Elimination of Cholesterol Ester from Macrophage Foam Cells by Adenovirus-mediated Gene Transfer of Hormone-sensitive Lipase*

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Cholesterol ester (CE)-laden foam cells are a hallmark of atherosclerosis. To determine whether stimulation of the hydrolysis of cytosolic CE can be used as a novel therapeutic modality of atherosclerosis, we overexpressed hormone-sensitive lipase (HSL) in THP-1 macrophage-like cells by adenovirus-mediated gene delivery, and we examined its effects on the cellular cholesterol trafficking. We show here that the overexpression of HSL robustly increased neutral CE hydrolase activity and completely eliminated CE in the cells that had been preloaded with CE by incubation with acetylated low density lipoprotein. In these cells, cholesterol efflux was stimulated in the absence or presence of high density lipoproteins, which might be at least partially explained by the increase in the expression of ABCA1. Importantly, these effects were achieved without the addition of acyl-CoA:cholesterol acyltransferase inhibitor, cAMP, or even high density lipoproteins. Furthermore, the uptake and degradation of acetylated low density lipoprotein was significantly reduced probably by decreased expression of scavenger receptor A and CD36. Notably, the cells with stimulated CE hydrolysis did not exhibit either buildup of free cholesterol or cytotoxicity. In conclusion, increased hydrolysis of CE by the overexpression of HSL leads to complete elimination of CE from THP-1 foam cells not only by increasing efflux but also by decreasing influx of cholesterol.

Cholesterol ester (CE)[1]-laden macrophage foam cells are a hallmark of fatty streak lesions in atherosclerotic plaques.

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[1] The abbreviations used are: CE, cholesterol ester; TG, triacylglycerol; FC, free cholesterol; PBS, phosphate-buffered saline; SR, scavenger receptor; ACAT, acyl-CoA:cholesterol acyltransferase; NCEH, neutral cholesterol ester hydrolase; ABCA1, ATP-binding cassette

Besides cleaning up extracellularly deposited atherogenic lipoproteins, macrophage foam cells secrete a wide variety of substances, which include inflammatory cytokines, chemokines, growth factors, and proteases (1). Furthermore, lipid-rich lesions that are characterized by a plethora of macrophage foam cells are associated with massive infiltration with activated T lymphocytes and prone to rupture, thereby leading to thrombotic coronary occlusion (2). Thus, elimination of CE from foam cells is potentially a promising therapeutic strategy to stabilize rupture-prone atherosclerotic plaques.

Foam cells are generated by the uptake of modified lipoproteins through scavenger receptors, such as scavenger receptor A (SR-A) and CD36 (3). Hydrolysis of the lipoprotein-associated CE by lysosomal acid lipase liberates free cholesterol (FC), which is subsequently re-esterified by acyl-CoA:cholesterol acyltransferase 1 (ACAT1) to form CE for storage in the cytoplasmic lipid droplets (4, 5). The cytoplasmic CE is in turn hydrolyzed by neutral CE hydrolase (NCEH) to generate FC, which is transported to a compartment for re-esterification by ACAT1. The rest of the FC is released out of the cells primarily through ATP-binding cassette transporter A1 (ABCA1) (6, 7). Thus, the balance between synthesis and hydrolysis of CE conceivably governs the level of CE in macrophages.

Inhibition of ACAT activity is shown to suppress CE accumulation with concomitant promotion of the net hydrolysis of CE in mouse peritoneal macrophages (8). Supporting these *in vitro* results, ACAT inhibitors have been reported to suppress atherosclerosis in many animal models (9). Consistently, we have reported that atherosclerotic lesion areas as well as aortic CE contents are moderately reduced in mice that are deficient in ACAT1 in the setting of apoE or the LDL receptor deficiency (10). Paradoxically, however, Fazio *et al.* (11) reported that macrophage-specific ablation of ACAT1 aggravated atherosclerotic lesions. It was speculated that inhibition of ACAT was detrimental because of cytotoxicity of excess FC in macrophages, as has been reported in cells treated with ACAT inhibitors (12, 13). Together, it remains unknown whether selective

transporter A1; LDL, low density lipoproteins; HDL, high density lipoproteins; HSL, hormone-sensitive lipase; acLDL, acetylated LDL; BSA, bovine serum albumin; Ad-LacZ, recombinant adenovirus carrying β-galactosidase expression cassette; Ad-HSL, recombinant adenovirus carrying HSL expression cassette; m.o.i., multiplicity of infection; RT, reverse transcriptase; FBS, fetal bovine serum; RPMI, Roswell Park Memorial Institute; apo, apolipoprotein; CYP27, cholesterol 27-hydroxylase; PPAR, peroxisome proliferator-activated receptor; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

inhibition of ACAT1 in macrophages is useful as a therapeutic tool.

