Association of ACAT1-Positive Vesicles with Late Endosomes/Lysosomes in Cholesterol-Rich Human Macrophages

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Aim: Acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1) is an endoplasmic reticulum (ER)-resident enzyme that catalyzes the conversion of cholesterol into cholesteryl esters. We previously showed that cholesterol-loaded macrophages produce numerous ER-derived vesicles with elevated ACAT1 enzyme activity; some of these vesicles were shown to be closely associated with Golgi-related organelle(s). The aim of this study was to investigate the translocation of ACAT1 vesicle in cholesterol-loaded macrophages.

Methods: To demonstrate association of ACAT1 with late endosomes/lysosomes (LE/LS), primary human macrophages with or without cholesterol-loading was subjected to confocal microscopy, immunoelectron microscopy, subcellular fractionation, and immunoadsorption assay. Furthermore, cholesterol esterification assay was also carried out to investigate function of ACAT1 associated LE/LS.

Results: Confocal fluorescence microscopy revealed that no significant ACAT1 signal was associated with the signal for LAMP2, a marker protein for LE/LS, in cholesterol non-loaded macrophages; however, approximately 20% of the total ACAT1 signals colocalized with the LAMP2 signal in cholesterol-loaded macrophages. ACAT1-positive membranes isolated by immunoadsorption using ACAT1-specific antibody contained LAMP2, demonstrating the association of ACAT1 and LE/LS. In addition, in macrophages phagocytosing latex beads, the close association of ACAT1 with LE/LS can be demonstrated in phagosomes isolated from cholesterol-loaded macrophages, not from non-loaded macrophages. Furthermore, cholesterol-loaded macrophages re-esterified aggregated LDL-derived cholesteryl ester even in the presence of U18666A, a reagent known to block egression of cholesterol from LE/LS.

Conclusion: Our results indicated that cholesterol-loaded human macrophages produce LE/LS in close association with ACAT1, and may promote efficient esterification of modified LDL-derived free cholesterol on LE/LS.


Key words; Human macrophage, ACAT1, Cholesterol, Cholesterol esterification, Late endosome/lysosome

Introduction

Macrophages bearing numerous lipid droplets are known as foamy transformed macrophages. The lipid droplets in foamy transformed macrophages are mainly composed of cholesteryl esters and triglycerides. The accumulation of foamy transformed macrophages in the aortic intima is a pathological hallmark of the early phase of atherosclerosis. Foamy transformed macrophages secrete various biologically active molecules and promote vascular remodeling with lipid deposition, eventually resulting in catastrophic vascular events, such as acute myocardial infarction and cerebral ischemia¹. Genetic or pharmacological intervention in foam cell formation has been shown to effectively ameliorate atherosclerotic plaque¹, ². Macro-
phages derived from peripheral blood monocytes internalize modified LDL via various scavenger receptors. They also take up native LDL by fluid-phase pinocytosis. Internalized LDL is transferred to endosomes/lysosomes (LE/LS) where the cholesteryl esters are hydrolyzed and released as free cholesterol. Cholesterol is released from LE/LS compartments in a Niemann-Pick type C1/C2 protein (NPC1/2)-dependent manner, and is transferred to the plasma membrane for utilization; excess free cholesterol is transferred to the endoplasmic reticulum (ER) to be re-esterified by ACAT. Using fatty acyl-coenzyme A as the fatty acyl donor, two ACAT genes encode two different enzymes, ACAT1 and ACAT2. In macrophages, ACAT1 is the major isoenzyme. In various cell types examined, ACAT1 is a tubular ER membrane-resident enzyme. Results from genetic and pharmacological inhibition studies suggest that partial inhibition of ACAT1 should be beneficial, while total inhibition of ACAT1 may be harmful for combating atherosclerosis. Previously, we have demonstrated that upon cholesterol loading, foamy macrophages produce numerous ACAT1-positive, small ER-derived vesicles, 80–150 nm in diameter. Others demonstrated that in mouse macrophages, HDL2 treatment caused the translocation of ACAT1 from the perinuclear area to the cell periphery. Taken together, these findings suggest that in macrophages, various lipoproteins can regulate ACAT1-mediated cholesterol esterification, in part by altering the intracellular location of ACAT1. Further examination revealed that these ER-derived vesicles have elevated ACAT1 activity; some of these vesicles are closely associated with Golgi-related organelle(s). In the current work, we further examined the ACAT1 translocation process in cholesterol-loaded macrophages, and made the unexpected finding that a significant amount of these ACAT1-positive, ER-derived vesicles are also closely associated with LE/LS, and these particular LE/LS perform efficient cholesterol esterification in loco. We also provide pathophysiological relevance of these findings by demonstrating the differential associations of ACAT1 with LE/LS in normal and in foamy transformed macrophages in vivo.

