Cholesteryl Ester Efflux from Extracellular and Cellular Elements of the Arterial Wall

Model Systems in Culture With Cholesteryl Linoleyl Ether

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Cholesteryl ester (CE) accretion in human atheroma is a slow process during which lipoproteins infiltrate the arterial extracellular space and then gain entry into the cellular components. The present aim was to simulate this process in model systems in culture to learn whether cholesteryl ester transfer protein (CETP) may promote CE efflux at different stages of atheroma formation. To simulate CE efflux from arterial interstitium, cationized LDL labeled with $^3$H-cholesteryl linoleyl ether ($^3$H-CLE) was added to fixed aortic smooth muscle cells (SMC) or to extracellular matrix. To study efflux of $^3$H-CLE taken up by cells via receptor-mediated endocytosis of LDL, the SMC cultures were fixed and permeabilized prior to the determination of CE efflux. The cellular model included macrophages, which had ingested acetylated LDL labeled with $^3$H-CLE. Efflux of $^3$H-CLE and $^{14}$C-CE was studied during postincubation of the labeled cultures with human lipoprotein deficient serum (LPDS) or partially purified CETP. As controls, we used SMC cultures incubated with albumin. In all systems, a 3- to 12-fold increase of $^3$H-CLE or $^{14}$C-CE efflux was found in the postincubation medium containing human LPDS or partially purified CETP when compared to controls. Permeabilization of the cells with saponin enhanced cellular $^3$H-CLE and $^{14}$C-CE efflux in the presence of human LPDS. The findings indicate that CETP may promote CE efflux from aortic interstitium or disintegrating cells. We propose that CETP may play an important role in aortic CE homeostasis under physiological and pathological conditions. (Arteriosclerosis 6:70-78, January/February 1986)

Accumulation of cholesteryl ester (CE) in the arterial wall is among the prominent features of atherosclerosis. It appears$^1$-$^3$ that plasma lipoproteins are the main source of the CE which can be located both in intracellular and extracellular compartments of the artery.$^4$$^5$ This accumulation of the esterified cholesterol reflects an imbalance between inflow and outflow, and the mechanism of the latter has not been elucidated. Efflux of cholesterol from the artery could occur in the form of free cholesterol after intracellular hydrolysis of ingested lipoprotein CE. Alternately, one could envisage the efflux of whole lipoprotein particles or, as another possibility, the mediation of CE efflux by cholesteryl ester transfer protein (CETP).

In a recent study,$^6$ we described a model system in culture to investigate the putative role of CETP in promoting CE efflux from an extracellular compartment. In that study, we used CE and cholesteryl linoleyl ether (CLE) incorporated in trace amounts into unilamellar liposomes. Presently we report results obtained in several model systems in culture using both lipoprotein and liposome CE. We designed these models to mimic the various compartments within the aortic wall in which CE accumulates during the development of atheroma and from which it could be amenable to removal during regression of atherosclerosis.
Methods

Cell Cultures

Bovine aortic smooth muscle cells (SMC) were prepared from explants of bovine aortas.7 The cells were cultured in modified Dulbecco-Vogt medium containing 5% fetal bovine serum and 5% newborn calf serum. The cells were seeded in 35 mm petri culture dishes (4·10^4 cells/dish) and grown for 5 to 7 days until completely confluent. Human skin fibroblasts were cultured in Eagle's Minimal Essential Medium supplemented with 10% bovine fetal serum.8 The macrophage cell lines J774 and its CT2 variant were cultured as described before.9

