Mobilization of cytoplasmic CE droplets by overexpression of human macrophage cholesteryl ester hydrolase

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Abstract **The obligatory first step in the removal of cholesterol from foam cells is the hydrolysis of stored cholesteryl esters (CEs) to release free cholesterol (FC). Neutral cholesteryl ester hydrolase (CEH) catalyzes this hydrolysis, and limiting levels of CEH could play a role in determining the susceptibility to atherosclerosis. We have recently reported the first identification and cloning of cDNA for human macrophage CEH. In the present study, we tested the hypothesis that systematically varied levels of overexpression of human macrophage CEH results in a proportional degree of reduction in cellular CE content in a cell system with known and reproducible amounts of CE accumulation. CEH expression was confirmed by demonstrating the presence of CEH mRNA and protein with an increase in CEH activity. A significant reduction in intracellular lipid droplets was observed in CEH-expressing cells, together with a decrease in cellular CE mass and a 2-fold increase in FC efflux. These results demonstrate that when human macrophage CEH is expressed in lipid-laden cells, hydrolysis and mobilization of CE (stored as lipid droplets) occur. These data establish the possibility that increased CE hydrolysis, mediated by CEH up-regulation, could represent an important mechanism to reduce the cholesterol burden of foam cells.**—Ghosh, S., R. W. St. Clair, and L. L. Rudel. **Mobilization of cytoplasmic CE droplets by overexpression of human macrophage cholesteryl ester hydrolase.** ***J. Lipid Res.*** **2003.** 44: **1833–1840.**

Supplementary key words reverse cholesterol transport • cholesterol efflux • atherosclerosis • macrophage foam cells • cholesteryl ester

Atherosclerotic lesions begin as fatty streaks resulting from the recruitment of blood monocytes to the arterial wall, where they differentiate into macrophages and ultimately accumulate cholesterol from the uptake of modified cholesteryl ester (CE)-rich lipoproteins. Unregulated uptake of modified forms of LDL (oxidized, aggregated, etc.) via scavenger receptors, e.g., scavenger receptor A and CD36, leads to the development of macrophage "foam cells" that contain massive amounts of cytoplasmic CE inclusions. Lipoprotein-associated CEs are first hydrolyzed in the lysosomes with the resulting free cholesterol (FC) being transferred out of the lysosome. The FC content of cells is maintained under tight control in order to prevent excessive enrichment with FC of the plasma membranes, which can alter membrane function (1). Two processes working in tandem are involved in maintaining the homeostatic balance between intracellular FC and CEs: esterification of excess FC by acyl CoA: cholesterol acyltransferase (1) (ACAT1), and hydrolysis of CE by a neutral cholesteryl ester hydrolase (CEH). This "futile" cycle of CE and hydrolysis is known as the CE cycle and serves to maintain appropriate FC availability for cell membranes (2).

Efficient cholesterol efflux from macrophages is critical for the prevention of foam cell formation and for subsequent protection against atherosclerosis. Because there is no significant release of intact CE from cells, including macrophages, for efflux to occur, CE must first be hydrolyzed to FC. FC leaves the cell by being picked up by one of several cholesterol acceptors in the extracellular space. Ultimately, FC is transferred into HDL, where much of it is esterified by lecithin:cholesterol acyltransferase to form CE. In this form, it is removed from plasma during either HDL particle clearance after transfer by cholesteryl ester transfer protein to other lipoproteins that are cleared from plasma by the liver, or after interaction of HDL with the scavenger receptor class B type I (CLA-1) resulting in selective CE uptake. Once in the liver, cholesterol can be catabolized to bile acids, and these detergents, together with phospholipids and cholesterol, are secreted in bile. The directional movement of cholesterol from peripheral tissues to the liver is known as reverse cholesterol transport. The obligatory first step in reverse cholesterol transport is, therefore, the hydrolysis of CE to release FC, a reaction catalyzed by a neutral CEH. Because the rate of CE hydrolysis is often slower than FC movement to the extracellular acceptor,

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Abbreviations: CE, cholesteryl ester; CEH, cholesteryl ester hydrolase; FC, free or unesterified cholesterol; PC, phosphatidylcholine.

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CE hydrolysis is increasingly recognized as the rate-limiting step in cellular cholesterol efflux (3, 4). Thus, macrophages with high neutral CEH activity do not accumulate CE in the presence of atherogenic lipoproteins, such as β-migrating VLDLs, in comparison to macrophages with low CEH activity (5). In addition, animal models of atherosclerosis, such as the hypercholesterolemic rabbit and the White Carneau pigeon, appear to possess macrophages in which stored CE is resistant to hydrolysis and subsequent mobilization (6, 7).