**Methods**

**Materials**

RPMI1640 medium, protease inhibitor mixture, SDS, BSA, saponin, DMSO, EDTA, methyl-β-cyclodextrin (MβCD), paraformaldehyde, sodium hydroxide, and amphipathic amine U18666A were purchased from Sigma. Penicillin-streptomycin solution for cell culture was from Invitrogen. μMACS Protein A MicroBeads was from Miltenyi Biotech, and DTT and ECL reagent were from Pierce. Rabbit anti-human ACAT1 antibodies (DM102) and mouse anti-human ACAT1 monoclonal antibody (Mab) were produced previously. Anti-human lysosome-associated membrane protein 2 (LAMP2) and β-actin monoclonal antibody were from Santa Cruz Biotechnology. Anti-rabbit IgG Alexa Fluor 488 and antimouse IgG Alexa Fluor 568 were from Molecular Probes. Fluorescent mounting medium was from Vector Laboratories. Density gradient solution for subcellular fractionation, Optiprep, was from Axis-Shield. Fluoresbrite YG carboxylate microspheres, 0.75 μm, were from Polyscience Inc. Radioactive compounds, [3H]cholesterol linolate and [3H]oleoyl-CoA were from American Radiolabeled Chemicals.

**LDL Isolation and Aggregation**

Human LDL was isolated from plasma by sequential ultracentrifugation (1.019<d<1.063) as previously described and stored at 4°C until use. Isolated LDL was vortexed at maximum speed for 2 min for aggregated LDL (agLDL) preparation.

**Cell Culture**

Because cellular functions of mouse macrophages as well as various human macrophage cell lines are not completely identical to those of primary cultured human monocyte-derived macrophages, we chose human primary macrophages as the cell system for current experiments. Peripheral blood monocytes were obtained from healthy volunteers and cultured for differentiation into macrophages, as described previously. Informed consent was obtained from all blood donors in accordance with protocols approved by Kumamoto University Hospital Review Board. Mature macrophages after 10 days, incubation in medium A (RPMI1640 medium containing 10% FBS, 100 units/mL penicillin G, and 0.1 mg/mL streptomycin sulfate) were used for all experiments. To introduce cholesterol overload, macrophages were incubated with medium B (medium A with 100 μg/mL agLDL), or medium C (medium A with 250 μM MβCD containing 16 μM cholesterol; MβCD/cholesterol complex) for 48 hrs, respectively.

**Subcellular Fractionation**

To examine the close association of ACAT1 with late endosomal marker protein LAMP2, subcellular fractionation was employed as described previously. Briefly, macrophages were harvested and homogenized in homogenization buffer (0.25 M sucrose, 5 mM...
EDTA, 20 mM Tris (pH 7.4), protease inhibitor cocktail. After low speed centrifugation, the postnuclear supernatant was loaded at the top of a continuous 7.5−19.5% Optiprep gradient. Membranes in the sample were separated by sedimentation velocity ultracentrifugation at 200,000 × g for 2 hrs, and 19 fractions were collected from top to bottom. To obtain further fractionated ACAT1-containing membrane, two-step sequential fractionation was carried out. ACAT1-rich fraction after first step ultracentrifugation was prepared as 20% Optiprep solution. The prepared sample was placed at the bottom of the 7.5−19.5% Optiprep gradient, and centrifuged at 200,000×g for 2 hrs for buoyant density ultracentrifugation. After two-step sequential ultracentrifugation, 19 fractions were collected from top to bottom.