Isolation, Labeling, and Modification of Lipoproteins

Low density lipoprotein (LDL) was isolated from human plasma containing 0.1% of EDTA by ultracentrifugation at d = 1.019 to 1.063 g/ml at 190,000 g for 24 hours at 4°C and washed once by refloation at the same density. Human and rat lipoprotein-deficient serums (LPDS) were prepared from plasma after removal of lipoproteins at d = 1.21 or 1.25 g/ml at 192,000 g for 48 hours. The LPDS were routinely heated at 56°C for 30 minutes to inactivate LCAT. LDL was labeled with 3H-CLE or 14C-cholesteryl linolate (CL) by transfer from sonicated dispersions of Intralipid. Trace amounts of 3H-CLE and 14C-CL were dried in a glass tube and sonicated twice for 30 seconds in 4 ml 0.9% NaCl containing Intralipid, 125 μg triacylglycerol. The Intralipid had been washed by refloation in a three-layered discontinuous sucrose gradient to reduce the surplus of phospholipid.8 The sonicate was then incubated under sterile conditions with 5 mg protein of LDL and human LPDS to give 30 mg protein/ml. After 18 hours at 37°C under N2, the incubation mixture was brought to d = 1.019 g/ml with solid KBr and centrifuged for 5 hours at 190,000 g in a SW41 rotor. The upper 1 ml containing the Intralipid was removed with the help of a tube slicer. The clear subnatant was brought to d = 1.063 g/ml, and the LDL was isolated by centrifugation at 190,000 g for 24 hours. The yield of the label obtained in several experiments ranged between 30% and 40%, and the lipid composition of the labeled LDL was not significantly different from the parent LDL. Under these conditions, i.e., 125 μg triacylglycerol and 5 mg of LDL protein, the transfer of 14C-triolein from the Intralipid to LDL was negligible, <2%. The labeled LDL was used as such, or modified by acetylation10 or cationized11 as described previously.8,12

Preparation of Labeled Compounds and Liposomes

Synthesis of 3H-CLE was carried out as described previously.13 The 14C-CE was synthesized according to the method of Goodman.14 Nonradioactive 1,2-dioleyl ether-sn-glycero-3-phosphocholine (DOEPC) and DOEPC 14C-choline were synthesized as detailed previously.15 Unilamellar liposomes were prepared from DOEPC as described before.16 Briefly, 2.66 μmol of phospholipid and trace amounts of 3H-CLE or 14C-CL in 4 ml of 0.15 M NaCl were subjected to ultrasonic irradiation for five periods of 1 minute each at room temperature under N2, by using the Braun-Sonic 3000 instrument at 100 W output and a 9 mm probe. Liposomes were prepared also from DOEPC 14C-choline. The clear-to-opalescent sonicate was diluted with 10 ml F10 culture medium containing 4% fatty acid poor bovine serum albumin (BSA), left overnight at 4°C, and subjected to ultracentrifugation in a SW41 rotor at 190,000 g for 3 hours. The clear infranatant was aspirated and sterile-filtered through a 0.45 μ Millipore filter. Recovery of the labeled phospholipid was determined by measuring of lipid phosphorus.17

Partial Purification of Cholesteryl Ester Transfer Protein

Intralipid particles were purified by ultracentrifugal flotation in a three-layered, discontinuous sucrose gradient, according to the method of Erkelens et al.18 The freshly prepared Intralipid was added to the d > 1.21 g/ml fraction of human plasma (heated for 30 minutes at 56°C) in order to adsorb the CETP. The volumes used, time of incubation and ultracentrifugation, and desorption of the CETP were according to the method of Albers et al.19 except that the Intralipid CETP complex that was separated by ultracentrifugation was diluted again with Tris buffer containing 10% sucrose (5 ml), overlayed with Tris buffer (5 ml), and centrifuged under the same conditions. This step raised the specific activity of the partially purified CETP markedly. The partially purified CETP was compared to the original d > 1.21 g/ml plasma fraction on the basis of protein content.