Despite the obvious significance of CE hydrolysis in cellular FC efflux, the identity of neutral CEHs in macrophages remains controversial. Several lines of evidence suggest that the enzyme responsible for CE hydrolysis in murine macrophages is similar to hormone-sensitive lipase (HSL), which is present in adipose and steroidogenic tissues (8–10). Overexpression of adipose tissue HSL in murine (RAW 264.7) macrophages resulted in a 2- to 3-fold increase in CE hydrolysis (11). Recently, however, Osuga et al. (12) and Contreras (13) observed identical levels of CE hydrolysis in peritoneal macrophages isolated from wild-type or HSL knockout mice, which suggests that an enzyme other than HSL is responsible for catalyzing CE hydrolysis in murine macrophages. Species-specific differences in HDL-induced cholesterol efflux and CE hydrolysis has been documented by Ishii et al. (14), Graham et al. (15), and Hakamata et al. (16), while the identity of CEH in human macrophage continues to be a subject of debate. While Reue, Cohen, and Schotz detected HSL mRNA in the human monocyte cell line THP-1 (17), and Johnson, Jang, and Bernard (18) demonstrated the presence of the testicular isoform of HSL in THP-1 monocyte/macrophage, Contreras and Lasuncion (19) failed to detect HSL mRNA in human macrophages. Li and Hui (20) recently reported the absence of HSL in human macrophages and demonstrated the expression of bile salt-stimulated CEH, similar to secretory pancreatic CEH. Because this enzyme was secreted from the cells, Li and Hui proposed that it was unlikely to play a role in intracellular cholesterol metabolism and suggested that another CEH may be responsible for CE metabolism in human macrophages. Ghosh (21) has recently reported the cloning and characterization of the human macrophage CEH cDNA and demonstrated the expression of the mRNA for this enzyme in the human monocyte/macrophage cell line, THP-1, as well as in human peripheral blood monocyte/macrophages. This enzyme-hydrolyzed CE is present in lipid droplets, the physical state of stored CE in macrophage foam cells. Furthermore, not only did overexpression of this enzyme increase CEH, but also the FC that was generated decreased the expression of the sterol-regulated LDL receptor gene in the same cells (21).

In the present study, we examined the potential function of the newly cloned CEH in attenuating intracellular CE accumulation. In order to accomplish this, we overexpressed the human monocyte/macrophage CEH cDNA in a cell line stably expressing ACAT1, where predictable accumulation of CE-rich lipid droplets occurs in the cytoplasm. This system allows demonstration of a possible rate-limiting role of CEH in cellular cholesterol efflux under conditions of continual reesterification of FC by ACAT1. Our results demonstrate that despite constitutive overexpression of ACAT1 in these cells, increasing levels of the expression of CEH caused a proportional reduction in lipid droplets and intracellular CE content without increasing cellular FC levels. The data suggest that in the future, it may become possible to alter the extent of foam cell formation through the combined targeting of CEH up-regulation and ACAT1 down-regulation.

EXPERIMENTAL PROCEDURES

Materials

All cell culture media and reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA). Effectene™ and RNeasy™ Mini Kit were obtained from Qiagen (Valencia, CA). GeneScreen™ hybridization membrane, [^{3}H]cholesterol, cholesterol [1-^{14}C]oleate, and [α-^{32}P]dCTP were purchased from NEN Life Science Products (Boston, MA). Alamar Blue™ was obtained from BioSource International (Camarillo, CA), and Alexa Fluor® 546 conjugated goat anti-rabbit IgG was obtained from Molecular Probes (Eugene, OR). Stigmasterol, cholesterol, cholesterol oleate, and cholestenone were from Steraloids (Newport, RI), and phosphatidylcholine (PC) was obtained from Avanti Polar Lipids (Alabaster, AL). PerfectHyb™ hybridization solution, cholesterol oxidase, Oil Red O, and all other chemicals were from Sigma-Aldrich (St. Louis, MO). All solvents were HPLC grade and purchased from Fisher Scientific.

Methods

Cell culture and transfections. The stable expression of ACAT1 in agmACAT1 cells leads to visible accumulation of cytoplasmic lipid droplets in these cells (22) and constitutes a suitable cell system to evaluate the role of CEH overexpression in the mobilization of lipid droplets and cellular CE content. agmACAT1 cells were maintained in Ham's F12 medium and supplemented with 10% FBS, penicillin/streptomycin, and 200 μg/ml Geneticin. Cells were plated in 24-well tissue culture plates at a density of 2×10^5 cells/well and were either transfected with vector alone (pCMV) or CEH expression vector, pCMV-CEH. Effectene™ was used for transfections. The growth medium containing 10% FBS was replaced twice (24 h and 48 h after transfection), and analyses were performed 72 h after transfection unless specified otherwise.

Cell viability after transfection. The viability of the cells transfected with pCMV or pCMV-CEH was assessed using Alamar Blue™, a nontoxic Redox indicator metabolically reduced by viable cells. The reduction of the dye results in the conversion of resazurin to resarufin, which is measured at 530 nm excitation and 590 nm emission (23) using a microplate fluorometer. The percent reduction of Alamar Blue™ reflects cell viability. Alamar Blue™ was added to a final concentration of 10% to transfected cells after 24 h, 48 h, or 72 h. Reduction of the dye was quantified following a 4 h incubation at 37°C in a CO_2 incubator. Percent viability was calculated by considering the cells transfected with pCMV vector alone as 100% viable. Data are presented as mean ± SD for three independent experiments.

