ACAT1 deficiency resulted in a functional deficit in the esterification of cholesterol, sterol mass in macrophages was measured by gas chromatography. Macro-
phages were treated for 28 hours with 50 µg/mL acLDL in DMEM/10% FBS. ACAT1<sup>−/−</sup> macrophages treated with acLDL showed an 88% decrease in CE mass compared with wild-type macrophages (3.6±2.0 versus 29.5±0.7 µg CE/mg cell protein, *P*<0.001, *n*=3). Under these mild cholesterol-loading conditions, the mass of FC was not significantly different between genotypes (24.4±1.7 versus 25.1±1.6 µg FC/mg cell protein, *P*=0.68, *n*=3).

**Cholesterol-Induced Cytotoxicity**

To determine whether the loading conditions could stress cholesterol homeostasis in ACAT1<sup>−/−</sup> macrophages without inducing toxicity, the release of <sup>14</sup>C-adenine was measured during treatment with modified lipoproteins. As shown in the Table, there was no significant difference in toxicity between ACAT1<sup>−/−</sup> macrophages and wild-type macrophages treated with acLDL.

**Efflux of Cellular Cholesterol Versus Lipoprotein-Derived Cholesterol**

Because the efflux of both cellular and lipoprotein-derived sterols directly affect foam cell formation, efflux was measured from macrophages labeled by 2 different methods of sterol delivery. The method to label cellular (membrane) cholesterol pools resulted in decreased <sup>3</sup>H-cholesterol efflux from ACAT1<sup>−/−</sup> macrophages (Figure 1A). The method to label lipoprotein-derived cholesterol pools resulted in increased <sup>3</sup>H-cholesterol efflux from ACAT1<sup>−/−</sup> macrophages (Figure 1B).

<sup>3</sup>H-cholesterol efflux was compared with changes in total cholesterol mass during the efflux period. Measurement of cholesterol mass represents bulk cholesterol whereas <sup>3</sup>H-cholesterol efflux measures changes only in the targeted pool. Regardless of the labeling method, the cholesterol mass remaining after the efflux period showed that ACAT1<sup>−/−</sup> macrophages retain more cholesterol mass (Figure 1A and 1B). Cholesterol esters were <1% of the cholesterol mass in either cell type, indicating very mild loading conditions.

**Cholesterol Efflux and ABCA1 Expression**

The efflux of cellular and lipoprotein-derived cholesterol were measured in experiments where ABCA1 was also measured. Efflux of cellular cholesterol to apoAI was decreased by 25% in ACAT1<sup>−/−</sup> macrophages compared with wild-type macrophages (*P*<0.005, *n*=4) (Figure 2A). Efflux of lipoprotein-derived cholesterol to apoAI was increased by 32% in ACAT1<sup>−/−</sup> macrophages compared with wild-type macrophages (*P*<0.05, *n*=4) (Figure 2A).

In light of the decreased efflux of cellular cholesterol in ACAT1<sup>−/−</sup> macrophages, we measured the expression of ABCA1, which is responsible for most of the cholesterol efflux to apoAI. Unexpectedly, ACAT1<sup>−/−</sup> macrophages had a 236% increase in ABCA1 mRNA compared with wild-type macrophages (Figure 2B). The large increase in expression was associated with a minor increase of ABCA1 protein compared with wild-type macrophages (Figure 2C).

**Uptake, Turnover, and Storage of Lipoprotein-Derived Cholesterol**

In addition to changes in cholesterol efflux, other aspects of cholesterol homeostasis can be affected by a defective cholesterol esterification process. Because the uptake, turnover, and storage of lipoprotein-derived sterols directly affect foam cell formation, these processes were measured after the delivery of <sup>3</sup>H-CE to lysosomes via acLDL.
Uptake of $^3$H-CE/acLDL was increased by 38% in ACAT1$^{-/-}$ macrophages compared with wild-type macrophages ($P<0.005$, n=4) (Figure 2D).