Experimental Design

Experiments with Cationized LDL

The SMC were grown for 5 days and used when fully confluent. The medium was removed, the cell layer was washed twice with phosphate-buffered saline (PBS), and then formaldehyde 0.2% or 1% in PBS was added and fixation was carried out for 15 minutes at room temperature. After removal of the fixative, the cell layer was washed twice with 0.1 M glycine in PBS (pH 7.4) and incubated in serum-free medium containing 0.1 M glycine to quench residual formaldehyde. Cationized LDL, 25 to 60 μg protein labeled with 3H-CLE and with 14C-CL, was added to 1 ml medium without serum containing 1% BSA, and the cells were incubated for 2 or 24 hours at 37°C. During that time, the cationized protein became bound to the cell surface and to the extracellular matrix. Thereafter, the medium was removed, the cell layer was washed twice with PBS and then incubated in serum-free medium containing 1% BSA at
37°C for 24 hours, to remove loosely attached lipoprotein. To study the efflux of 3H-CLE and 14C-CL, the cells were then incubated in medium containing either 1% BSA alone or supplemented with LPDS from rat or human plasma or partially purified CETP isolated from human plasma. In these experiments, we also used petri dishes coated with matrix, which were a gift of Israel Vlodavsky, Hadassah University Hospital. The preparation of the matrix has been described in detail in the treatment of cultures of corneal endothelial cells with 0.5% Triton X-100 (vol/vol). Matrix-coated dishes were carried through the same protocol as the cell-containing dishes. In some experiments, labeled cationized LDL was added to empty culture dishes.

The efflux experiments were carried out for 24 hours. The amount of radioactivity recovered in the culture medium was expressed as the percentage of pulse value. The latter was defined as the amount of radioactivity present in the petri dish after 2 or 24 hours of labeling followed by the 24-hour wash in serum-free medium. After removal of the medium, the cell layer was scraped with methanol, and after the addition of chloroform, the lipids were extracted and the amount of radioactivity was determined.

**Experiments with LDL Labeled with 3H-CLE or 14C-CL**

Uregulation of the LDL receptor was carried out by exposure of the SMC to medium containing d > 1.25 g/ml fraction of serum, 10 mg protein/ml for 48 or 72 hours. In some experiments, 100 μM chloroquine was added 2 hours before labeling in order to ensure sequestration of the labeled CE in the lysosomal compartment. Labeling of the cells was carried out by incubation in medium containing 1% BSA and 3H-CLE- or 14C-CL-labeled LDL, 20 to 40 μg protein/ml, for 24 hours at 37°C.

After the labeling period, the medium was removed and the cell layer was washed three times with PBS. At that stage, triplicate control dishes, which were designed to represent “pulse value,” were terminated by releasing of the cells from the petri dish with trypsin. Following inactivation of the trypsin with serum-containing medium, the cells were pelleted by centrifugation, and lipid radioactivity was extracted with chloroform/methanol 1:1 (vol/vol). The sum of radioactivity recovered in the cell pellet and in the trypsin supernatant was designated “pulse value” and served as 100% for the calculation of 3H and 14C-efflux in the experimental dishes. Cholesterol ester, free cholesterol, and CLE efflux in the experimental dishes were studied after the treatment of the cells, as detailed below. To stop metabolic processes, all cells were fixed with 1% formaldehyde for 15 minutes, washed twice with 0.1 M glycine in PBS, and incubated in serum-free medium containing 0.1 M glycine at 37°C for 30 minutes, to quench formaldehyde. In each experiment, part of the dishes was also permeabilized by incubation of the fixed cells with 0.5% saponin in PBS for 15 minutes at room temperature. The saponin treatment was introduced after the first 0.1 M glycine wash, was followed by an additional glycine wash, and then by a 30-minute wash with serum-free medium.

Efflux was determined during a 24-hour incubation of the fixed and, where appropriate, permeabilized cells. The efflux medium consisted of culture medium containing 1% BSA and the appropriate additions. The amount of efflux was determined in the culture medium in which the lipid radioactivity was analyzed (see below) to determine free and esterified cholesterol. The efflux was expressed as the percentage of pulse value as defined above. In addition, lipid radioactivity was determined on cell pellets, removed from the dish with methanol, and extracted with chloroform.

**Experiments with Liposomes**

The liposomes used in this study were prepared from DOEPC and were labeled either with 3H-CLE and 14C-CL, or with 3H-CLE and 14C-DOEPC. The labeling of the cells was carried out by addition of the liposomes (5 μg phospholipid/ml) to medium containing 4% BSA and bovine milk lipoprotein lipase (LPL), 2.5 μg protein/ml. After 24 hours of labeling, the medium was removed, the cell layer was washed with PBS, and the labeled cells were incubated in complete culture medium containing 10% serum for one or two periods of 48 hours; in the latter instance, there was also a change of medium. The purpose of this protocol was to maximally reduce the surface-associated label and to increase the relative proportion of intracellular label. Thereafter, the protocol detailed in the previous section was followed.