Staining of cytoplasmic lipid droplets. Cells in culture dishes were fixed in 10% formaldehyde in PBS for 10 min, followed by washing twice with PBS and staining with Oil Red O (0.2% in 60% 2-propanol) for 30 min. Following extensive washing (at least three times), cells were imaged using an Olympus model IX70™

inverted phase microscope fitted with a MagnaFire™ digital camera. At least two different fields per sample were imaged and analyzed using Image-Pro Plus 4.0 software. Briefly, cell outlines were traced following digital color extraction. A minimum of 10–15 cells per field were analyzed. Filter range thresholds were applied to define oil droplets as bright objects and counted. Population density command was then used to determine droplets per cell using the previously traced cell outlines. The average number of droplets for 10–15 cells per field was calculated. Data are reported as mean ± SEM for six fields (two fields/sample for n = 3).

Determination of cholesterol and CE mass. Total lipids were extracted from parallel sets of cells with 2-propanol-containing stigmasterol as an internal standard. The lipid extract was analyzed for total cholesterol and FC content by gas-liquid chromatography via the procedure of Ishikawa et al. (24) as modified by Klansek et al. (25). Esterified cholesterol was determined as the difference between total and FC and, where indicated, esterified cholesterol mass was multiplied by 1.67 to convert to CE mass. Following lipid extraction, cellular proteins were solubilized in NaOH and quantified using the Pierce BCA™ Kit.

Measurement of cholesterol efflux. The intracellular FC and CE pools were labeled with [^{3}H]FC by incubating the cells for 48 h with growth medium containing 2 μCi/ml [^{3}H]cholesterol according to the method of Mahlberg et al. (26). The cells were then washed and incubated with serum-free medium containing 2 mg/ml BSA for 24 h to allow all pools of cholesterol to equilibrate. Total lipids were extracted from representative cells, and specific radioactivity of cellular FC and CE was determined to ensure equilibration of the [^{3}H]cholesterol label within these two forms of cellular cholesterol. Cells then were transfected with pCMV or pCMV-CEH in growth medium containing 10% FBS as described above. After 24 h, the culture medium was changed to growth medium containing 10% FBS plus 250 μg/ml PC (as lipid vesicles), which acts as an additional cholesterol acceptor. In order to measure the efflux of FC from cells, an aliquot of the medium was withdrawn at the indicated times and centrifuged for 10 min at 14,000 *g* to remove cell debris. The radioactivity associated with the supernatant fluid was determined by liquid scintillation counting. Total cell-associated radioactivity was determined from the 2-propanol lipid extract of the cells at zero time. Efflux of [^{3}H]FC from the cells into the medium is expressed as the percentage of the total [^{3}H]cholesterol in the cells at time zero. Data are expressed as mean ± SD for three independent experiments.

Northern blot analysis. Cells were plated in 35 mm tissue culture plates at a density of 8×10^5 cells/well and were transfected with either the vector alone or the CEH expression vector. After 24 h, total RNA was extracted using the RNeasy™ Mini Kit. Twenty micrograms of total RNA were separated on formaldehyde-agarose gels, transferred to GeneScreen™ hybridization membranes, and probed with a [^{32}P]-labeled full-length CEH cDNA probe. Following a high-stringency wash with 0.2 × SSC and 0.1% SDS at 65°C for 20 min, the blot was exposed to Kodak X-OMAT LS film overnight at −70°C.

Immunocytochemistry. Human macrophage CEH protein sequence was analyzed using the PCGENE program that selects antigenic regions of a protein based on three criteria, namely antigenicity, flexibility, and β-turn. Peak values were assigned for each criterion, with overlapping regions of the three criteria and peak values greater than 1.0 being chosen. Using these criteria, polyclonal antibodies were raised in rabbits to a 15 amino acid-long peptide (residues of 532–545, plus an N-terminal cysteine for conjugation). The antiserum was used for immunocytochemical detection of CEH protein in cells. Cells were plated on fibronectin-coated coverslips in 24-well tissue culture plates and transfected with either the vector alone or with the CEH expression vector. Forty-eight hours after transfection, culture media was aspirated and cells were fixed in 3.7% paraformaldehyde in PBS for 10 min at 4°C. Cells were permeabilized by exposure to 0.15% Triton X-100 in PBS for 3 min at 4°C, washed with PBS, and blocked with 0.5% BSA in PBS for at least 1 h at room temperature. Cells were then incubated with the rabbit polyclonal antibody to CEH overnight at 4°C. For detection, cells were subsequently incubated with Alexa Fluor® 546 conjugated goat anti-rabbit IgG in 0.5% BSA for 1 h at room temperature.

Cells were washed with PBS, covered with two drops of Crystal-Mount, and mounted with coverslips inverted on glass slides. Cells were imaged at 20× magnification using an Olympus model IX70™ inverted phase microscope fitted with a MagnaFire™ digital camera using a TRITC filter cube (530–560 nm excitation, 590–650 nm emission, Chroma Technology Corp.).

CEH activity. Cells were plated in 35 mm tissue culture dishes at a density of 8×10^5 cells/well and were transfected with either the vector alone or with the CEH expression vector. After 48 h, cells were harvested in cold homogenizing buffer, and CEH activity was measured as described (21).