**Phagosome Isolation**

To examine the presence of ACAT1 on the phagosome membrane, we isolated bead-containing phagosomes, as described previously with modification. Ten million mature human macrophages in 10 cm tissue culture dishes grown in medium A or medium C for 36 hrs were further incubated in segregated media containing 1% Fluoresbrite YG carboxylate beads (0.75 μm) for 12 hrs. The cells were washed, homogenized, and 9 mL diluted samples on 1 mL of a 20% Optiprep cushion were ultracentrifuged at 10,000×g for 20 min. After removal of the supernatant, pelleted bead-containing phagosomes were mixed with 20% Optiprep cushion, overlaid on a 4%−11% discontinuous Optiprep gradient, and then ultracentrifuged at 200,000×g for 2 hrs. The floated bead fraction was collected and subjected to immunoblot analysis and immunogold electron microscopy. The number of beads in each fraction was determined by absorbance at 475 nm.

**Immunoblot Analysis of ACAT1-Positive Membrane**

To obtain highly purified ACAT1-positive membrane, we employed the immunoblot analysis technique, as described previously. Postnuclear supernatant from mature macrophages with or without medium B or medium C treatment was directly reacted with anti-rabbit ACAT1 antibody-conjugated protein A beads (μMACS protein A MicroBeads) for 2 hrs. The sample/MicroBeads mixture was loaded onto a μMACS isolation column, and washed, and immunoreacted ACAT1-positive membranes were eluted using 10% SDS following the manufacturer’s instructions.

**Double Immunofluorescent Staining**

Buffer A, phosphate-buffered saline (PBS, pH 7.4) containing 0.5% BSA and 0.01% saponin, was used as washing/dilution buffer. Cells were fixed with 4% paraformaldehyde with 0.1 M phosphate buffer (pH 7.4) for 30 min on ice. After washing out the fixative and following preincubation with 5% goat serum, the samples were incubated with primary antibodies for 1 hr at room temperature. After rinsing, samples were sequentially incubated with anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Flour 568 for 60 min at room temperature inside a darkbox. After the removal of nonreacted secondary antibodies, coverslips were mounted on to the sample with fluorescent mounting medium and observed by a confocal laser scanning microscope FV300 (Olympus, Tokyo, Japan). Tissue samples containing foamy transformed macrophages or tissue-resident macrophages fixed with 4% paraformaldehyde for 12 hrs were washed in PBS with a graded series of sucrose, prepared as frozen sections, and immunostained as described above. For quantitative colocalization analysis, at least 30 images were scanned at random, and colocalization coefficient and relative fluorescent intensity were calculated using Fluoview software version 5.0c (Olympus, Tokyo, Japan).

**Immunogold Electron Microscopy**

Isolated phagosomes were incubated with homogenization buffer containing 1% BSA and 5% goat serum for 30 min. After brief centrifugation, the supernatant was removed and suspended phagosomes were incubated with ACAT1-specific antibody for 2 hrs. Phagosomes were intensely washed with homogenization buffer with 1% BSA, and further incubated with 15 nm colloidal gold-labeled goat anti-rabbit IgG overnight. After the removal of excess secondary antibody, the samples were fixed with 2.5% glutaraldehyde and 1% osmium tetroxide, embedded in resin, prepared as ultrathin sections with lead citrate and uranyl nitrate staining, and observed on a Hitachi H-7500 transmission electron microscope (Tokyo, Japan), as described previously.

**Immunoblot**

The isolated phagosome fraction and membrane fractions after subcellular fractionation or immunoblotting were prepared as samples containing 10% SDS and 0.1 M DTT, and incubated at 37°C for 30 min. Each sample was run on 10% SDS polyacrylamide gel electrophoresis and subjected to immunoblotting, as described previously.

**Cellular Cholesterol and Cholesteryl Ester Level**

Human macrophages incubated with or without
medium B or medium C for 48 hrs were rinsed with PBS three times and dried at room temperature. Cellular lipids were extracted with hexane: 2-propanol (3:2), and total cholesterol and free cholesterol were determined by a cholesterol determination kit following the manufacturer’s instructions (Wako Chemical Industries, Tokyo, Japan). The amount of cholesteryl ester was calculated by subtracting free cholesterol from total cholesterol. Cellular protein dissolved in 0.1 N sodium hydroxide was determined by a BCA Protein Assay Kit (Pierce) with BSA as the standard.