**Experiments with Acetylated 3H-CLE LDL and Macrophages**

The macrophages were cultured for 24 hours in serum-containing medium and then labeled for 24 hours with medium containing 30 μg protein/ml of acetylated LDL labeled with 3H-CLE. The pulse value was determined in cells removed from the petri dish with methanol, because the macrophages cannot be detached by trypsin. Other than that, the efflux was determined as detailed previously.

**Chemical and Radiochemical Determinations**

The cells were scraped from the dishes with a Teflon policeman by using 50% and 100% methanol. After the addition of chloroform, the lipids were extracted to give a methanol/chloroform ratio of 1:1 (vol/vol). The residue was pelleted and used for the determination of protein. After chloroform was added to give a methanol/chloroform ratio of 1:2, the lipids were purified according to the method of Folch et al. and used for liquid scintillation counting and for thin-layer chromatography (TLC). The protein was determined according to the method of Lowry et al.
using bovine serum albumin as the standard. Lipid phosphorus was determined according to the method of Bartlett.\textsuperscript{17} For separation of labeled free cholesterol and CE, aliquots of the chloroform extracts were analyzed on silicic acid plates by using ready plastic sheets F1500 (Schleicher and Schuell, Dessel, FRG), with a solvent system of 5% ethyl acetate in chloroform. The compounds were visualized with iodine vapors, and identified with the help of reference standards. Radioactivity was determined with a beta scintillation spectrometer (Tri-Carb 2660, Packard, La Grange, Illinois). The scintillation fluid was 20% Triton X-100, 0.005% 1,4-bis(2-(5-phenyl-oxazolyl) benzene (POPOP), and 0.4% 2,5-diphenyloxazole (PPO) in toluene.

**Materials**

Bovine milk LPL, isolated and purified as described,\textsuperscript{25} was a generous gift of Thomas Olivecrona, Umeå, Sweden. Bovine serum albumin was obtained from Sigma, St. Louis, Missouri. Culture media and sera were obtained from Gibco, Grand Island, New York. All radioactive compounds were purchased from Amersham International, UK. Intrapluid was purchased from A.B. Vitrum, Stockholm, Sweden.

**Results**

The aim of this study was to compare various model systems in culture with respect to the availability of CE for removal by CETP. The models were constructed to mimic various phases of the atheromatous process in the arterial wall.

**Extracellular Model**

In this model, we attempted to simulate removal of CE from arterial extracellular pools. To that end, we used cells fixed with formaldehyde (to prevent endocytosis) and cationized LDL labeled with \(^3\text{H}\)-cholesteryl linoleyl ether (\(^3\text{H}\text{-CLE}) the nonhydrolyzable analogue of CE. Of the added radioactivity, 70% to 80% was recovered in the petri dish after a 24-hour wash in serum-free medium at 37°C. The results presented in Figure 1 are from a representative experiment out of a series of four experiments.

![Figure 1](http://atvb.ahajournals.org/)

**Table 1. Comparison of Efflux of \(^3\text{H}\)-Cholesteryl Linoleyl Ether and \(^14\text{C}\)-Cholesteryl Ester from Cationized Low Density Lipoprotein Bound to Smooth Muscle Cells and Extracellular Matrix**

<table>
<thead>
<tr>
<th>Acceptor in medium</th>
<th>(^3\text{H}\text{-CLE})</th>
<th>(^14\text{C}\text{-CL})</th>
<th>(^3\text{H}\text{-CLE})</th>
<th>(^14\text{C}\text{-CL})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Smooth muscle cells</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>1.1 ± 0.1</td>
<td>1.2 ± 0.1</td>
<td>—</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Human LPDS (8 mg/ml)</td>
<td>4.5 ± 0.4</td>
<td>6.2 ± 0.6</td>
<td>2.3 ± 0.3</td>
<td>3.2 ± 0.4</td>
</tr>
<tr>
<td>Rat LPDS (9 mg/ml)</td>
<td>2.3 ± 0.4</td>
<td>2.2 ± 0.3</td>
<td>0.8 ± 0.1</td>
<td>1.0 ± 0.1</td>
</tr>
</tbody>
</table>