The role of CE hydrolysis in the development of atherosclerosis is less clear, because molecular identity of NCEH in macrophages has yet to be determined. Several investigators (14–16) have attributed the NCEH activity in macrophages to hormone-sensitive lipase (HSL), which is a multifunctional enzyme that catalyzes both triacylglycerol (TG) and CE in various organs including adipose tissue and testis (17). This is apparently consistent with the results that treatment with cAMP, which is known to activate HSL, stimulated the CE hydrolysis in macrophages (18, 19). However, cAMP stimulates the expression of ABCA1 (20, 21), another rate-limiting step for cholesterol efflux, making it difficult to conclude that CE hydrolysis and cholesterol efflux are primarily mediated by endogenous HSL. Furthermore, we showed that NCEH activity was not reduced in peritoneal macrophages obtained from HSL knockout mice (22), indicating that another enzyme that is distinct from HSL mediates NCEH activity in macrophages. In this context, it is noteworthy that a new NCEH has been cloned from the human macrophages library (23). Thus, neither molecular identity nor characteristics of NCEHs in macrophages are completely elucidated.

Previously, Escary *et al.* attempted to achieve increased hydrolysis of CE by overexpression of HSL using macrophage-specific enhancer/promoter of SR-A in RAW 264.7 cells (24) and transgenic mice (25). However, they were not successful in obtaining sufficient increases in CE hydrolysis. In the transfected cells, without the addition of ACAT inhibitor, increased hydrolysis of CE was not observed (24). In macrophages from HSL transgenic mice, CE was paradoxically increased when incubated with acetylated LDL (acLDL) *in vitro*, which was ascribed to the compensatory activation of ACAT1 with low level of increase in NCEH activity (25). Thus, it still remains unclear whether stimulation of CE hydrolysis suppresses foam cell formation.

To achieve higher expression of HSL in macrophages, we have used adenovirus-mediated gene delivery. We show here that the overexpression of HSL caused a robust increase in NCEH activity, which accompanied complete elimination of CE from the cells loaded with CE without affecting cell viability in THP-1 macrophages. These results implicate that adenovirus-mediated overexpression of HSL can be used as a therapeutic strategy to regress and stabilize rupture-prone foam cell lesions.

EXPERIMENTAL PROCEDURES

Materials—Phorbol 12-myristate 13-acetate, triolein, lecithin, bovine serum albumin fraction V (BSA), and leupeptin were purchased from Sigma. Cholesterol esterase from *Pseudomonas* sp., horseradish peroxidase, *p*-hydroxyphenylacetic acid, and sodium taurocholate were purchased from Wako Pure Chemicals (Osaka, Japan). Cholesterol oxidase was purchased from Roche Molecular Biochemicals. Tri[³H]oleoylglycerol and cholesterol [1-¹⁴C]oleate, Na¹²⁵I, [*cholesteryl*-1,2,6,7-³H]cholesteryl linoleate, [*cholesteryl*-1,2,6,7-³H]cholesteryl oleate, [1-¹⁴C]oleic acid, and [1-¹⁴C]oleoyl-CoA were purchased from Applied Biosystems (Foster City, CA). MTT assay kit was purchased from Chemicon International, Inc. (Temecula, CA). Adenovirus plasmid carrying β-galactosidase (pAd-LacZ) was described previously (26).

Construction of Recombinant Adenoviruses—Recombinant adenovirus that carried murine HSL cDNA under the control of cytomegalovirus promoter, designated as Ad-HSL, was constructed using the cDNA cloned by reverse transcriptase-PCR (RT-PCR) from mouse white adipose tissue (22) as described previously (27). The recombinant adenoviruses were expanded in HEK293 cells and purified by cesium chloride ultracentrifugation. The purified viruses were stored in 10% (v/v) glycerol/phosphate-buffered saline (PBS) at −80 °C. In our preparations, 1 multiplicity of infection (m.o.i.) corresponded to 25 particles of adenovirus per cell, and cells were infected at 100 m.o.i., unless otherwise stated.

Preparation of Lipoproteins—After an overnight fast, blood was collected from normolipidemic volunteers to isolate plasma. LDL (*d* 1.019–1.063 g/ml) and high density lipoproteins (HDL) (*d* 1.063–1.21 g/ml) were isolated from the plasma by sequential density ultracentrifugation (28). LDL was acetylated by repetitive additions of acetic anhydride (29). acLDL was radioiodinated with Na¹²⁵I by the iodine monochloride method (30). CE in acLDL was reconstituted with [*cholesteryl*-1,2,6,7-³H]cholesteryl linoleate as described previously (31).

Cell Culture—THP-1 cells were cultured in RPMI-1640 containing 10% (v/v) FBS and differentiated to THP-1 macrophages by the treatment with 100 nmol/liter phorbol 12-myristate 13-acetate for 48 h. P388D1 cells were cultured in RPMI 1640 containing 10% (v/v) FBS. J774, Raw 264.7, and HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) FBS. Mouse peritoneal macrophages were harvested as described previously (22). Mononuclear cells, which were isolated from peripheral blood of healthy donors using Lymphoprep (NYCOMED, Roskilde, Denmark) and adhered to plastic dishes, were used as human monocytes.

Cells were loaded with cholesterol by incubation with RPMI 1640 containing 100 µg/ml acLDL and 5 mg/ml BSA for 24 h at 37 °C. After transduction with Ad-HSL or Ad-LacZ for 72 h at 37 °C in RPMI containing 5 mg/ml BSA and 100 µg/ml acLDL, cells were harvested.

Adenovirus Transduction Efficiency—Cells were plated at 0.5×10^6 cells per well in 12-well tissue culture plates and infected with Ad-LacZ at 100 m.o.i. On day 3, β-galactosidase activity in the cellular proteins was measured by standard kits (Promega, Madison, WI). Cellular protein was determined by BCA protein assay (Pierce).