RESULTS

Cell viability following CEH overexpression

Cell viability was monitored following transfection with either pCMV or pCMV-CEH after 24 h, 48 h, and 72 h. No significant change in cell viability (116 ± 4.2% at 24 h, 105.2 ± 8.8% at 48 h, and 95.8 ± 4.8% at 72 h) was observed at any given time point, indicating no deleterious/toxic effects of CEH overexpression on these cells.

CEH overexpression in agmACAT1

In order to determine if there was an increase in CEH expression following transfection, Northern blot analysis of total cellular RNA was performed. No CEH mRNA was detected in vector-transfected cells (lane 1, **Fig. 1A**). In contrast, the cells transfected with CEH expression vector

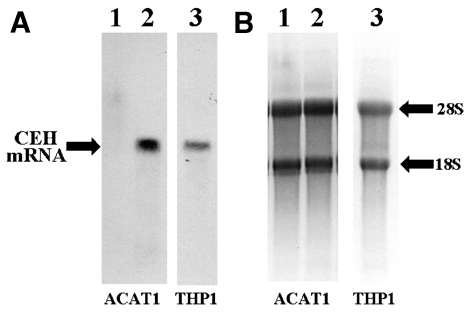


Fig. 1. Northern blot analysis. Twenty micrograms of total RNA from cells transfected with either vector alone (lane 1) or with cholesteryl ester hydrolase (CEH)-expression vector (lane 2) was separated on formaldehyde-agarose gel. Following transfer to GeneScreen™ membrane, hybridization was carried out at 65°C using PerfectHyb™ and [^{32}P]-labeled human macrophage CEH cDNA as a probe. After an initial low-stringency wash in 2 × SSC + 0.1% SDS at room temperature for 10 min, the blot was washed in a high-stringency solution (0.2 × SSC + 0.1% SDS) at 65°C for 20 min and exposed to Kodak X-Omat™ LS film. A: Autoradiogram showing positive hybridization. B: Ethidium bromide-stained gel showing equal loading and integrity of the RNA samples; 18*S* and 28*S* rRNA bands are marked. THP-1 RNA was used as a positive hybridization control (lane 3).

(lane 2, Fig. 1A) showed a prominent band of positive hybridization demonstrating the expression of CEH. Total RNA from THP-1 macrophages was used as a positive control and is shown in lane 3 (Fig. 1A). Equivalent loading and integrity of the RNA samples was demonstrated by ethidium bromide staining (Fig. 1B).

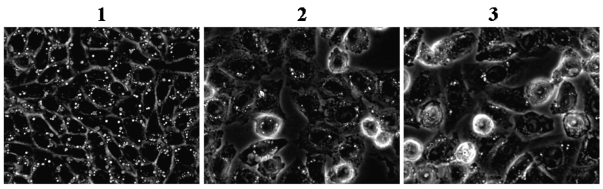
Expression of CEH protein in cells was determined by immunocytochemistry. Bright cell-associated fluorescence, indicative of increased CEH protein expression, was evident in cells transfected with CEH expression vector pCMV-CEH (**Fig. 2**) compared with cells transfected with vector alone (pCMV). The presence of increased CEH protein was associated with a greater than 2-fold increase in CEH-specific activity (3.87 ± 0.27 nmol/h/mg protein vs. 1.81 ± 0.07 nmol/h/mg protein, $P < 0.05$, n = 3). Dilution of the radioactive cholesteryl oleate, used as a substrate by substantial endogenous CEs present in the cell lysates, probably accounts for the lower fold increase in activity compared with the observed increase in CEH protein and mRNA. An 8-fold increase in CEH activity was observed earlier in transfected CHO-K1 cells (21), the parent cell line of agmACAT1 cells that do not contain substantial amounts of endogenous CEs.

Mobilization of intracellular lipid droplets

Because of the constitutively high as well as constant level of ACAT activity and accumulation of visible lipid droplets in the cytoplasm, agmACAT1 cells were ideally suited to evaluate the role of CEH in the mobilization of intracellular CE droplets. For this, CEH-expressing cells were stained with Oil Red O 72 h posttransfection, and the number of intracellular lipid droplets was determined microscopically as described in Experimental Procedures. Representative images and quantification of intracellular lipid droplets are shown in **Fig. 3A** and B, respectively. Numerous lipid droplets (>13 per cell) were clearly visible in pCMV-transfected cells incubated with medium containing 10% FBS, as shown in panel 1 (Fig. 3A). A significant reduction ($P < 0.05$) in the number of intracellular Oil Red O-stained droplets (down to 3.3–3.7 per cell) was observed following the CEH transfection (panels 2 and 3, Fig. 3A). Thus, an increase in the activity of CEH resulted in mobilization of intracellular, CE-rich lipid droplets.