**Non-Reconstitutive ACAT Assay**

To monitor ACAT activity in prepared subcellular membrane fractions, we performed the non-reconstitutive ACAT assay; cellular cholesterol associated with the membrane served as the enzyme substrate. The assay was initiated by adding 20 μL of solution containing 10 nmol of [3H]oleoyl-CoA and 10 nmol fatty acid-free BSA to the samples, and the reaction mixture was incubated at 37°C for up to 30 min. Enzyme activity was linear with time within 30 min. The reaction was terminated by adding methanol-chloroform mixture, the extracted lipid was separated on thin layer chromatography, and radioactivity from [3H]cholesteryl oleate was determined by liquid scintillation counter.

**Modified LDL-Derived Cholesterol Re-Esterification at LE/LS**

To demonstrate modified LDL-derived cholesterol re-esterification at LE/LS in cholesterol-rich human macrophages, we performed the cholesterol re-esterification assay using [3H]cholesteryl linoleate-labeled aggregated LDL ([3H]CL-agLDL) with or without amphipathic amine U18666A. [3H]CL-LDL with specific radioactivity of 5 × 10^4 cpm/μg protein was prepared as previously described, and then [3H]CL-LDL was aggregated by vortex mixing. Human macrophages were incubated in a 24-well tissue culture plate at the density of 0.3 million cells/well. Mature human macrophages were preincubated with medium A or medium C for 2 days, washed with PBS three times, and further treated with medium A containing 5 μM amphipathic amine U18666A for 30 min to inhibit the egression of free cholesterol from LE/LS. The cells were further incubated with medium A containing 50 μg/mL of [3H]CL-agLDL plus 5 μM U18666A for 18 hrs. Total lipid in the cells was extracted with hexane: 2-propanol (3:2), and separated on silver nitrate-impregnated thin layer chromatography plates, which separate cholesteryl oleate from cholesteryl linoleate, using the procedure described. The radioactivity of [3H]cholesteryl oleate was determined using a liquid scintillation counter.

**Statistics**

Data are presented as the means ± SD. Statistical analyses of results were performed using the Mann-Whitney U-test. A p value < 0.05 was regarded as significant.
Results

Cellular Free Cholesterol and Cholesteryl Ester Level

To induce cholesterol loading on to peripheral monocyte-derived human primary macrophages, we treated cells with 100 μg/mL agLDL (medium B), or MβCD/cholesterol complex (medium C) for 48 hrs. Changes in cellular cholesterol and cholesteryl ester levels are summarized in Fig. 1. The results show that for each treatment, the free cholesterol level was increased approximately 3 times, and the cellular cholesteryl ester level was increased 20 times. Additional results showed that free cholesterol or cholesteryl ester levels in cells grown in medium B and medium C were not significantly different.

Immunofluorescent Confocal Microscopic Analysis

To demonstrate the close association of ACAT1 with LE/LS in cholesterol-rich human macrophages, we carried out immunofluorescent confocal microscopy (Fig. 2). Quantitative colocalization coefficient analysis using FluoView software revealed that only approximately 5% of the ACAT1 signal and the LE/LS marker LAMP2 signal apparently overlapped in medium A (Fig. 2B). However, more than 20% of LAMP2 signals were apparently colocalized with the ACAT1 signal in cholesterol-rich human macrophages (Fig. 2B right panel); similarly, at least 15% of ACAT1 signals were colocalized with the LAMP2 signal in cholesteryl-rich macrophages (Fig. 2B left panel). These results showed that cholesterol loading in human macrophages results in a significant amount of ACAT1 closely associated with LAMP2-related membranes, suggesting that a significant portion of ACAT1 and LAMP2 signals is present in one single organelle, or in two separate, but very closely associated organelles. Additional results showed that the total ACAT1 signal was not significantly changed regardless of medium B or medium C treatment, whereas the LAMP2 signal was significantly increased after medium B/C treatment (Fig. 2C). The increase of the LAMP2 expression level and the lack of increase of the ACAT1 expression level were confirmed by immunoblot analysis (Fig. 2D).