Western Blot Analysis—Cells were sonicated in buffer A (50 mmol/liter Tris-HCl, pH 7.0, 250 mmol/liter sucrose, 1 mmol/liter EDTA, 2 µg/ml leupeptin) and centrifuged at $100,000 \times g$ for 45 min at 4 °C. The supernatant was used for Western blot analysis as described previously (22) using an anti-HSL antibody that was raised according to the described method (32).

NCEH and TG Lipase Activities—Cells were sonicated in buffer A and centrifuged at $100,000 \times g$ for 45 min at 4 °C. The supernatant was used for the enzyme assay. NCEH and TG lipase activities were measured as described previously (22).

Cholesterol Determination—Cellular lipids were extracted by hexane/isopropyl alcohol, and cholesterol contents were determined by enzymatic fluorometric microassay according to the method of Heider and Boyett (33), with minor modifications (10).

Oil Red O Staining—THP-1 macrophages were plated in 4-chamber plates at 0.5×10^6 cells per well and treated with acLDL and recombinant adenovirus, as described above. Cells were washed twice with PBS, fixed with 3% (w/v) paraformaldehyde in PBS, and stained with Oil Red O in 60% (v/v) isopropyl alcohol, and hematoxylin.

MTT Assay—Colorimetric MTT assay for cell survival and proliferation was performed following manufacturer's protocol.

CE Formation—CE formation from [1-¹⁴C]oleic acid was determined as described previously (34) with minor modifications.

Microsomal ACAT Activity—Cells were sonicated in buffer A and centrifuged at $100,000 \times g$ for 45 min at 4 °C. The precipitates were resuspended and used for the assay. ACAT activity in microsomes was determined by the rate of incorporation of [1-¹⁴C]oleoyl-CoA into the CE fraction according to Yagyu *et al.* (10).

Cholesterol Efflux—Cholesterol efflux assays were performed as described previously (31). Cells were incubated for 24 h at 37 °C in culture media containing 5 mg/ml BSA and 100 µg/ml acLDL whose CE was reconstituted with [*cholesteryl*-1,2,6,7-³H]cholesteryl linoleate. Cells were then infected with recombinant adenoviruses at indicated m.o.i. at 37 °C for additional 72 h in RPMI 1640 containing 5 mg/ml BSA and 100 µg/ml acLDL in the presence or absence of 250 µg/ml HDL. An aliquot of the medium was removed and centrifuged at $15,000 \times g$ for 2 min, and the radioactivity in the supernatant was determined by a liquid scintillation counter. Total cell-associated radioactivity was determined after dissolving the cells at time 0 in 0.1 N NaOH. Cholesterol efflux of radioactive cholesterol from the cells into the medium was determined as a percentage to the total radioactivity in the cells at time 0.

Uptake and Degradation of Lipoproteins—Cells were incubated in RPMI containing 5 mg/ml BSA and ¹²⁵I-labeled acLDL at the indicated concentrations at 37 °C for 6 h. The amounts of ¹²⁵I-labeled acLDL associated with or degraded by the cells were determined as described (30). Nonspecific values were determined by adding a 50-fold excess of unlabeled acLDL. Specific values were calculated by subtracting the nonspecific value from the total value.

Northern Blot Analyses—Five µg of total RNA, which was isolated from the cells by TRIzol reagent (Invitrogen), was used for Northern blot analysis as described (22). Probes for human adipophilin, CD36,

SRBI, SR-A, ABCA1, human ABCG1, human apoE, and cholesterol 27-hydroxylase (CYP27) were constructed from cDNA fragments amplified by RT-PCR using cDNA obtained from THP-1 monocyte/macrophages as a template. Probes for murine adipocyte lipid-binding protein (aP2) and HSL (exon 8 probe) were constructed from cDNA fragments amplified by RT-PCR using cDNA obtained from mouse adipose tissue as a template (22).

Statistical Analyses—Results are presented as means ± S.D. Student's *t* test was employed to compare the means. All calculations were performed with STAT view version 5.0 for Macintosh (SAS Institute Inc.).

RESULTS

To identify macrophages or macrophage-like cell lines that were effectively transduced with recombinant adenovirus, we compared the expression of LacZ in macrophage-like cell lines (THP-1, J774, RAW 264.7, and P388D1), primary cultures of macrophages such as murine peritoneal macrophages and human monocyte-derived macrophages, and HEK293 cells, after infection with Ad-LacZ. Compared with HEK293 cells, macrophage-like cells expressed extremely low activity of LacZ as follows: THP-1, 0.71%; J774, 0.01%; RAW 264.7, <0.01%. LacZ activity was undetectable in the following cells: P388D1, murine peritoneal macrophages, and human monocyte-derived macrophages. Among these cells, THP-1 macrophages were transduced with adenovirus most effectively. Thus, we used 30, 100, and 300 m.o.i., which correspond to 0.21, 0.71, and 2.13 copies of the virus infected into a THP-1 cell, respectively.