Ten percent FBS alone versus 10% FBS with 1 mg/ml PC in the growth medium appeared to be equally effective in their extracellular cholesterol acceptor capabilities,

A



B

	1	2	3
Transfected with	pCMV	pCMV-CEH	pCMV-CEH
Extracellular Acceptor	10% FBS	10% FBS	10% FBS + PC
Droplets per Cell Mean ± sem	13.9 ± 0.63	3.3 ± 0.92	3.7 ± 0.42

Fig. 3. CEH overexpression in agmACAT1 cells; effect on mobilization of intracellular lipid droplets. Cells were transiently transfected with vector alone (pCMV, panel 1) or with CEH-expression vector (pCMV-CEH, panels 2 and 3). Fresh medium containing 10% FBS as extracellular cholesterol acceptor was added after 24 h. After an additional 24 h, a medium was replaced with either medium containing 10% FBS (panel 2) or with medium containing 10% FBS + 1 mg/ml phosphatidylcholine (PC) (panel 3). Cells were fixed after an additional 24 h, and intracellular lipid droplets were stained with Oil Red O and imaged as described in Experimental Procedures. Representative images (from n = 3) are shown (A). Lipid droplets appear as bright punctate spots distributed within the cell. Droplets per cell (10–15 cells/field) were counted in at least two fields per sample and mean ± SEM for each group is reported (B).

with both allowing the efficient efflux from the cells of the FC liberated after hydrolysis of CE droplets (cf. panels 2 and 3, Fig. 3A), which leads to decreased intracellular droplets.

Changes in cellular esterified cholesterol

In order to confirm that the decrease of intracellular lipid droplets in CEH-expressing cells resulted in a decrease in cellular CE content at 72 h posttransfection, lipids were extracted and analyzed for FC and esterified cholesterol mass by gas-liquid chromatography, and the results are expressed per milligram of cell protein. A significant ($P < 0.05$) >75% decrease in CE mass was observed following CEH overexpression (**Fig. 4A**). As was true for the depletion of lipid vesicles with the expression of CEH, the addition of PC to the FBS-containing culture medium did not further increase the clearance of cellular CE. Cellular CE mass was significantly correlated ($P < 0.01$)

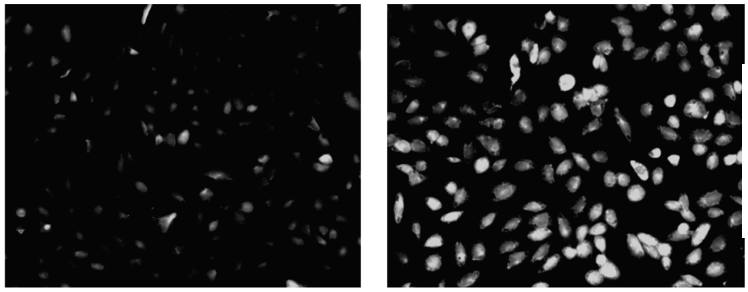


Fig. 2. Immunocytochemical detection of CEH protein in transfected cells. Cells were transiently transfected with vector alone (pCMV) or CEH-expression vector (pCMV-CEH), and CEH protein was visualized by using polyclonal antibodies to CEH and Alexa Fluor® 546 conjugated secondary antibody as described in Experimental Procedures. Cells were imaged at 20× magnification and representative images of cells (from n = 3) transfected with vector alone (pCMV) or with CEH-expression vector (pCMV-CEH) are shown here. Bright fluorescence distributed within the cells transfected with pCMV-CEH demonstrates the immunological detection of CEH protein expressed within these cells.

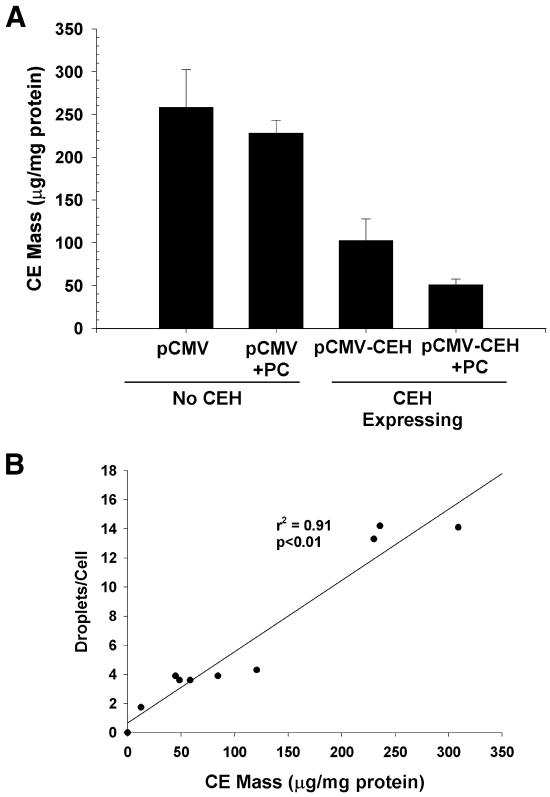


Fig. 4. Effect of CEH overexpression on cellular cholesteryl ester (CE) mass. Cells were transiently transfected with vector alone (pCMV) or with CEH-expression vector (pCMV-CEH). Fresh medium containing 10% FBS as extracellular cholesterol acceptor was added after 24 h. After an additional 24 h, the medium was replaced with either medium containing 10% FBS or with medium containing 10% FBS + 1 mg/ml PC. Total lipid and proteins were extracted as described under Experimental Procedures. Total and free cholesterol (FC) was determined by gas chromatography and esterified cholesterol mass calculated by difference. CE mass (after a total of 72 h posttransfection) was normalized to total cellular protein. Intracellular lipid droplets were stained with Oil Red O from a parallel set of experiments and droplets/cell determined as described under Experimental Procedures. A: CE mass, data are shown as mean ± SD (n = 3). B: Regression line plot to show correlation between CE mass and intracellular Oil Red O-stained lipid droplets per cell.

with the number of Oil Red O-stained lipid droplets/cell with $r^2 = 0.91$ (Fig. 4B).

