REVIEW

Role of ACAT1-positive late endosomes in macrophages : Cholesterol metabolism and therapeutic applications for Niemann-Pick disease type C

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Abstract : Macrophages in hyperlipidemic conditions accumulate cholesterol esters and develop into foamy transformed macrophages. During this transformation, macrophages demonstrate endoplasmic reticulum fragmentation and consequently produce acyl coenzyme A : cholesterol acyltransferase 1 (ACAT1)-positive late endosomes (ACAT1-LE). ACAT1-LE-positive macrophages effectively esterify modified or native low-density lipoprotein-derived free cholesterol, which results in efficient cholesterol esterification as well as atherosclerotic plaque formation. These macrophages show significant cholesterol ester formation even when free cholesterol egress from late endosomes is impaired, which indicates that free cholesterol is esterified at ACAT1-LE. Genetic blockade of cholesterol egress from late endosomes causes Niemann-Pick disease type C (NPC), an inherited lysosomal storage disease with progressive neurodegeneration. Induction of ACAT1-LE in macrophages with the NPC phenotype led to significant recovery of cholesterol esterification. In addition, *in vivo* ACAT1-LE induction significantly extended the lifespan of mice with the NPC phenotype. Thus, ACAT1-LE not only regulates intracellular cholesterol metabolism but also ameliorates NPC pathophysiology. J. Med. Invest. 61 : 270-277, August, 2014

Keywords **:** *ACAT1, late endosomes, macrophages, cholesterol metabolism, Niemann-Pick disease type C*

INTRODUCTION

Macrophages are the principal cell of innate immunity. They show active phagocytosis, produce cytokines, present antigens to lymphocytes, and play an important role in host defense against various microorganisms. In addition, macrophages actively metabolize cholesterol and contribute to atherogenesis by producing various biologically active molecules (1). Macrophages in atheromatous plaques commonly accumulate numerous lipid droplets in their cytoplasm. These unique macrophages are called foamy transformed macrophages. Macrophages in hyperlipidemic conditions actively internalize low-density lipoprotein (LDL), with or without chemical modification, via scavenger receptor-mediated endocytosis or fluid-phase pinocytosis (2, 3). Internalized modified or native LDL is hydrolyzed at acid lipase-positive late endosomes, which results in the generation of free cholesterol *in loco* (4, 5). Free cholesterol generated at late endosomes is transferred to plasma membranes by Niemann-Pick disease type C1/C2 protein (NPC1/2), and excess amounts of transferred free cholesterol are further transported

Received for publication February 4, 2014 ; accepted June 8, 2014.

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to the endoplasmic reticulum (ER), where acyl coenzyme A : cholesterol acyltransferase (ACAT) resides. ACAT catalyzes the esterification of free cholesterol, so that modified or native LDL-derived free cholesterol is re-esterified at the ER, released into the cytoplasm, and stored as lipid droplets. These processes cause foamy transformation of the macrophages (4, 5).

During our morphological analysis of foamy transformed macrophages, we discovered a unique, cholesterol-induced, functional endosomal organelle : ACAT1-positive late endosomes (ACAT1-LE). In this review article, we describe our discovery of ACAT1-LE and discuss the functional significance of this unique cholesterol-induced organelle. In addition, we describe the clinical significance of ACAT1-LE induction on the congenital endosomal storage disease--Niemann Pick disease type C (NPC).

ACAT

The presence of a cholesterol-esterifying enzyme in mammalian cells has been known since the 1960s. Its enzymatic activity was usually detected in microsomal fractions, so ACAT is said to reside in the ER. Chang *et al.* first cloned ACAT in 1993 (6), and extensive genetic investigations then produced the cloned ACAT isozyme ACAT2 (after which the first cloned ACAT was renamed ACAT1) (7). Most human somatic cells express ACAT1 ; in particular, macrophages, adrenal cortex cells, and sebaceous cells express a significant amount of ACAT1 (8). In contrast, ACAT2 expression is limited to the apical region of enterocytes, fetal hepatocytes, and well-differentiated macrophages (9-11). Under physiological conditions, ACAT functions in intracellular cholesterol homeostasis in mammalian cells, especially in macrophages and steroid hormone-producing cells ; in addition, it regulates lipoprotein synthesis in hepatocytes and enterocytes (5). Artificial inhibition of ACAT enzymatic activity therefore causes impaired lipoprotein synthesis as well as ER stress-mediated apoptosis via free cholesterol-induced cell toxicity (12).