To verify the expression of exogenous HSL after the infection with Ad-HSL, we performed Western blot analysis and measured activities for NCEH and TG lipase in the cells (Fig. 1). HSL protein was expressed in THP-1 macrophages infected with Ad-HSL in a dose-dependent manner but not in cells infected with Ad-LacZ (Fig. 1*A*). In parallel, NCEH activity was robustly stimulated after infection with Ad-HSL in cells treated with acLDL in a dose-dependent manner; 120-fold increase was observed at 300 m.o.i. (Fig. 1*B*). Similarly, TG lipase activity was stimulated after infection with Ad-HSL in cells treated with acLDL in a dose-dependent manner; 4-fold increase was observed at 300 m.o.i. (Fig. 1*C*). Fold increase was apparently more prominent in NCEH activity than in TG lipase activity, because basal level of endogenous NCEH activity was much lower than that of endogenous TG lipase activity.

To examine whether the increased NCEH activity hydrolyzes cellular CE, we measured CE and FC contents in THP-1 macrophages infected with various doses of Ad-LacZ or Ad-HSL (Fig. 2). Incubation with acLDL increased both CE and FC in the cells. The net amount of these lipids was not decreased by the addition of HDL, which is a physiological acceptor of FC that is released from cells and conceivably stimulates the efflux of FC from the cells to the medium (Fig. 2, compare *A* with *C* and *B* with *D*). Infection with Ad-LacZ did not change the contents of either CE or FC. In contrast, infection with Ad-HSL significantly decreased the CE contents in a dose-dependent manner; CE was almost undetectable at 300 m.o.i. Importantly, the elimination of CE did not accompany an increase in FC. HDL had no significant effect on the CE contents (Fig. 2, compare *A* with *C*).

In agreement with these results, Oil Red O staining revealed that the number and size of intracellular lipid droplets, which were increased by incubation with acLDL, were remarkably reduced by the infection with Ad-HSL but not by the infection with Ad-LacZ (Fig. 3*A*). To rule out the possibility that the overexpression of HSL is cytotoxic and thereby decreases the lipid accumulation, we performed MTT assay (Fig. 3*B*). Incubation with acLDL increased MTT activity by 51%. Infection with either Ad-LacZ or Ad-HSL decreased MTT activity by 41 and 33%, respectively; there was no
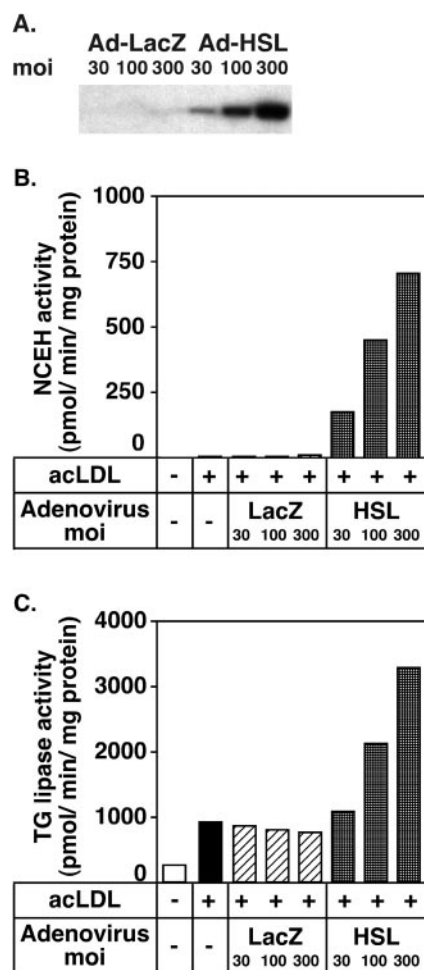

FIG. 1. **Expression of HSL protein (*A*), NCEH (*B*), and TG lipase activities (*C*) in THP-1 macrophages infected with recombinant adenoviruses.** THP-1 macrophages were infected with Ad-HSL or Ad-LacZ at the indicated m.o.i. On day 3 after the infection, 10 μg of cytosolic proteins were subjected to Western blot analysis (*A*). THP-1 macrophages were infected with Ad-HSL or Ad-LacZ at the indicated m.o.i. 24 h after incubation with acLDL. On day 3 after the infection, cytosolic proteins were extracted for the measurements of NCEH activity (*B*) and TG lipase activity (*C*). Data are expressed as means of duplicate measurements.

significant difference between the cells infected with two viruses.

To determine how Ad-HSL decreased cellular CE contents, we compared CE formation from [^{14}C]oleic acid between Ad-LacZ and Ad-HSL (Fig. 4*A*). The CE formation, which was stimulated by acLDL, was substantially inhibited by the infection with Ad-HSL but not by Ad-LacZ. It is of note that these results were nearly identical to those on CE contents. To determine whether the decreased CE formation resulted from decreased ACAT activity, we measured microsomal ACAT activities (Fig. 4*B*). Incubation with acLDL stimulated microsomal ACAT activity. Neither Ad-HSL nor Ad-LacZ reduced the activities.

Because FC contents were not increased despite the increased hydrolysis of CE, we hypothesized that FC, which is generated by the breakdown of CE, was effectively transported out of the cells to the media. To verify the possibility, we measured the amounts of FC released from the cells to the media (cholesterol efflux) (Fig. 5, *A* and *B*). Ad-HSL stimulated the cholesterol efflux in a dose-dependent manner. HDL increased the cholesterol efflux by 50% (compare Fig. 5, *A* and *B*).