Association of ACAT1 with Lower-Density, LAMP2-Positive Membranes

To provide support for data obtained using confocal microscopy, we sought biochemical evidence for the close association of ACAT1 with LAMP2-positive membranes in cholesterol-rich human macrophages. Using Optiprep ultracentrifugation analysis, we previously demonstrated that cholesterol-rich human macrophages produced lower density, ER-derived membrane fractions; these fractions contained elevated ACAT1 activity. Here we used the same technique and isolated these ACAT1-positive, lower density membrane fractions. These low-density ACAT1-positive membrane fractions had high ACAT enzymatic activity in vitro (Fig. 3A, medium B), confirming our previous results. When these membranes were subjected to second-step ultracentrifugation (described in Methods), the ACAT1-positive fraction still had the LAMP2 signal. The result of the negative control experiment showed that the LAMP2 signal could not be detected in higher-density, ACAT1-positive fractions isolated from macrophages grown in medium A (Fig. 3A, medium A).

We next performed immunoadsorption, using magnetic beads coated with ACAT1-specific antibodies, and isolated the highly purified ACAT1-positive membrane fraction. We tested the presence of the LAMP2 signal in these membranes. The results showed that no LAMP2 signal was detectable in the immunoadsorbed pellet fraction from human macrophages grown under normal conditions (Fig. 3B medium A), or from the pellet fraction using non-immunized rabbit IgG as an antibody for immunoprecipitation (Fig. 3B control). In contrast, significant LAMP2 signal was detected in the pellet fraction from medium B-treated cholesterol-rich human macrophages (Fig. 3B medium B). The same results were obtained from medium C-treated, cholesterol-rich human macrophages (result not shown). These results support the results obtained using confocal microscopic analysis and Optiprep ultracentrifugation analysis.

Association of ACAT1 with LAMP2-Positive Phagosomes

To obtain more evidence for the close association of ACAT1 with LAMP2-positive LE/LS, we tested the presence of ACAT1 in phagosomes. It is known that materials phagocytosed by macrophages initially appear in immature phagosomes, which then transform into mature phagosomes by undergoing sequential fusion with early endosomes, late endosomes and lysosomes. If ACAT1 is associated with LE/LS in cholesterol-rich macrophages, we should be able to detect the ACAT1 signal in mature phagosomes prepared from cholesterol-rich macrophages, but not in phagosomes prepared from non-cholesterol loaded macrophages. We used medium C as a cholesterol donor, for fear that aggregated LDL may interfere with macrophages’ ability to phagocytose beads. The results demonstrated that the ACAT1 signal was amply present in the LAMP2-positive phagocytosed bead fraction iso-
Fig. 2. Analysis of expression level and colocalization coefficient of ACAT1 and LAMP2 in human macrophages with or without cholesterol loading

Day 10 human macrophages with or without cholesterol loading for 48 hrs were fixed and immunostained as described in the Methods. A. Representative immunofluorescent images from control (medium A) and cholesterol-loaded macrophages (medium B and medium C). Green signal indicates ACAT1, magenta is LAMP2, and the colocalization signal is white. Details of the findings are described in the text. DIC, differential interference contrast. B. Quantitative evaluation of colocalization coefficient between ACAT1 and LAMP2 signals. Immunofluorescent signals from all samples were scanned and analyzed as described in Methods. Results are representative of three separate experiments. C. Semiquantitative evaluation of immunofluorescent signals from ACAT1 and LAMP2. Immunostained samples were scanned and analyzed as described in Methods. Data are expressed as relative signal intensity against medium A-grown macrophages, and are representative of three individual experiments. D. Immunoblot to examine expression levels of ACAT1 and LAMP2 proteins in human macrophages with or without cholesterol loading. Total cell lysate of 20 μg protein was prepared as immunoblot samples, and subjected to SDS-PAGE and immunoblot as described in Methods. Data are from identical results from two independent experiments.
lated from cholesterol-rich macrophages (Fig. 4A medium C), while no ACAT1 signal could be detected in phagosomes from macrophages without cholesterol loading (Fig. 4B medium A). To provide further evidence for the presence of ACAT1 associated with mature phagosomes in cholesterol-rich human macrophages, we carried out immunogold-labeled electron microscopy, using colloidal gold-labeled ACAT1-specific antibodies as the probe. The results showed that phagosomes isolated from macrophages grown in medium A were not labeled by colloidal gold (Fig. 4B); however, phagosomes from medium C-treated macrophages were significantly labeled with the gold signal (Fig. 4C); some of the colloidal gold signal was detected in small vesicles closely associated with phagosomes (Fig. 4D). The data presented here strongly indicated that in cholesterol-rich human macrophages, a significant portion of ACAT1-positive small vesicles is associated with LE/LS.