In pathological situations, ACAT, by esterifying cholesterol, serves a critical function as a protector against free cholesterol-induced cell toxicity. The presence of foamy transformed macrophages in atherosclerotic plaques therefore indicates ACAT-dependent macrophage protection against massive cholesterol loading. Foamy transformed macrophages in atheromatous plaques, *i.e.* macrophages with massive cholesterol loading, continuously internalize modified LDL via scavenger receptors. The key issue for foamy transformed macrophages in atheromatous plaques relates to sufficient ACAT1 expression (mature macrophages express both ACAT1 and ACAT2, and 90% of enzymatic activity derives from ACAT1) (11). In fact, a high-fat diet fed to atherogenic mice with ACAT1-null macrophages led to a reduced number of foamy transformed macrophages and consequently severe inflammation, which resulted in increased atheromatous plaque formation (13). These data prompted us to question how macrophages protect themselves from cholesterol-induced cell toxicity. The answer to this basic question is ACAT1-LE.

ACAT1 VESICLES AND ACAT1-LE

As noted above, ACAT is an ER-resident enzyme. We confirmed the location of ACAT1 to be the tubular ER membrane in normal human macrophages (Figure 1A, intact Mφ) (14). Next, we tried to detect the ACAT1 signal in macrophages under cholesterol-rich conditions. Treatment with a cholesterol donor (acetylated LDL) caused the ACAT1 signal in human macrophages to appear on small vesicles approximately 100 nm in diameter (Figure 1A, cholesterol-rich Mφ). The ER marker protein GRP78 also appeared on small vesicles after cholesterol donor treatment (Figure 1B, cholesterol-rich Mφ). Furthermore, both ACAT1 and GRP78 signals correlated with each other before and after foamy transformation (Figure 1C), and the expression level of ACAT1 did not change before and after cholesterol donor treatment (data not shown). These data thus indicate that cholesterol-rich, foamy transformed macrophages produce ACAT1-positive vesicles via ER fragmentation.

ACAT is a representative allosteric enzyme whose enzymatic activity increases after cholesterol binds to its allosteric site. We thus believed that formation of ACAT1 vesicles would be related to allosteric regulation. A series of investigations of the significance of ACAT1 vesicle formation led to the discovery of ACAT1-LE. As Figure 2 shows, separate ACAT1 signals and signals of the late endosomal marker protein lysosomal-associated membrane protein 2 (LAMP2) were detected in macrophages with no lipid accumulation (Figure 2A, intact Mφ). In contrast, approximately 20% of the ACAT1 signal corresponded to the