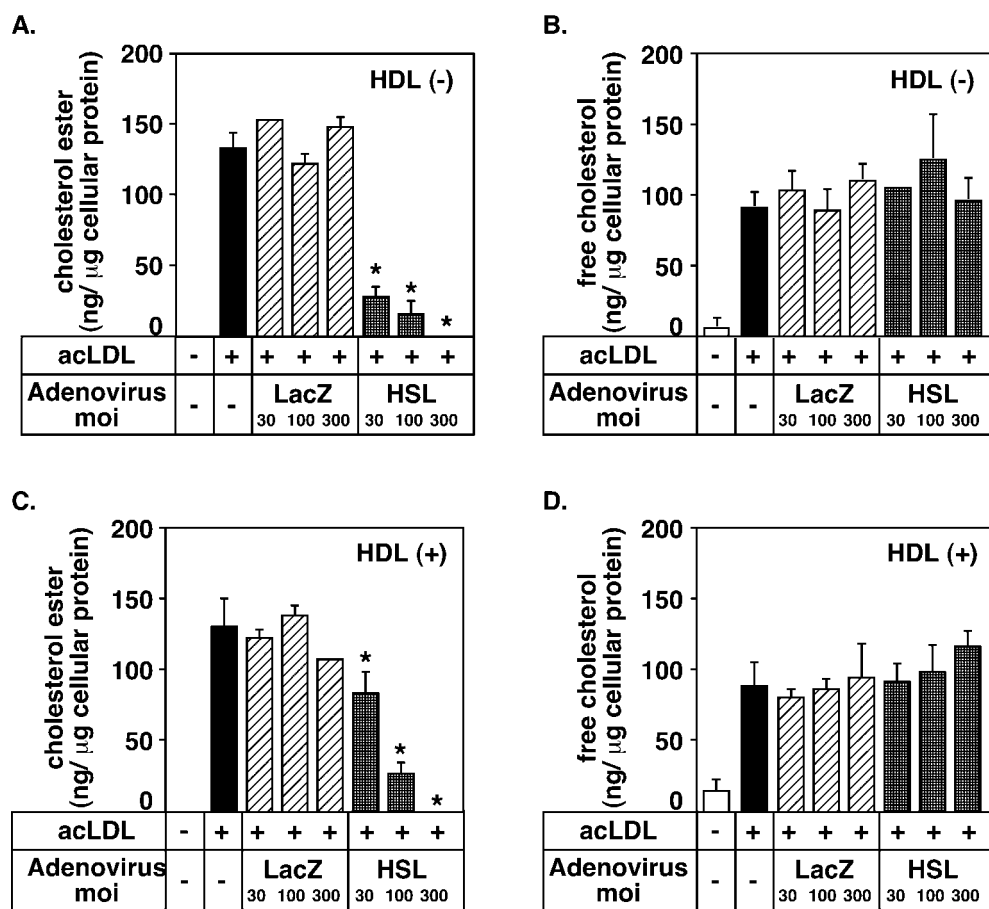Suppression of the uptake of acLDL may explain the de-

FIG. 2. **Cellular contents of CE (*A* and *C*) and FC (*B* and *D*) in THP-1 macrophages infected with recombinant adenoviruses in the absence (*A* and *B*) and presence (*C* and *D*) of HDL in the medium.** Twenty four h after the incubation with acLDL, THP-1 macrophages were infected with recombinant adenoviruses, with (*C* and *D*) or without (*A* and *B*) the addition of 250 µg/ml HDL. On day 3 after infection, intracellular contents of CE (*A* and *C*) and FC (*B* and *D*) were measured by an enzymatic fluorometric microassay. Data are expressed as means ± S.D. of triplicate wells and are representative of four independent experiments. *, $p < 0.001$, Ad-HSL *versus* Ad-LacZ.

crease in CE content of Ad-HSL-infected cells. To test this possibility, we measured the amounts of ^{125}I-labeled acLDL associated with or degraded by the cells infected with the viruses (Fig. 5, *C* and *D*). Even the infection with Ad-LacZ reduced the amounts of ^{125}I-labeled acLDL degraded by or associated with the cells by 2-fold. The infection with Ad-HSL further decreased the uptake and degradation by 30.2 and 50.8%, respectively.

To investigate the changes in the expression of genes that govern cholesterol trafficking in the cells, we have performed Northern blot analyses (Fig. 6). The overexpression of HSL accompanied an ~2-fold increase in the mRNA expression of ABCA1 but not the expression of other genes involved in cholesterol efflux, such as *ABCG1, apoE,* and *CYP27*. Apparently consistent with the reduced uptake and degradation of acLDL, the mRNA expression of the members of scavenger receptor family such as SR-A, CD36, and SRBI was decreased by ~2-fold in the cells infected with Ad-HSL. Finally, we found no changes in the mRNA expression of the molecules whose expression was reported to be increased in response to oxidized LDL, such as adipophilin (35) and adipocyte lipid-binding protein (36).