CEH cDNA concentration-dependent decrease in intracellular lipid droplets and CE levels

Cells were transfected with increasing concentration of pCMV-CEH to establish a correlation between the amount of CEH cDNA used for transfection and the observed decrease in intracellular lipid droplets and CE mass. The total DNA concentration was maintained constant at 400 ng/well using the empty vector pCMV. The culture medium contained 10% FBS as the cholesterol acceptor and was changed 24 h and 48 h posttransfection. One set of cells was stained with Oil Red O and the other set was processed for lipid extraction and CE mass analysis. Representative Oil Red O-stained images are shown in **Fig. 5**. A dose-dependent decrease in intracellular lipid droplets is apparent with increasing pCMV-CEH DNA concentration from 50 ng to 400 ng/well. The intracellular CE levels were inversely correlated ($P < 0.001$) with a concentration of pCMV-CEH cDNA used for transfection with $r^2 = -0.95$ (**Fig. 6**), establishing the role of this CEH in intracellular CE hydrolysis and removal.

Effect of CEH overexpression on cellular FC levels

In order to determine the effects of CEH overexpression on cellular FC concentrations, cells were transfected and subsequently grown in either serum-free medium containing 0.5% BSA (which is a poor extracellular acceptor of cholesterol and would be expected to limit the amount of FC that could efflux from the cells) or medium containing 10% FBS as the extracellular cholesterol acceptor. Total lipids were extracted from cells 24 h after transfection, and esterified cholesterol and FC mass were determined. As shown in **Fig. 7**, regardless of the presence of a limiting or sufficient extracellular cholesterol acceptor, there was no FC accumulation in CEH-expressing cells. Cellular CE levels, however, decreased by only 12.5% in the presence of a medium containing 0.5% BSA, wherein FC efflux would be limited, but decreased by 26% ($P < 0.05$) in the presence of 10% FBS, which is a potent extracellular cholesterol acceptor. These data suggest that the FC generated by increased CEH-mediated CE hydrolysis in "ACAT-sufficient" cells is either reesterified (under limiting acceptor concentration) or effluxed (in the presence of a sufficient extracellular acceptor) from the cells, preventing buildup of FC, which ultimately could be cytotoxic.

Efflux of FC from cells

Cellular FC and CE pools were labeled with [^{3}H]cholesterol for 48 h prior to transfection of the cells with either pCMV or pCMV-CEH to examine whether the CEH-mediated decrease in cellular CE levels is due to an increase in the efflux of FC from the cells. As shown in **Fig. 8**, a 2-fold higher rate of FC efflux was observed in CEH-expressing cells (pCMV-CEH) when compared with vector-transfected control cells (pCMV). Consistent with the proposed rate-limiting role of CEH in FC efflux, these data demonstrate that CEH overexpression results in an increase of FC efflux that leads to the mobilization of cellular lipid droplets and a decrease in CE mass, as shown in Figs. 3 and 4.

DISCUSSION

The data presented here demonstrate, for the first time, that overexpression of human macrophage CEH in lipid-laden cells results in mobilization of intracellular CE-rich lipid droplets with a corresponding decrease in cellular CE mass. The significant inverse correlation observed between intracellular CE levels (Fig. 6) and concentration of pCMV-CEH cDNA used for transfection further establishes the role of this CEH in intracellular CE hydrolysis

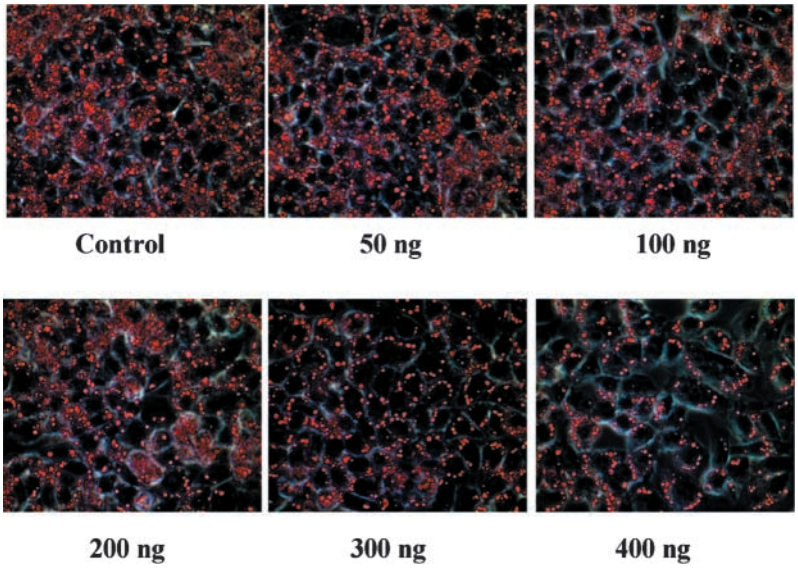


Fig. 5. Effect of increasing CEH cDNA concentration on intracellular lipid droplet mobilization. Cells were transiently transfected with either pCMV vector alone (control) or with increasing concentrations (50–400 ng/well) of pCMV-CEH. The culture medium contained 10% FBS as the extracellular cholesterol acceptor and was replaced 24 and 48 h posttransfection. Cells were fixed, stained with Oil Red O and imaged as described under Experimental Procedures. Representative images are shown. Intracellular lipid droplets are stained red.