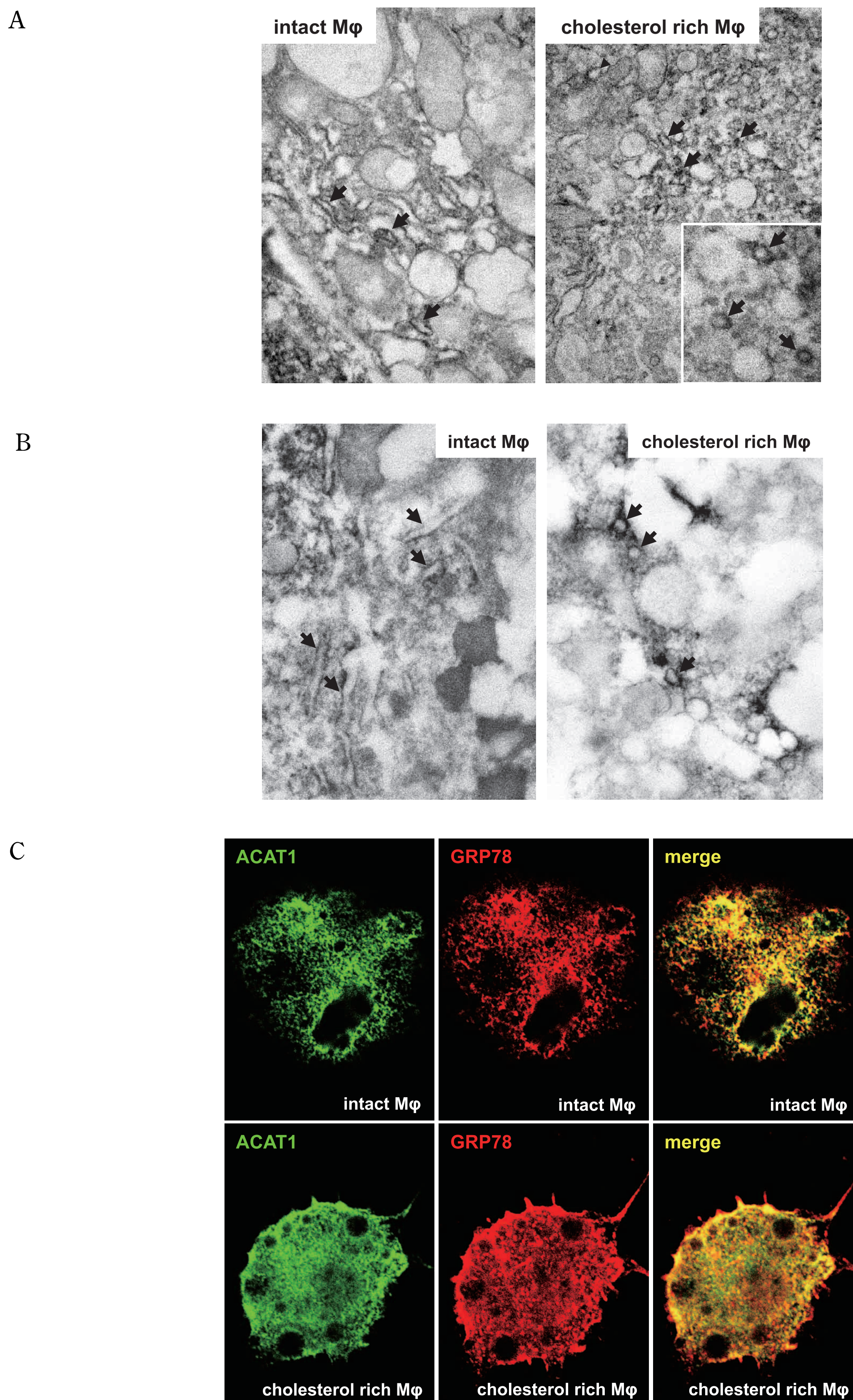

Figure 1 ACAT1 vesicle formation in cholesterol-rich macrophages
Human macrophages derived from peripheral blood monocytes with or without acetylated LDL treatment were subjected to immunoelectron microscopy (post-embedding method) and confocal laser scanning microscopy. (A) Immunoelectron microscopy after using ACAT1-specific antibody. (B) Immunoelectron microscopy after using GRP78 (ER marker protein)-specific antibody. The inset in A is an enlarged image. Arrows indicate specific signals. (C) Confocal laser scanning microscopy after using ACAT1-specific antibody (green) and GRP78-specific antibody (red). Yellow in the merged image indicates a colocalization signal. Details are provided in the text. Adapted from references 9 and 14.

LAMP2 signal in foamy transformed macrophages (Figure 2A, cholesterol-rich Mφ, Figure 2B). The purified ACAT1-positive organelles from cholesterol-rich macrophages contained LAMP2, but those from intact macrophages did not (Figure 2C), and immunoelectron microscopy confirmed the presence of the ACAT1 signal on beads containing phagosomal membranes from only foamy transformed macrophages (Figure 2D), not intact macrophages. These data thus indicate that foamy transformed macrophages form ACAT1-LE via ER fragmentation (14, 15).