DISCUSSION

Foam cell formation, accumulation of CE in monocyte/macrophages, is an initial step in the development of atherosclerotic lesions. Thus, elimination of CE from foam cells would be of considerable therapeutic benefit. This study was designed to determine whether the hydrolysis of CE is the rate-limiting

step in the removal of CE from foam cells. We show here that overexpression of HSL leads to the complete elimination of CE from THP-1 macrophage foam cells without affecting cellular viability. These changes are associated not only with an increase in cholesterol efflux but also with a decrease in lipoprotein uptake, which may effectively protect foam cells from excessive accumulation of FC.

Previously, Escary *et al.* (24) attempted to overexpress HSL in macrophages. They took advantage of plasmid transfection to RAW 264.7 macrophage-like cells (24) as well as transgenic mice using SR-A enhancer/promoter (25). Unfortunately, however, the levels of the expression were relatively low (5-fold in *in vitro* study, and 7-fold in *in vivo* study), and did not sufficiently overcome the compensatory activation of endogenous ACAT activity (2-fold increase in both studies). Indeed, the macrophages isolated from the transgenic mice showed paradoxical accumulation of CE when incubated with acLDL. Similar paradoxical worsening of atherosclerotic lesions was observed in the transgenic mice. In this regard, it is noteworthy that our study was the first to achieve a significant increase in the hydrolysis of CE in macrophages by virtue of adenovirus-mediated gene delivery (Fig. 1), which allowed us to investigate the role of CE hydrolysis in foam cell formation without the need for concomitant addition of ACAT inhibitors and/or cAMP analogues, which were required to demonstrate the increased CE hydrolysis in the studies of Escary *et al.* (24, 25).

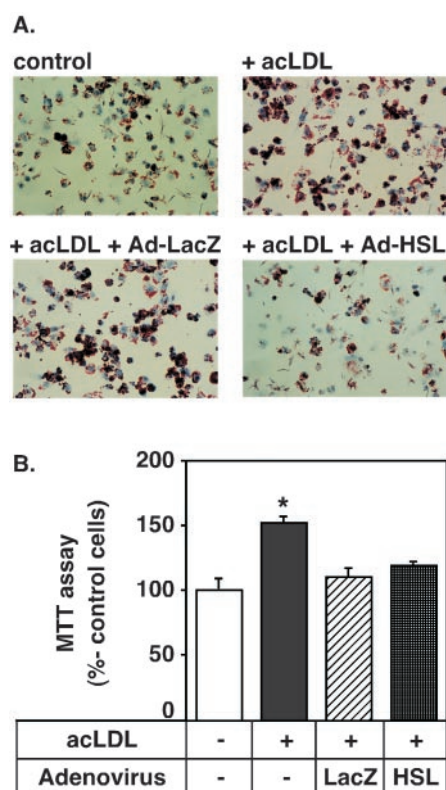The resultant increased activity of NCEH led to complete elimination of CE accumulated in THP-1 macrophages that

FIG. 3. **Accumulation of lipid droplets (*A*) and cell viability (*B*) of THP-1 macrophages infected with recombinant adenoviruses.** Twenty four h after the incubation with acLDL, THP-1 macrophages were infected with recombinant adenoviruses and were incubated for the following 72 h. *A*, cells were fixed and stained with Oil Red O and hematoxylin (×100 magnification). The number of cells was ~100 for each group. *B*, MTT assay was performed to assess cell viability. Data are expressed as percentages to control cells. Data are expressed as means of triplicate wells and representative of three experiments. *, $p < 0.005$, compared with other groups.

had been preloaded with CE by incubation with acLDL (Fig. 2). The elimination of CE was not caused by inhibition of cholesterol esterification, because microsomal ACAT activity was not decreased (Fig. 4). The suppression of CE formation from [^{14}C]oleic acid (Fig. 4) could be explained not only by the increased efflux (Fig. 5, *A* and *B*) but also by the decreased influx of cholesterol (Fig. 5, *C* and *D*).

It is noteworthy that FC contents were not increased in these cells overexpressing HSL (Fig. 2). FC that was generated by the hydrolysis of CE might be effectively removed out of the cells by the increase in cholesterol efflux (Fig. 5), indicating that CE hydrolysis is the rate-limiting step in foam cell formation of THP-1 macrophages. In comparison with murine macrophages such as mouse peritoneal macrophages and J774.2 cells, human macrophages including THP-1 macrophages are reportedly resistant to cholesterol efflux; neither cAMP nor the addition of HDL enhanced the cholesterol efflux (37, 38). This particular nature of THP-1 macrophages has been ascribed to the low activity of endogenous NCEH, which may in part account for our success in obtaining the significant increase in NCEH activity above the endogenous level after the transduction with Ad-HSL. The increased cholesterol efflux was at least partly in agreement with the up-regulation of the expression of ABCA1, which is a key transporter facilitating cellular cholesterol efflux (7) (Fig. 6). Hydrolysis of CE generates cholesterol and fatty acids, both of which conceivably stimulate the expression of ABCA1, because fatty acids, their oxidized forms, and oxysterols might activate PPARα, PPARγ, and liver X receptor α, respectively, which increase the expression of ABCA1 in turn (7).
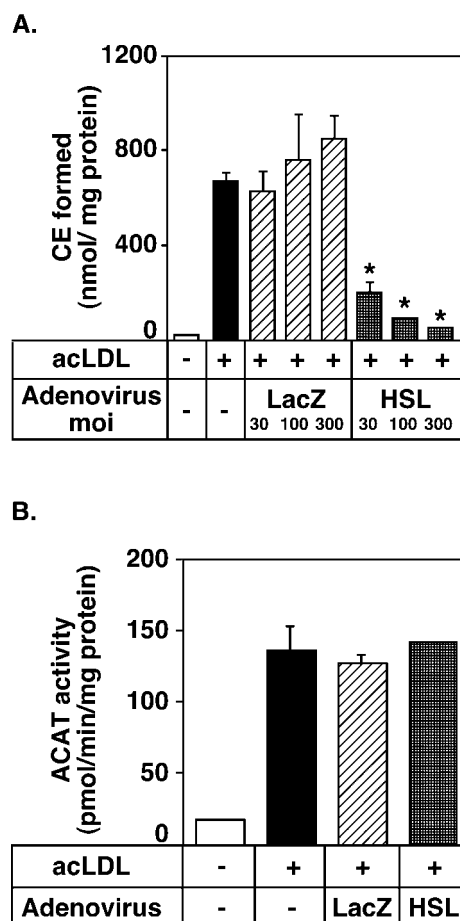