The cells were incubated for 24 hours in 1 ml of culture medium containing 1% albumin and 60 \(\mu\)g of protein-cationized LDL labeled with \(^3\text{H}\text{-CLE}\) and \(^14\text{C}\text{-CL}\) in medium containing 1% albumin for 24 hours. Triplicate control dishes were terminated, and the radioactivity recovered of each label added initially to the culture medium amounted to 70% to 80% and was taken as 100%, i.e., pulse value. Medium containing 1% albumin and where appropriate lipoprotein-deficient serum (LPDS) was added to the experimental dishes. After 24 hours of incubation, the radioactivity recovered in the medium was expressed as a percentage of pulse value. Data are means ± SE of three experiments (\(n = 9\)). In one experiment, labeled cationized LDL was added to empty dishes, and the amount of radioactivity recovered in the presence of human LPDS was 3.0% ± 0.3% for \(^3\text{H}\text{-CLE}\) and 4.7% ± 0.3% for \(^14\text{C}\text{-CL}\); in the presence of rat LPDS, it was 1.0% ± 0.1% and 1.7% ± 0.1% for \(^3\text{H}\) and \(^14\text{C}\), respectively.
periments that compared the efflux of \(^{3}H\)-CLE into the culture medium in the presence of various acceptors. The amount of label recovered in the albumin-containing medium was less than 1% of pulse value, and it was increased threefold by the addition of human LPDS (5 mg protein/ml). A similar effect to that obtained with human LPDS was also observed by the addition of 21 \(\mu\)g/ml of partially purified CETP. Addition of Intralipid to the LPDS-containing medium enhanced the \(^{3}H\)-CLE efflux.

This basic observation was studied in further experiments in which cationized LDL labeled with \(^{14}C\)-CL and \(^{3}H\)-CLE was added to the culture dish. The culture dish contained either SMCs or extracellular matrix derived from corneal endothelial cells (see Methods) that had been fixed with formaldehyde, washed, and then incubated with labeled cationized LDL. In Table 1 it can be seen that the efflux of CL was consistently higher in the presence of human LPDS than with equivalent amounts of rat LPDS. More \(^{14}C\)-CL or \(^{3}H\)-CLE was recovered in the human of LPDS-containing medium added to dishes containing SMCs than from dishes containing the extracellular matrix only.

**Cellular Model**

**Labeling with Liposomes**

In a previous study, we used bovine milk LPL to transfer labeled CLE from liposomes to cells and study efflux of \(^{3}H\)-CLE from a surface-associated compartment of the cultured cells. Presently we have extended this model to also study the availability of CLE present in an intracellular compartment. To that end, we made several modifications to the previously used model. Human skin fibroblasts were incubated with liposomes labeled with \(^{3}H\)-CLE and \(^{14}C\)-DOEPC and milk LPL for 24 hours, and 30% to 40% of the added label were taken up by the cells. Thereafter, the labeling medium was removed, and the cells were incubated in culture medium containing 10% fetal bovine serum for 48 or 96 hours. During that time, most of the surface-associated label had been internalized, and 90% of the cellular \(^{3}H\)-CLE was recovered in a trypsin-resistant compartment, which was interpreted to be mainly intracellular, as was evidenced by the hydrolysis of 80% to 85% of \(^{14}C\)-CL.

The other modification consisted of permeabilizing cells with saponin; this step was carried out on formaldehyde-fixed cells to reduce cell loss. Thereafter, efflux of \(^{3}H\)-CLE was studied during a 24-hour incubation in the presence of acceptors in the medium. The data presented in Table 2 and Figure 2 are de-