**Association of ACAT1 with LE/LS in Foamy Macrophages within the Human Atherosclerotic Aorta**

Our data described above demonstrated that human macrophages in cholesterol-rich condition pro-
Fig. 4. Presence of ACAT1 on phagoendosomes from cholesterol-rich human macrophages

Day 10 human monocyte-derived macrophages with or without medium C treatment were incubated with Fluorobrite beads for 12 hrs and beads containing phagoendosome fractions were isolated and analyzed as described in Methods. **A.** Association of ACAT1 in phagoendosomes prepared from cholesterol-rich human macrophages. Prepared phagoendosomes in each fraction were analyzed by absorbance at 475 nm and immunoblot. Details of findings are described in the text. Data are representative of three individual experiments. **B−D.** Immunogold electron microscopy to demonstrate ACAT1-associated phagoendosomes. Phagoendosomes were immunostained and prepared as ultrathin sections for electron microscopy. **B.** Phagoendosomes from macrophages grown in medium A. **C, D.** From macrophages after medium C treatment. Scale bars in **B, C, D.** 250 nm.

Fig. 5. Presence of ACAT1-associated late endosomes/lysosomes in foamy transformed human macrophages in atherosclerotic aorta

Tissue samples from human atherosclerotic aorta and lymph node were immunostained and analyzed as described in Methods. **A.** Representative images of immunofluorescent confocal microscopy. **B.** Results of quantitative colocalization coefficient analysis. Data are representative of two individual experiments.
duce small, ACAT1-positive vesicles that are closely associated with late endosomes/lysosomes. To test the in vivo evidence that such an association may exist in the human atherosclerotic aorta, we carried out a confocal microscopic experiment. The results showed that the ACAT1 signal was not associated with the LAMP2 signal in lymph node sinus macrophages, which are representative of non-loaded macrophages. On the other hand, for macrophages in atherosclerotic plaque, a significant number of ACAT1 signals were closely associated with the LAMP2 signal (Fig. 5A), suggesting that a significant amount of ACAT1 is closely associated with LE/LS in human atherosclerotic plaque. Fig. 5B summarizes the colocalization coefficients of ACAT1 and LAMP2 signals in tissue macrophages.

Re-Esterification of ag-LDL-Derived Cholesterol in Cholesterol-Rich Human Macrophages

We investigated the functional significance of the association of ACAT1 with LE/LS in cholesterol-rich macrophages. Earlier studies showed that NPC1/2 plays crucial roles in assisting the egression of free cholesterol from LE/LS. Cells lacking functional NPC1 show free cholesterol accumulation in LE/LS. Treating normal cells with amphipathic amine U18666A induces the NPC1-deficient phenotype. We hypothesized that the close association of ACAT1 with LE/LS, produced in cholesterol-rich macrophages, might facilitate the re-esterification of modified LDL-derived cholesterol, even under conditions when cholesterol transport out of LE/LS is functionally defective. To test this possibility, we fed human macrophages with \(^{3}H\)CL-agLDL, and carried out a cholesteryl linoleate re-esterification assay in the absence or presence of U18666A. The results (Fig. 6) show that U18666A at 5 \(\mu M\) significantly inhibited re-esterification of \(^{3}H\) cholesterol derived from \(^{3}H\)CL-agLDL in macrophages without cholesterol loading, but failed to cause significant inhibition of re-esterification in cholesterol-loaded macrophages. These results support the idea that in cholesterol-rich human macrophages, the close association of ER-derived, ACAT1-positive vesicles with LE/LS facilitates the re-esterification of cholesterol derived from LE/LS.