FIG. 4. **CE formation from [^{14}C]oleic acid (*A*) and microsomal ACAT activity (*B*) in THP-1 macrophages infected with recombinant adenoviruses.** *A*, 24 h after the incubation with acLDL and 1% (w/v) [^{14}C]oleate-BSA complex, THP-1 macrophages were infected with recombinant adenovirus at the indicated m.o.i. and incubated for the following 72 h. Radioactivity of intracellular CE formed from [^{14}C]oleate was determined. Data are expressed as means ± S.D. of triplicate wells and are representative of four independent experiments. *, $p < 0.005$, Ad-HSL *versus* Ad-LacZ. *B*, microsomal protein was extracted from the cells and used for measurement of microsomal ACAT activity. Data are expressed as means ± S.D. of triplicate assays.

It is important to note that cholesterol efflux was increased even in the absence of HDL in the medium (Fig. 5). Thus, the increased hydrolysis of CE stimulated cholesterol efflux via a pathway that is distinct from ABCA1, which requires HDL, in addition to cholesterol efflux via the ABCA1-mediated pathway. Thus far, it has been reported that the HDL-independent efflux of cholesterol is mediated either by apoE (39) or by CYP27, an enzyme that catalyzes hydroxylation of cholesterol to form 27-hydroxycholesterol for elimination as bile acids from the liver (40, 41). Recently, PPARδ has been shown to regulate the expression of CYP27 at the transcriptional level (42). Therefore, it is tempting to speculate that FC released by the CE hydrolysis might be directed to a distinct subcellular compartment where FC is preferentially metabolized by this enzyme, thus facilitating cholesterol efflux. In this context, it is interesting to note that inhibition of ACAT results in accumulation of FC in endoplasmic reticulum, not in cytosol (43).

Increased cholesterol efflux should have a therapeutic benefit, because accumulation of excess FC might be toxic to the cells. For example, many investigators (12, 13) have reported the cytotoxicity of ACAT inhibition in macrophages, whereas there are some who disagree (37). In this context, it is noteworthy that the LDL receptor knockout mice whose bone marrow
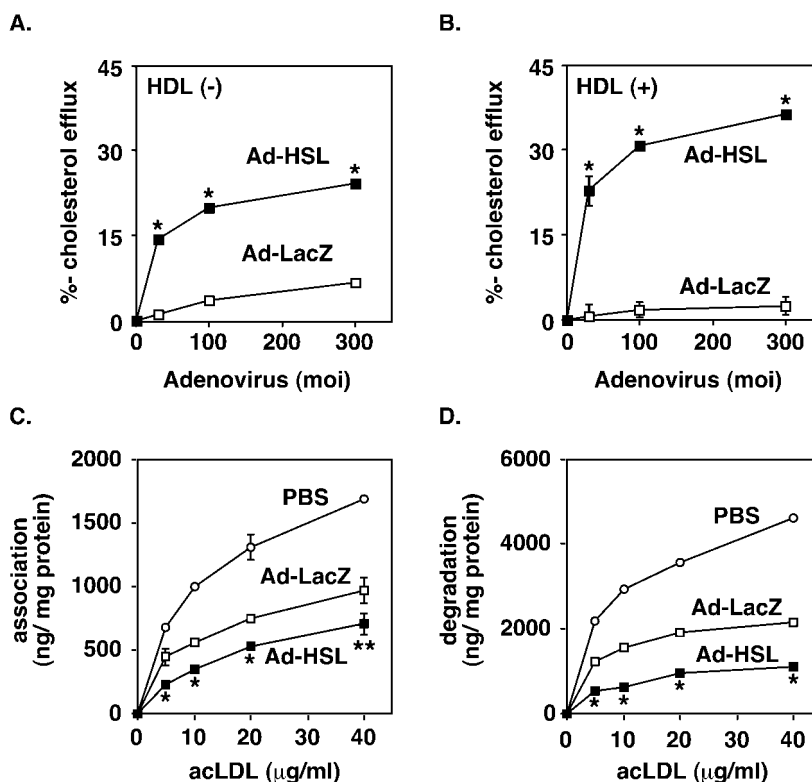
FIG. 5. **Stimulation of cholesterol efflux in the absence (*A*) and presence (*B*) of HDL in the media, and specific uptake (*C*) and degradation (*D*) of ¹²⁵I-labeled acLDL in THP-1 macrophages infected with recombinant adenoviruses.** THP-1 macrophages were incubated with 100 µg/ml acLDL whose CE was reconstituted with [*cholesteryl*-1,2,6,7-³H]cholesteryl linoleate. After 24 h, the cells were washed, infected with recombinant adenoviruses at the indicated m.o.i., and incubated for the following 72 h, in the absence (*A*) or presence of HDL (*B*). Radioactivities in the medium and cells were measured. Cholesterol efflux to the medium was determined as a percentage to the total radioactivity in the cells at time 0. The values for the non-infected cells were subtracted from those for the infected cells to obtain cholesterol efflux that was specifically stimulated by the infection with adenoviruses. Data are expressed as means ± S.D. of triplicate wells. *, $p < 0.005$, Ad-HSL *versus* Ad-LacZ. Twenty four h after the incubation with acLDL, THP-1 macrophages were infected with recombinant adenoviruses and incubated for the following 72 h. On day 3 after the infection, cells were washed extensively and incubated with ¹²⁵I-labeled acLDL at the indicated concentrations at 37 °C for 6 h. The amounts of ¹²⁵I-labeled acLDL associated with (*C*) and degraded by (*D*) the cells were determined. Nonspecific values were obtained by adding a 50-fold excess of unlabeled acLDL. Specific values were calculated by subtracting the nonspecific value from the total value. Data are expressed as means ± S.D. of triplicate wells. *, $p < 0.005$, and **, $p < 0.05$, Ad-HSL *versus* Ad-LacZ.
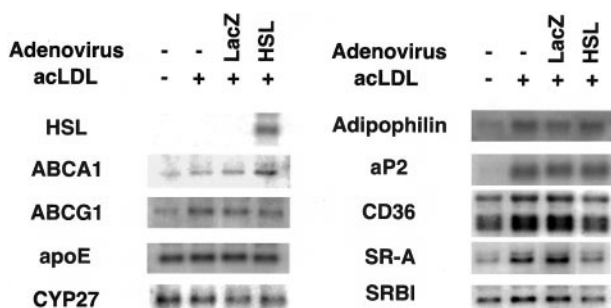