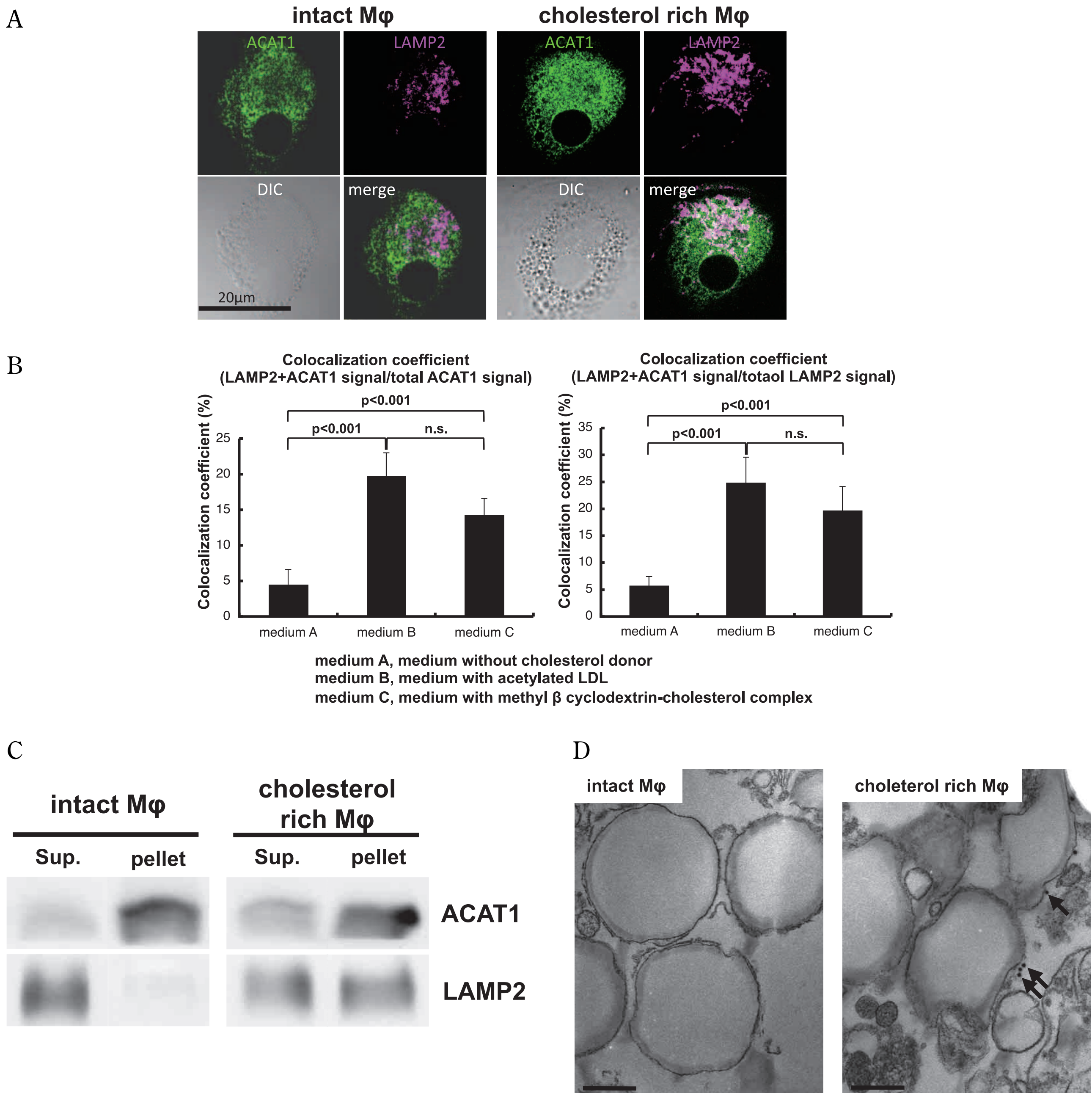

Figure 2 ACAT1-LE in intact and cholesterol-rich macrophages
(A, B) Human macrophages derived from blood-borne monocytes with or without aggregated LDL treatment were subjected to immunofluorescence staining and quantitative colocalization analysis. The green signal indicates ACAT1, the magenta signal indicates LAMP2, and the white signal indicates colocalization. DIC indicates differential interference contrast microscopy. (C) Macrophages with or without aggregated LDL treatment were homogenized and subjected to immunoadsorption with beads conjugated to ACAT1-specific antibody. Immunoadsorbed ACAT1-positive particles were analyzed by means of immunoblotting. Sup., supernatant. (D) Macrophages with or without methyl-β-cyclodextrin-cholesterol complex (mβCD-cho) treatment (cholesterol donor) were incubated with fluorescent beads to investigate phagocytosis. Macrophages after phagocytosis were homogenized and subjected to ultracentrifugation to collect beads containing phagosomes, and the phagosomal fraction was further subjected to immunoelectron microscopy (pre-embedding technique). Arrows indicate ACAT1-positive colloidal gold signals. Details are provided in the text. Adapted from references 14 and 15.