<table>
<thead>
<tr>
<th>Acceptor in medium</th>
<th>Radioactivity recovered in postincubation medium (% of pulse value)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Exp 1</td>
</tr>
<tr>
<td>Albumin</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Human LPDS</td>
<td>47.6 ± 1.7</td>
</tr>
<tr>
<td>Rat LPDS</td>
<td>2.2 ± 0.3</td>
</tr>
<tr>
<td>CETP</td>
<td>31.9 ± 2.4</td>
</tr>
<tr>
<td>Human apo HDL</td>
<td>---</td>
</tr>
<tr>
<td>Rat apo HDL</td>
<td>---</td>
</tr>
<tr>
<td>Dog apo A-I</td>
<td>---</td>
</tr>
</tbody>
</table>

Confluent cultures of human skin fibroblasts or smooth muscle cells (Experiment 4) were used. The cells were incubated for 24 hours with 2.5 \(\mu\)g milk lipoprotein lipase and DOEPC liposomes (5 \(\mu\)g phospholipid) labeled with \(^{3}H\)-cholesterol linoleyl ether (6 \(\times\) 10^4 dpm). The labeling medium was removed, and the cells were incubated with medium containing 10% serum for 48 hours (Experiment 1) or 96 hours (Experiments 2–4). Thereafter, triplicate control dishes were terminated, and the radioactivity recovered in the cell pellet and trypsin supernate was taken as 100%, i.e., pulse value (which amounted to 40% of added dpm). The experimental dishes were washed, fixed, permeabilized, and incubated in medium containing 1% albumin with acceptor, where appropriate. Values are means ± SE of triplicate dishes. Human lipoprotein-deficient serum (LPDS) and rat LPDS were 8 and 9 mg protein/ml, respectively. Partially purified cholesteryl ester transfer protein (CETP) was 50 \(\mu\)g protein/ml. Human and rat delipidated high density apolipoprotein (apo HDL) was 50 \(\mu\)g protein/ml. Dog apoprotein A-I (apo A-I) was 20 \(\mu\)g protein/ml.
CHOLESTERYL ESTER EFFLUX FROM ARTERIAL WALL

Stein et al.

4% to 6% of the medium label were taken up by the SMC, and 95% of this label was recovered with the cell pellet after trypsinization of control dishes. As in the previous experiments before the onset of efflux, the cells in the petri dish were fixed with formaldehyde and permabilized with saponin to increase the availability of the intracellular \(^{3}H\)-CLE. Two representative experiments are shown in Table 3. In both experiments, the efflux of \(^{3}H\)-CLE was highest in the presence of human LPDS; the addition of rat LPDS resulted in a more variable increase in efflux as compared to albumin only, while CETP was intermediate between the rat and human LPDS. When the cells were treated only with formaldehyde but not with saponin, the efflux in the presence of human LPDS was reduced 4.2-fold, but was still four times higher than in the presence of albumin. Another variant of the cellular model was the model that used cells incubated with LDL and chloroquine to enhance intralysosomal accumulation of CE. After 24 hours of incubation with 50 \(\mu\)g LDL labeled with \(^{3}H\)-CLE and 100 \(\mu\)M chloroquine, about 4% of the medium label was recovered with the cells. The cells were then treated with formaldehyde and saponin and the outflow of \(^{14}C\)-CL (expressed as percentage of pulse value) was 8.2 ± 0.3 in the presence of albumin alone; it was 34.7 ± 2.0 and 16.8 ± 1.0 with human LPDS and with rat LPDS, respectively.

**Labeling with Acetylated LDL (the Macrophage Model)**

In view of the heterogeneity of the cellular population of atheroma, we extended the present experiments to macrophages, known to avidly ingest acetylated LDL. We used the macrophage cell line, J774, a CT2 variant. The cells were incubated with acetylated LDL, labeled with \(^{3}H\)-CLE for 48 hours, and thereafter subjected to fixation and permeabilization. The data presented in Table 4 show that treatment with saponin markedly enhanced the efflux of \(^{3}H\)-CLE from cells loaded with \(^{3}H\)-CLE-acetylated LDL when human LPDS was present in the medium. The efflux of \(^{3}H\)-CLE was three- to sevenfold higher in the presence of human LPDS than at equivalent amounts of rat LPDS.