and removal. This CEH is expressed in human monocyte/macrophage cell line THP-1 (Fig. 1) as well as primary human blood monocyte-derived macrophages (21), and based on the results shown here, it could potentially represent a target to attenuate CE accumulation during macrophage foam cell formation.

agmACAT1 cells that stably express a high level of ACAT1 were employed as a model cell system to permit a direct evaluation of the role of human macrophage CEH under conditions where an abundance of CE substrate would be continuously available. Constitutive expression of ACAT1 in these cells leads to a high degree of accumulation of cytoplasmic CE droplets that are analogous to the CE-rich cytoplasmic lipid inclusions seen in macrophage foam cells (22). Furthermore, CHO-K1 (the transformed parent cell line of agmACAT1 cells) has also been used extensively to study cellular cholesterol efflux (27–29). Under the conditions of functional intracellular ACAT1 in agmACAT1 cells, the FC released after CEH-mediated hydrolysis can either be effluxed or reesterified. If indeed CEH-mediated hydrolysis is the rate-limiting step in cellular cholesterol efflux, then CEH overexpression should result in increased CE mobilization despite functional ACAT1 in these cells.

The data presented here demonstrate that overexpression of human macrophage CEH results in significant mo-

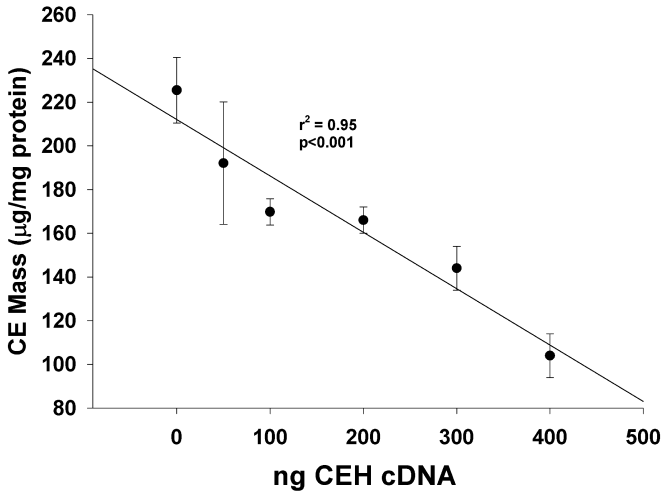


Fig. 6. Effect of increasing CEH cDNA concentration on intracellular CE levels. Cells were transiently transfected with either pCMV vector alone (control) or with increasing concentrations (50–400 ng/well) of pCMV-CEH. The culture medium contained 10% FBS as the extracellular cholesterol acceptor and was replaced 24 h and 48 h posttransfection. Total lipid and proteins were extracted as described under Experimental Procedures. Total cholesterol and FC were determined by gas chromatography, and esterified cholesterol mass was calculated by difference. Regression analysis of the data (CE mass, µg/mg protein, mean ± SD, n = 3) revealed a highly significant negative correlation ($r^2 = -0.95$, $P < 0.001$) between pCMV-CEH cDNA concentration added for transfection and cellular CE mass.

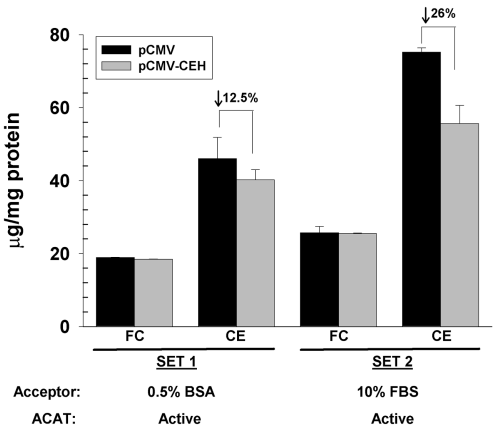


Fig. 7. Effect of CEH overexpression on cellular FC levels. Cells were transiently transfected with vector alone (pCMV) or CEH-expression vector (pCMV-CEH). The culture medium contained either 0.5% BSA (limiting cholesterol acceptor) or 10% FBS (sufficient cholesterol acceptor). Lipids and proteins were extracted after 24 h as described in Experimental Procedures. Total cholesterol and FC were determined by gas chromatography, and esterified cholesterol mass was calculated by difference. FC and CE mass are expressed per milligram cellular protein. Data (FC and CE mass values, 24 h posttransfection) are shown as mean ± SD (n = 3).