ACAT1-LE AND CHOLESTEROL METABOLISM

As described above, the late endosome is an organelle that contains free cholesterol derived from internalized LDL, and ACAT1 is an enzyme that esterifies free cholesterol. ACAT1-LE would therefore be an organelle that would re-esterify LDL-derived free cholesterol *in loco*. To demonstrate efficient cholesterol esterification at endosomes, we investigated cholesterol esterification kinetics in macrophages with ACAT1-LE. Figure 3A illustrates that intact macrophages required at least 2 hours to re-esterify LDL-derived free cholesterol because of the complex intracellular cholesterol trafficking system described here, in the Introduction. In contrast, foamy transformed macrophages with ACAT1-LE esterified a significant amount of LDL-derived cholesterol within 30 minutes. This finding suggests efficient cholesterol esterification in ACAT1-LE-positive macrophages. To demonstrate directly that ACAT1-LE is the site of cholesterol esterification, we performed an experiment in which cholesterol egress was inhibited. U18666A is an amphiphilic amino-steroid that blocks free cholesterol egress from late endosomes by interfering with the cholesterol trafficking protein NPC1 (5). Macrophages treated with U18666A showed negligible cholesterol esterification because no cholesterol reached the ER, where ACAT1 resides. In contrast, foamy transformed ACAT1-LE-positive macrophages esterified a significant amount of free cholesterol even in the presence of U18666A (Figure 3B). These data are direct evidence of cholesterol esterification at the ACAT1-LE, *i.e.* this unique organelle effectively esterified LDL-derived free cholesterol just after LDL hydrolysis at late endosomes. This finding stimulated us to pursue the therapeutic application of ACAT1-LE to NPC.

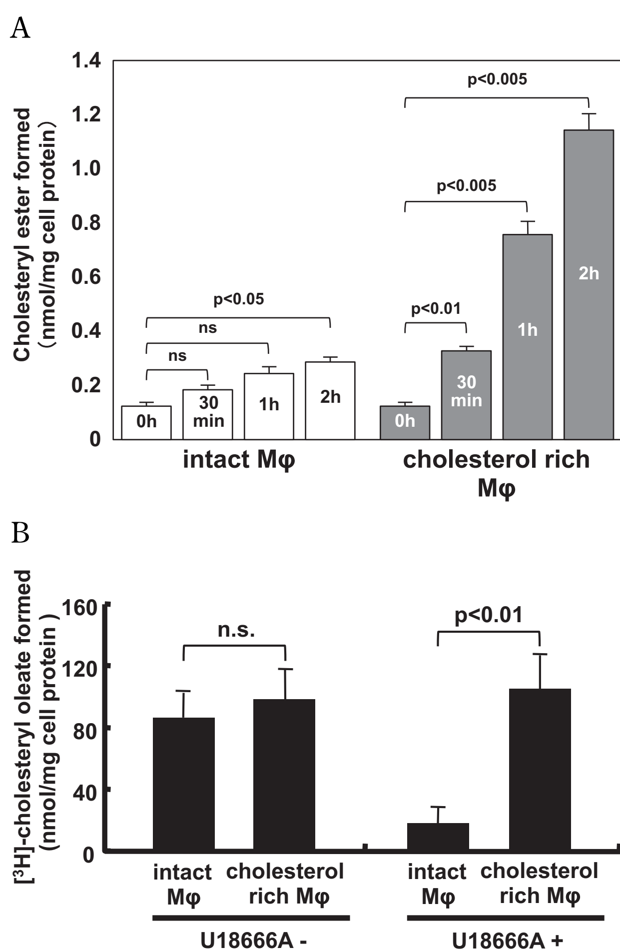

Figure 3 Functional analysis of ACAT1-LE
(A) Human macrophages derived from blood-borne monocytes with or without mβCD-cho (cholesterol donor) treatment were incubated with [^{3}H]cholesterol-labeled acetylated LDL. Macrophages after incubation with radiolabeled acetylated LDL were subjected to the [^{3}H]cholesteryl ester formation assay. (B) Human macrophages with or without treatment with mβCD-cho and the amphiphilic amino-steroid U18666A were incubated with [^{3}H]cholesterol-labeled acetylated LDL. They were then subjected to the [^{3}H]cholesteryl ester formation assay. Details are provided in the text. Adapted from references 14 and 15.