**Discussion**

In a previous study in which the influx of plasma CE into atheromatous aorta of cholesterol-fed rabbits was determined with the help of \(^{3}H\)-CLE, the influx was designated as “minimum” influx. We used this term because we could not exclude some cholesteryl ether egress from the artery. We have hypothesized that the loss of CE could occur by efflux of intact lipoproteins trapped in the extracellular matrix back into the circulation. Another possibility envisaged was that plasma- or macrophage-derived CETP could have been involved in the efflux of extra-cellular cholesteryl ether. In the present study, we have described several model systems in which the
Table 4. Efflux of 3H-Cholesteryl Linoleyl Ether from Internalized Acetylated LDL after Permeabilization (the Macrophage Model)

<table>
<thead>
<tr>
<th>Acceptor in medium</th>
<th>Exp 1 + Saponin</th>
<th>Exp 1 - Saponin</th>
<th>Exp 2 + Saponin</th>
<th>Exp 2 - Saponin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Albumin</td>
<td>0.8 ± 0.1</td>
<td>0.6 ± 0.1</td>
<td>1.8 ± 0.1</td>
<td>1.4 ± 0.2</td>
</tr>
<tr>
<td>Human LPDS (8 mg/ml)</td>
<td>10.4 ± 0.4</td>
<td>1.0 ± 0.3</td>
<td>10.7 ± 0.5</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>Rat LPDS (9 mg/ml)</td>
<td>1.3 ± 0.1</td>
<td>1.0 ± 0.3</td>
<td>3.2 ± 0.3</td>
<td>1.0 ± 0.3</td>
</tr>
</tbody>
</table>

Macrophages were cultured for 48 hours in the presence of acetylated LDL (20 μg protein/ml) labeled with 3H-CLE (4 × 10⁶ dpm). The medium was removed, the cells were incubated for 30 minutes at 37°C in serum-free medium, fixed and permeabilized with 0.5% saponin, and postincubated for 24 hours in medium containing 1% albumin and acceptors where appropriate. The uptake of 3H-CLE determined in control dishes terminated at the end of the labeling period was 30% to 50% of added label and served as 100%, i.e., pulse value. Values are means ± SE of triplicate dishes. Abbreviations are as in Table 2.

latter possibility could be tested in vitro. In the first series of experiments, the binding of LDL labeled with 3H-CLE to the cell surface and extracellular matrix was achieved by the use of cationized LDL, which was previously shown to accumulate to a large extent in an extracellular compartment.12 The recovery of significantly more labeled CLE or CL in the postincubation medium containing human LPDS or partially purified CETP than with albumin alone or Intralipid indicated that the efflux of CL from cationized LDL trapped in the interstitial can be enhanced by CETP.

The present results indicated a possible role of CETP in the removal of lipoprotein CE and CLE from an extracellular location in an analogy to liposomal CLE,8 hence it seemed of interest to devise a model in which the availability for efflux of intracellular CLE could be tested. This model consisted of cultured cells in which internalization of liposomal CLE and CE was mediated by milk LPL.22 To decrease the extent of surface-bound label,22 an intermediate step was introduced into the experimental protocol. This entailed to 48- to 96-hour postincubation in nonlabeled serum containing medium before the measurement of 3H-CLE efflux. When efflux of 3H-CLE was determined from fixed cells without permeabilization, the amount of radioactivity removed was small and could be accounted for mainly by the label present at the cell surface, i.e., in a trypsin-releasable compartment.22 The very significant increase in 3H-CLE efflux in permeabilized cells provided evidence that CETP present in a trypsin-resistant, mainly intracellular, compartment can be made available for interaction with CETP. Saponin was chosen as a permeabilizing agent because it has been used in immunocytochemistry experiments in which visualization of intracellular antigens in intact cells was sought.21,26 Presently, we have used a higher concentration of saponin because the efflux at lower concentrations was more variable. This model also provided evidence that, while saponin treatment enabled the CETP to promote efflux of intracellular CLE, efflux of cellular phospholipid as determined by an ether analogue was increased to a much lesser extent. This finding supported the interpretation that the saponin treatment allowed a selective efflux of CE or its analogue. The intracellular location of the labeled CLE was presumably in the lysosomal compartment, as 80% to 85% of liposomal 14C-CE had undergone hydrolysis to free cholesterol, and the hydrolysis had been shown to be inhibited by chloroquine.22