FIG. 6. **Northern blot analyses for genes involved in cholesterol trafficking in THP-1 macrophages infected with recombinant adenoviruses.** Twenty four h after the incubation with acLDL, THP-1 macrophages were infected with recombinant adenoviruses. On day 3 after the infection, 5 µg of total RNA isolated from the cells was subjected to Northern blot analyses.

was repopulated with that from ACAT1-deficient mice developed more severe atherosclerosis than the LDL receptor knockout mice (11), which was ascribed to potential cytotoxic effects of FC accumulated in macrophages due to ACAT inhibition. We did not observe either FC accumulation or cytotoxicity in THP-1 cells overexpressing HSL (Figs. 2 and 3).

Unexpectedly, we found that THP-1 macrophages overexpressing HSL showed reduced ability to take up and degrade acLDL (Fig. 5). In agreement with this, the expression of SR-A, CD36, and SRBI was decreased (Fig. 6). SR-A (44) and CD36

(45, 46) are receptors for both acLDL and oxidized LDL; SRBI is a receptor for acLDL as well as for HDL (47). Down-regulation of these proteins may contribute to the decreased uptake of acLDL as observed in our study. Since thiazolidinediones were reported to down-regulate the expression of SR-A (48), it is possible that the HSL overexpression generates oxidized fatty acids, which suppress the expression of SR-A by activating PPARγ. If this is the case, it is difficult to explain the suppression of the expression of CD36, which was reported to be upregulated by the activation of PPARγ (49). Intracellular cholesterol content may play a more central role in the regulation of CD36 than fatty acids (50).

Ghosh (23) has recently reported the cloning of a novel CE hydrolase expressed in macrophages. There may be multiple NCEHs in macrophages: HSL, the CEH cloned by Ghosh (23) and possibly others (22). It is yet to be known which enzyme is predominant in macrophages.

Although the successful elimination of CE from macrophage foam cells was shown only in the artificial system at cellular level, adenovirus-mediated gene transfer of HSL can be applied to the treatment of foam cell lesions in atherosclerotic plaques after solving issues at the *in vivo* level, such as gene delivery, tissue-specific expression, level of expression, side effects, etc.

In summary, we show here that the overexpression of HSL robustly increases NCEH activity and completely eliminates CE in foam cells. In these cells, cholesterol efflux was stimulated in the absence or presence of HDL, which might be at least partially explained by the increase in the expression of

ABCA1. Furthermore, the uptake and degradation of acLDL were significantly reduced probably by decreased expression of scavenger receptors such as SR-A and CD36. These changes in cholesterol trafficking, which are associated with the increased CE hydrolysis, may protect cells from cytotoxic effects of FC buildup. Thus, increased hydrolysis of CE by the overexpression of HSL leads to complete elimination of CE from THP-1 foam cells not only by increasing efflux but also by decreasing influx of cholesterol. Further studies are needed to clarify the mechanisms underlying the changes in cholesterol trafficking in cells with increased hydrolysis of CE.

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