Turnover of lipoprotein-derived cholesterol, which is the cumulative function of many processes, is indicated by the appearance of $^3$H-FC in the growth media of macrophages during incubation with lipoprotein associated $^3$H-CE. ACAT1$^{-/-}$ macrophages treated with $^3$H-CE/acLDL released increased amounts of $^3$H-FC into the loading media compared with wild-type macrophages (Figure 2E). Media $^3$H-FC was increased by 78% in ACAT1$^{-/-}$ macrophages ($P<0.005$, n=4).

Storage of intracellular $^3$H-CE in ACAT1$^{-/-}$ macrophages was decreased by 81% in ACAT1$^{-/-}$ macrophages ($P<0.005$, n=4) (Figure 2F). However, storage of intracellular $^3$H-FC was increased by 26% in ACAT1$^{-/-}$ macrophages ($P<0.005$, n=4) (Figure 2F).

Macrophage Morphology
Electron microscopy was performed to determine whether changes in cholesterol homeostasis caused by ACAT1 deficiency were associated with morphological changes. ACAT1$^{-/-}$ macrophages appeared to have increased surface activity as characterized by cytoplasmic extensions and intracellular vesicles (Figure 3B). In untreated macrophages, total vesicle volume was 74% larger in ACAT1$^{-/-}$ macrophages compared with wild-type macrophages ($P<0.005$, n=20). A similar trend was measured in macrophages treated with acLDL, in which total vesicle volume was 28% larger in ACAT1$^{-/-}$ macrophages compared with wild-type macrophages but this difference did not reach statistical significance ($P=0.10$, n=20). In these measurements, all membrane-limited vesicles were counted, including endosomes, lysosomes, and secretory vesicles.

Immunofluorescence of EEA1 revealed that ACAT1$^{-/-}$ macrophages have significantly more endosomes than wild-type macrophages (Figure 3D). Treatment with acLDL significantly increased EEA1 fluorescence in both cell types. Immunofluorescence of LAMP1 revealed that ACAT1$^{-/-}$ macrophages have significantly more lysosomes than wild-type macrophages (Figure 3E). Treatment with acLDL increased LAMP1 fluorescence in wild-type cells but levels in ACAT1$^{-/-}$ macrophages stayed at the same high level.

Discussion
To address the question of whether the absence of ACAT1 results in altered cholesterol homeostasis, we studied cholesterol ester formation and free cholesterol efflux in ACAT1$^{-/-}$ macrophages. We found that ACAT1$^{-/-}$ macrophages have decreased efflux of the cellular cholesterol and increased efflux of the lipoprotein-derived cholesterol. These changes were associated with an accumulation of lipoprotein-derived free cholesterol and with an expanded pool of intracellular vesicles, but were not a consequence of the toxic effects of free cholesterol.

For lipoprotein-derived cholesterol, ACAT deficiency increased efflux to apoAI, which is the main cholesterol acceptor for ABCA1-mediated efflux (Figure 2A). This is in
Figure 3. ACAT1-deficient macrophages have altered morphology and increased cellular vesicle volume. Macrophages were treated with 0 or 70 μg/mL acLDL in DMEM/1% FBS for 48 hours. Macrophages were fixed and visualized by transmission electron microscopy (5600× magnification). Electron micrographs of (A) an ACAT1(+/+) macrophage and (B) an ACAT1(−/−) macrophage treated with acLDL with a magnified insert in the upper right to show smaller vesicles. The black scale bars represent 2 μm. C, Intracellular vesicle volumes were determined from electron micrographs by point counting and is expressed as a percentage of total cellular volume. Bars and error bars represent the mean (n=20) and standard error of ACAT1(+/+) and ACAT1(−/−) macrophages treated with control or acLDL media. Asterisks (*) denote a statistically significant difference (P<0.05) compared with the appropriate ACAT1(+/+) group as determined by Student t test. D and E, Immunofluorescence microscopy was performed on macrophages treated with primary antibodies against the endosomal marker, LAMP1, or the lysosomal marker, LAMP1, with TRITC-conjugated secondary antibody. The area of fluorescence is expressed as pixels per cell. Bars and error bars represent the mean (n=5 random fields) and standard error of ACAT1(+/+) (WT) or ACAT1(−/−) (KO) macrophages. Asterisks (*) denote a statistically significant difference (P<0.05) compared with the appropriate ACAT1(+/+) group and pound signs (#) denote a statistically significant difference (P<0.05) compared with untreated groups as determined by Student t test.