NPC AND ACAT1-LE INDUCTION

Niemann-Pick disease is a congenital lysosomal storage disease characterized by massive sphingomyelin accumulation. This disease was traditionally classified as type A to type E according to its clinical manifestations, but molecular analysis of the disease led to reclassification of types according to two criteria : Niemann-Pick disease type A, with an acid sphingomyelinase deficiency, and NPC, with an NPC1 protein deficiency (16). Deletion of the NPC1 protein causes free cholesterol accumulation in late endosomes, which results in inhibition of acid sphingomyelinase. As a result, NPC accumulates free cholesterol as well as sphingomyelin, with consequent cell injury. One possible therapeutic strategy for NPC is using methyl-β-cyclodextrin (mβCD), which is a cyclic oligosaccharide, to incorporate hydrophobic molecules such as cholesterol. mβCD is internalized via pinocytosis, remains at late endosomes, and removes accumulated free cholesterol from late endosomes and plasma membranes of cells with the NPC phenotype (*npc-/-*) (17). Administration of mβCD to *npc-/-* mice ameliorated tissue injury and prolonged survival, and this beneficial effect was also observed in patients with NPC (18, 19).

In contrast to the approach of removing cholesterol by using mβCD, we believed that inducing ACAT1-LE in *npc-/-* macrophages would also effectively reduce the intracellular cholesterol level by means of cholesterol esterification. Indeed, treatment with mβCD-cholesterol complex (mβCD-cho), a cholesterol donor, effectively produced ACAT1-LE-positive *npc-/-* macrophages. These ACAT1-LE-positive *npc-/-* macrophages showed significant cholesterol esterification and effective reduction of the intracellular cholesterol level (Figure 4A). In addition, administration of mβCD-cho to *npc-/-* neonate mice effectively extended their survival to the same level as did conventional cholesterol remover treatment (Figure 4B) (20). These data thus show that induction of ACAT1-LE in patients with NPC may be an alternative therapeutic approach (Figure 5).