Discussion

LDL is the major cholesterol carrier in the blood. In early stages of atherosclerosis, continuous uptake of denatured and native LDLs by macrophages occurs in the subendothelial layer of the intima. The build-up of free cholesterol in membranes can cause membrane deformation, leading to cellular toxicity. To defend against the build-up of free cholesterol in membranes, the major esterification enzyme ACAT1 converts free cholesterol into cholesteryl esters (CE), which are neutral lipid molecules that do not partition well in membranes; instead, CEs aggregate to form lipid droplets in the cytosolic compartment. Normally, ACAT1 is a resident enzyme located in the ER. We have shown that upon cholesterol loading, macrophages use a novel mechanism to defend the build-up of free cholesterol. They produce small, ER-derived vesicles, 80–150 nm in diameter; these vesicles have elevated ACAT1 enzyme activity and are in close proximity to Golgi-related organelle(s). We and others have proposed that the close proximity between ACAT1 vesicles and various subcellular organelles, such as the Golgi and endosomal recycling compartments, may allow efficient esterification of free cholesterol derived from these organelles by ACAT1. In the experiments described in the current manuscript, we extended these previous studies by demonstrating that in cholesterol loaded macrophages, approximately 20% of ACAT1-positive, ER-derived vesicles are closely associated with LE/LS; this finding has not been reported in the literature. LE/LS is known to be the major organelle that digests LDLs and hydrolyzes the CEs stored in LDL; the free cholesterol released from LE/LS can be efficiently re-esterified to CEs by ACAT1. Normally, the egress of cholesterol from LE/LS requires NPC1/NPC2 proteins. Treating cells with U18666A caused cholesterol accumulation in LE/LS and caused the cells to exhibit a mutant NPC phenotype.
type. We showed that U18666A-treated cholesterol-rich human macrophages still efficiently re-esterify [3H]cholesterol derived from [3H]CL-agLDL, supporting the idea that the close proximity of ACAT1 to LE/LS facilitates the ability of ACAT1 to re-esterify cholesterol derived from LE/LS. We also found that the close association of ACAT1 with LE/LS occurred in foamy transformed macrophages present in human atherosclerotic plaque, but not in human macrophages without lipid loading, thus demonstrating the in vivo significance of our findings.

Similar to our work on human macrophages, Fazio and colleagues reported that ACAT1-/- mouse macrophages treated with acetylated LDL produced small vesicles and an increasing number of endosomal vesicle/vacuoles. This observation is consistent with our previous and current data reported in human macrophages, i.e., cholesterol-rich human macrophages produce many ACAT1-positive small vesicles, and that signals from LE/LS were significantly increased (Fig. 2C, 2D). In addition, others reported that mouse macrophages with sequential treatments of acetylated LDL and ACAT inhibitor resulted in the close contact of ER with lipid droplets and the formation of lamellar bodies surrounded by a limiting membrane, and these structures interact with endosomes in the presence of HDL. To explain the processes described above, we have suggested that a continuous ER fragmentation process may occur in intact macrophages, and that cholesterol loading may accelerate the formation of ACAT1-positive vesicles. Although the mechanism(s) that leads to the close association of ACAT1-positive vesicles with LE/LS in cholesterol-rich macrophages is unclear, formation of this particular LE/LS may regulate macrophage cellular function. We are now focusing on a comprehensive investigation regarding ACAT1-associated LE/LS in cholesterol-rich macrophages.

Acknowledgements

This work was supported by Grants in Aid for Scientific Research B-17390115 and C-20590384 (to N.S.), and B-16390108 and B-20390113 (to M.T.) from the Japan Society for the Promotion of Science, and by NIH grants HL60306 and HL36709 (to T.Y.C. and C.C.Y.C.). We thank E. Kiyoata, Y. Hayashita, and T. Nakagawa for their skilled assistance in this work.

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