The primary reason for using mild cholesterol loading conditions was to avoid the changes that are secondary to cholesterol toxicity. Shiratori et al have described the appearance of phospholipid whorls within the cytoplasm of heavily FC-loaded macrophages. Under the mild cholesterol loading conditions used in the current study, however, there was no morphological evidence of phospholipid whorls in ACAT1(−/−) macrophages (Figure 3B). Another characteristic of heavily FC-loaded macrophages is decreased ABCA1 levels caused by increased degradation of the protein. In contrast, the increased expression of ABCA1 mRNA and protein in ACAT1(−/−) macrophages in our studies supports our contention that we were successful in inducing only mild cholesterol loading (Figure 2B and 2C). However, the minor increase in protein relative to the large increase in mRNA may indicate reduced stability of ABCA1 protein. Also, it is interesting to note that macrophages treated with the ACAT inhibitor, MCC-147, have increases in ABCA1 expression that are similar to what we report for ACAT1(−/−) macrophages.

Atherosclerosis studies with hyperlipidemic mice show that macrophage ACAT1 deficiency increases necrosis and apoptosis in lesions. The rationale for treating atherosclerosis with inhibitors of ACAT1 is to stop intracellular CE mobilization and efflux from late endosomes/lysosomes.

In contrast to lipoprotein-derived cholesterol, absence of ACAT1 was associated with decreased efflux of cellular cholesterol to apoAI (Figure 2A). Warner et al reported decreased cholesterol efflux with ACAT inhibition under heavy loading conditions, in which FC accumulation induces cytotoxicity. In our study, we deliberately used mild cholesterol loading conditions that did not induce cytotoxicity. However, efflux was decreased in ACAT1(−/−) macrophages, indicating that cellular toxicity is not the reason why cholesterol efflux is affected. Another possibility is that under our mild loading conditions, cholesterol was sequestered in a nontoxic pool.

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Atherosclerosis studies with hyperlipidemic mice show that macrophage ACAT1 deficiency increases necrosis and apoptosis in lesions. The rationale for treating atherosclerosis with inhibitors of ACAT1 is to stop intracellular CE mobilization and prevent foam cell formation. The current studies confirm that the deficiency of ACAT1 increases the turnover of lipoprotein-derived cholesterol in macrophages by blocking cholesterol storage. This is viewed as an antiatherogenic mechanism. However, the decrease in the efflux of cellular cholesterol may represent a critical change in cholesterol homeostasis in macrophages.

The current studies add to previous reports in which ACAT activity was reduced by means of pharmacological inhibitors. Rodriguez et al reported increased efflux of lipoprotein-derived cholesterol during treatment with the ACAT inhibitor 58 to 035 in human monocyte-derived macrophages. ACAT inhibition has been reported to increase the efflux of cholesterol to various cholesterol acceptors. In our studies, ACAT1(−/−) macrophages had increased turnover of lipoprotein-derived CE as measured by the reappearance of processed CE in the media as FC (Figure 2E). However, ACAT1 deficiency reduced the efflux of cellular cholesterol from macrophages (Figure 2A). Although ACAT1 deficiency resulted in increased cholesterol turnover during acLDL...
In conclusion, these data show that the deficiency in macrophage ACAT1 disrupts the efflux of cellular cholesterol despite increased expression of ABCA1. The increased efflux of lipoprotein-derived cholesterol cannot counterbalance the accumulation rate of FC. These alterations in cholesterol homeostasis are associated with changes in cellular morphology, precede cholesterol-induced cytotoxicity, and may represent the basis for accelerated macrophage apoptosis in the growing plaque.

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