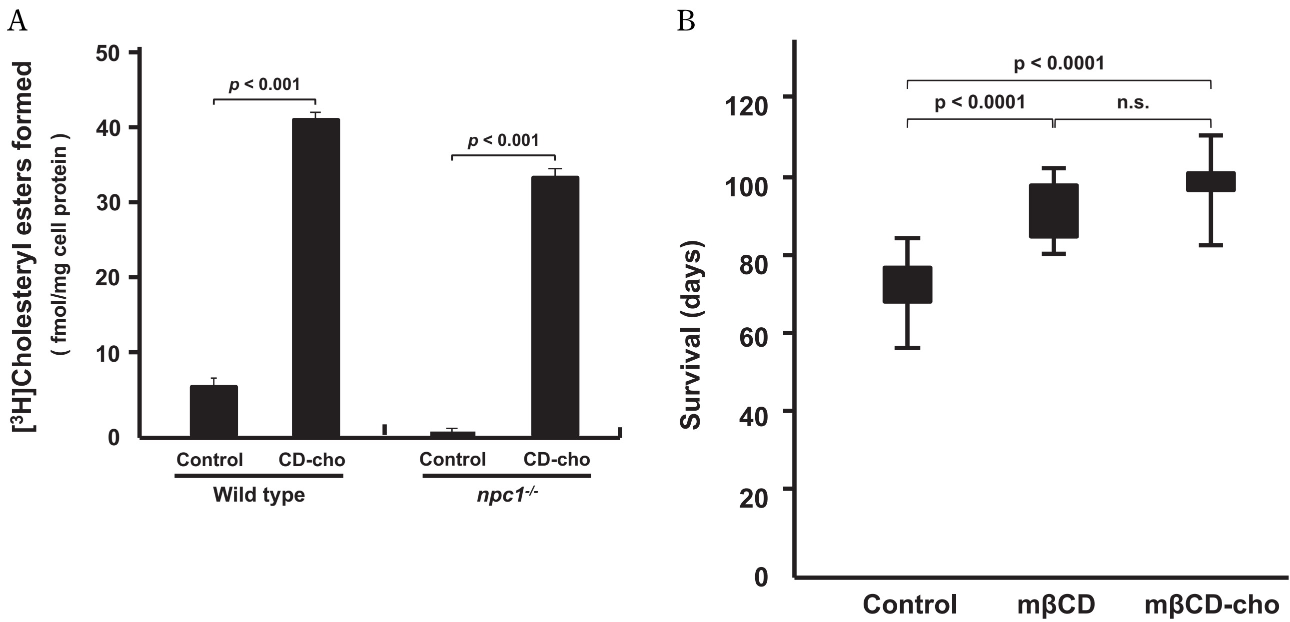

Figure 4 Therapeutic application of ACAT1-LE to NPC
(A) Bone marrow-derived macrophages with the NPC phenotype (*npc1-/-*) with or without mβCD-cho treatment were then incubated with [^{3}H]cholesterol-labeled acetylated LDL, after which they were subjected to the [^{3}H]cholesteryl ester formation assay. (B) At 7 days after birth, *npc1-/-* neonates were treated with mβCD-cho (cholesterol donor) or mβCD (cholesterol remover). Survival of *npc1-/-* mice after treatment was determined. Details are provided in the text. Adapted from reference 20.

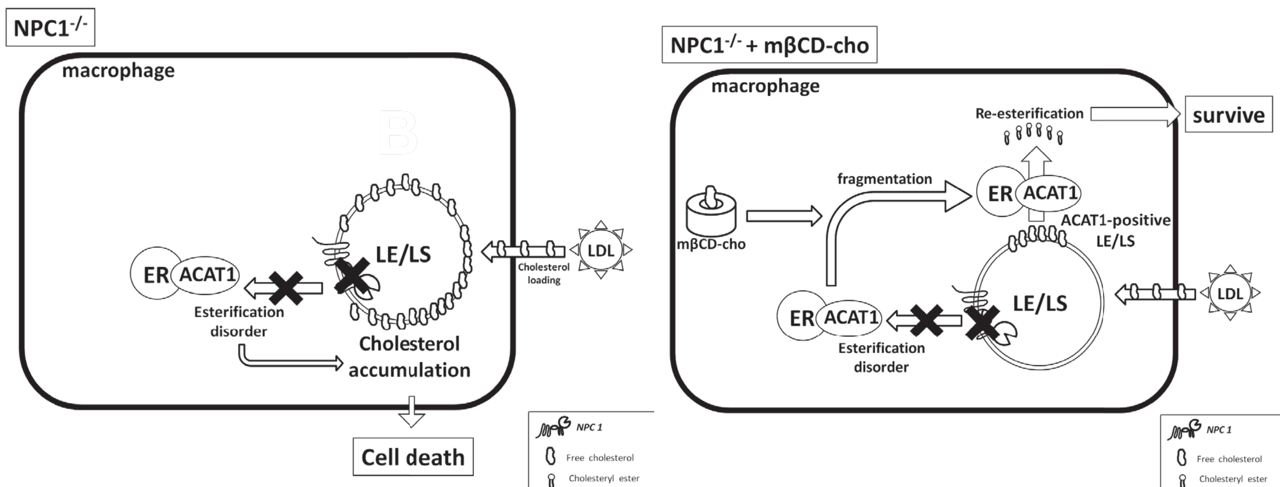

Figure 5 Schematic explanation of ACAT1-LE formation in NPC
In NPC, deficiency of the NPC1 protein causes massive accumulation of exogenous LDL-derived free cholesterol in late endosomes/lysosomes (LE/LS) in macrophages. This process results in significant cell injury and cell death. In contrast, pretreatment of macrophages with mβCD-cho induces ACAT1-LE and esterification of free cholesterol *in loco*, which results in reduced cell injury and greater cell survival. Adapted from reference 20.

CLOSING REMARKS

The question of whether intracellular ACAT1 localization would change under cholesterol-rich conditions (*i.e.* substrate-rich conditions for ACAT1) in human macrophages led us to discover ACAT1-LE and an alternative treatment strategy for NPC. Although in this review we did not mention the ACAT1-LE degradative pathway, ACAT1-LE has an impaired degradative pathway that depends on ACAT enzymatic activity. The late endosome is not only the principal organelle in the intracellular degradative pathway but is also the sorting compartment for protein synthesis. Therefore, ACAT1-LE-positive foamy transformed macrophages may manifest impaired protein sorting, which in hyperlipidemic conditions would result in disturbed protein synthesis such as cytokine production. Further research must clarify the details of ACAT1-LE function and its medical significance.

CONFLICT OF INTEREST

none

ACKNOWLEDGEMENT

This work was supported by Grants-in-Aid for Scientific Research C-23590448, C-20590384, and B-17390115 to N. S. from the Japan Society for the Promotion of Science (JSPS).

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