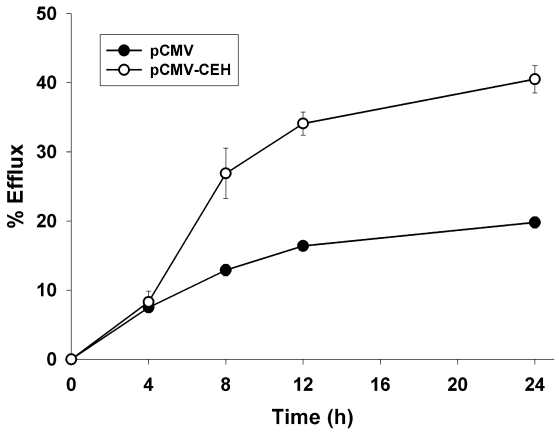


Fig. 8. FC efflux from CEH-expressing cells. Cellular cholesterol pools (FC and CE) were labeled with [^{3}H]cholesterol and FC efflux was measured following transfection with either pCMV or pCMV-CEH as described under Experimental Procedures. Efflux of [^{3}H]FC from the cells into the medium at each time point is expressed as the percentage of the total radioactivity in the cells at time zero. Data are expressed as mean ± SD for three independent experiments.

bilization of cytoplasmic lipid droplets (Figs. 3, 5) and a corresponding decrease in cellular CE levels (Figs. 4, 6) in cells that constitutively overexpress ACAT1. Thus, in the presence of an extracellular acceptor (10%, FBS), FC released by CEH-mediated hydrolysis of CEs is efficiently effluxed (cellular CE ↓26%, Fig. 7). In contrast, under conditions of limiting cholesterol acceptor (0.5% BSA, Fig. 7), excess FC generated by CEH is reesterified by ACAT1 (cellular CE ↓12.5%, Fig. 7). In either case, accumulation of FC within the cells is prevented. Similar results were also obtained with another ACAT1 stable cell line, hACAT1, expressing human ACAT1 (unpublished observations).

Several lines of investigation have established that hydrolysis of intracellular CE is the rate-limiting step in the net removal of cholesterol from cells (3, 4). Graham et al. observed impaired mobilization of cellular CE and attributed it to low CEH activity in THP-1 cells (15). Our results demonstrating increased CE mobilization and FC efflux under conditions of CEH overexpression not only provide support for the rate-limiting role ascribed to CEH, but also establish CEH as a new target for manipulation of cellular CE levels.

Pharmacological inhibition of ACAT as a means of preventing or attenuating foam cell formation has been extensively pursued (30–33); however, Perrey et al. showed increased plaque formation by preferential pharmacological inhibition of macrophage ACAT or ACAT1 in mouse and rabbit models of atherosclerosis (34). Recently, disruption of ACAT1 was shown to result in marked systemic abnormalities in lipid homeostasis in hypercholesterolemic apolipoprotein E-deficient and LDL receptor-deficient mice, leading to extensive deposition of FC in the skin and brain (35, 36). Further, Fazio et al. reported increased atherosclerosis in LDL receptor-null mice lacking ACAT1 in macrophages (37). Together, it is not clear at present whether inhibition of ACAT1 could be used as a potential therapeutic strategy.

Exploring the alternate strategy of reducing CE accumulation by overexpression of human macrophage CEH, our results demonstrate for the first time that a fundamental difference may result in the outcomes of the two strategies employed to reduce cellular CE burden. While ACAT1 inhibition often appears to result in FC buildup within the model foam cells in culture (38) and in the skin and brain of ACAT1-deficient mice (35, 36), overexpression of human macrophage CEH in "ACAT1-overexpressing" cells did not result in cellular FC accumulation. Under limiting the extracellular acceptor concentration where efflux of FC from the cells is attenuated, excess FC generated by CEH-mediated hydrolysis can be reesterified via the functional ACAT1 present in the cells, preventing cellular FC accumulation. These data suggest that up-regulation of CEH would be a better way to promote cholesterol removal from foam cells in atherosclerotic lesions than ACAT inhibition, although more information about the appropriate cellular systems is needed.

The CE-rich lipid core not only contributes to the growth of an atherosclerotic plaque but also decreases the plaque stability required to prevent thrombotic coronary occlusions. Thus, decrease in the CE burden of the plaque is a potentially promising strategy for reducing lesion growth and rupture explored previously by ACAT1 inhibition (39) and overexpression of adipose tissue HSL (40). The data presented here suggest that approaches to enhance expression or up-regulation of CEH in macrophages could provide a new therapeutic strategy to attenuate the CE burden of plaque-associated foam cells. While demonstration of significant reduction in cellular CE levels by overexpression of human macrophage CEH in cultured cells represents the first major step toward this goal, it remains to be seen whether a similar reduction in CE content of human macrophages would be achieved. CEH described here is expressed in human monocyte/macrophage cell line THP-1 (Fig. 1) as well as in primary human monocyte-derived macrophages (21). Viral vectors are currently being generated and evaluated to overexpress CEH in THP-1 cells and in primary human monocyte-derived macrophages and to examine whether CEH overexpression in human macrophages also results in a significant decrease in CE mass, as demonstrated by the data presented here. Future studies with the recently cloned and characterized proximal promoter for human macrophage CEH (41) will provide insight into the modes and mechanisms involved in regulating this enzyme in vivo and into how the endogenous expression of CEH can be manipulated to mimic the CE-lowering effects of CEH overexpression.

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