The availability for efflux of cholesteryl ether that had reached the lysosomal compartment via receptor-mediated endocytosis of native LDL and that remained sequestered in this compartment owing to its nonhydrolyzable ether bond was examined. Two conditions were studied, one in which the degradation of the apoprotein portion of the lipoprotein was not interfered with, the other when 3H-CLE was expected to remain as part of the lipoprotein particle, owing to inhibition of protein degradation by chloroquine. In an analogy to the liposomal model, the 3H-CLE taken up by the cell as part of the LDL was available for efflux in the presence of human LPDS, in the control as well as in the chloroquine-treated cells. As pointed out earlier, these model systems were designed to simulate various phases of atheroma formation. Efflux of CE could occur from arterial interstitium, and the model system using cationized LDL added to fixed cells suggests that interstitial CE could be available for efflux in the presence of CETP. The aortic SMCs that had taken up native 3H-CLE-LDL and then were fixed and permeabilized could be the counterpart of cellular elements of atheroma in various stages of disintegration. Thus, CE that had been internalized by arterial cells might also become available for efflux if the integrity of the cell membrane is impaired. Since the cellular population of the atheroma also includes macrophages, these cells were studied as well, and their known avidity for acetylated LDL proved convenient for the study of CE efflux from intracellular pools of damaged cells.

In all the experiments, the efflux of CLE and CL in the presence of albumin was considered as a control for that encountered with human LPDS. Addition of rat LPDS to the albumin-containing medium resulted in a variable increase in CLE or CL efflux. It appears
that rat LPDS also contains CETP activity (John Albers, personal communication), the variability of which was ascribed to the varying amounts of CETP inhibitor. The importance of the present findings could be related to the question of whether plasma (or tissue) levels of CETP in humans could influence the induction or reversibility of atherosclerosis. The availability of CETP might become important to catalyze CE efflux after postprandial hyperlipidemia, when the artery is exposed for brief periods of time to high concentrations of highly atherogenic lipoproteins.27,28 Since the development of atherosclerosis in humans is a process extending over decades, a continuous small efflux of the incoming lipoprotein CE could markedly influence its arterial concentration.

It is of interest that, in the animal kingdom, an inverse relation seems to exist between the plasma levels of CETP activity and the relative resistance to the induction of atherosclerosis. Thus, the rat, dog, sheep, and cow are relatively resistant to the development of atherosclerosis by cholesterol feeding and have a low CETP activity (with the possible exception of the pig, which has a low CETP activity but is resistant to atherosclerosis induced by cholesterol feeding).29 The reverse seems to be true in the rabbit, human, chicken, and turkey, species that are more prone to the induction of the atheromatous process and have relatively high plasma CETP values. The origin of human plasma CETP has not been ascertained, but it was recently reported that human macrophages secrete the CE transfer protein.30 Secretion of CETP by perfused rabbit liver,31 as well as the presence of intracellular CETP-like protein, was described in bovine liver microsomes;32 such a protein could also be present in the intestine. If plasma levels reflect tissue concentration of CETP, one may speculate that an intracellular CETP, perhaps in the intestinal cells, could enhance the transfer of intracellularly synthesized CE into chylomicrons during the intestinal absorption of lipids. This mechanism could aid in the formation and accumulation of CE-enriched atherogenic chylomicron remnants responsible for atheroma induction in the rabbit.27,33

Recently, Stender and Hjelms34 determined the influx of esterified plasma cholesterol into human ascending aorta without atherosclerotic lesions, and found it to be disturbingly high when related to the tissue content of cholesterol. The authors suggested that some removal mechanism for CE may exist to explain their data. Two possibilities were mentioned; one, net transfer of free cholesterol subsequent to cellular CE hydrolysis, the other, efflux of esterified cholesterol with plasma lipoproteins. Our findings in the model systems described here bring forth a third possibility: the putative role of CEPT in such a process.

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Index Terms: atherosclerosis • liposomes • lipoproteins • cholesterol • smooth muscle cells • lipoprotein lipase
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