J. Biochem. 128, 251–259 (2000)

Dual Pathways for the Secretion of Lysosomal Cholesterol into a Medium from Cultured Macrophages

Mamoru Ikemoto, Takemitsu Furuchi, Hiroyuki Arai,[1] and Keizo Inoue

Department of Health Chemistry, Graduate School of Pharmaceutical Sciences, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033

Received March 23, 2000; accepted May 26, 2000

The removal of cholesterol from macrophages is important for reversing foam cell formation. In a previous study, we demonstrated that mouse peritoneal macrophages in culture secrete significant amounts of unesterified cholesterol from the lysosomes into the medium during endocytosis and subsequent metabolism of cholesterol-containing liposomes [Furuchi, T., Aikawa, K., Arai, H., and Inoue, K. (1993) *J. Biol. Chem.* 268, 27345–27348]. In this study, we found that at least two distinct mechanisms are involved in this process. The efflux of unesterified cholesterol into the medium was greatly suppressed by pregnenolone, an inhibitor of lysosomal cholesterol transport, but an appreciable proportion of the unesterified cholesterol was still released into the medium. Analysis of the medium containing the secreted cholesterol by NaBr density gradient ultracentrifugation revealed that the unesterified cholesterol was distributed in two different density peaks (bottom and d = ~1.1). The d = ~1.1 peak material formed high-density lipoprotein (HDL)–like particles that were produced and secreted by the macrophages. The lipid components of these particles were phosphatidylcholine and sphingomyelin, while the sole protein component was apolipoprotein E (apo E). Treatment with pregnenolone completely abolished the production of these HDL-like particles but had little effect on the bottom fractions. These data indicate that macrophages release lysosomal cholesterol *via* both pregnenolone-sensitive and -insensitive pathways, and that only the cholesterol secreted through the pregnenolone-sensitive pathway is associated with endogenously synthesized apo E–containing HDL-like particles. Moreover, we found that the pregnenolone-sensitive pathway operated independently of the presence or absence of exogenous HDL, whereas secretion *via* the pregnenolone-insensitive pathway was greatly stimulated by exogenously added HDL.

Key words: cholesterol efflux, high-density lipoprotein, macrophage, pregnenolone, reverse cholesterol transport.

During atherogenesis, peripheral blood monocytes traverse the arterial endothelium and differentiate into mature macrophages. The scavenger receptor expressed by these macrophages mediates the binding and uptake of modified low-density lipoproteins (LDL) deposited in the intima of the arterial wall, leading to the accumulation of cholesteryl esters (CE) within these cells (*1–4*). The clustering of macrophages laden with massive amounts of CE (referred to as foam cells) in a fatty streak is the earliest sign of atherogenesis, this clustering being reversible (*5*, *6*). Since macrophages can not catabolize cholesterol themselves, efflux from the cells into the extracellular space is the only way by which intracellular cholesterol levels can be reduced. The studies of Brown and Goldstein (*7*) established that, after their uptake by macrophages, lipoprotein CE undergo lysosomal acid-lipase hydrolysis and then cytoplasmic re-esterification. The CE thus formed undergo continuous hydrolysis and esterification. Newly synthesized cholesterol, as well as cholesterol from lysosomes or cytosolic lipid droplets, can all be exported out of the cells, but the latter two cholesterol sources seem to be dominant in macrophages present in the intima of the arterial wall. Generally, the hydrolysis of cytosolic CE by neutral cholesterol esterase has a much slower half-time [about 12 h in mouse peritoneal macrophages (*8*)] than the hydrolysis of lysosomal CE by acid lipase (less than 1 h) (*9*). Thus, delineating the pathways and mechanisms by which lysosomal cholesterol is secreted from macrophages will be important for understanding the progression and regression of atherosclerosis.

After the uptake of modified LDL by macrophages, unesterified cholesterol (UC) liberated in the lysosomes is transported to other cellular sites, such as the endoplasmic reticulum (ER), for esterification. It has previously been demonstrated that the transport of lysosomal cholesterol is inhibited by certain steroids, such as progesterone, pregnenolone (*10*, *11*), and 3-β-[2-(diethylamino)-ethoxy]-androst-5-en-17-one (U18666A) (*12*). Taking advantage of the fact

[1] To whom correspondence should be addressed. Tel: +81-3-5841-4720, Fax: +81-3-3818-3173, E-mail: harai@mol.f.u-tokyo.ac.jp

Abbreviations: ACAT, acyl CoA cholesterol acyltransferase; apo E, apolipoprotein E; CE, cholesteryl ester; ER, endoplasmic reticulum; HBSS, Hank's-buffered salt solution; HDL, high density lipoprotein; LPDS, lipoprotein-deficient serum; LysoPC, lysophosphatidylcholine; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; UC, unesterified cholesterol.

© 2000 by The Japanese Biochemical Society.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

that this inhibition is reversible upon removal of the steroid, we characterized the post-lysosomal pathway of the endocytosed cholesterol in cultured mouse peritoneal macrophages. Using this system, we found that while some of the cholesterol accumulated within the lysosomes is esterified by acyl CoA cholesterol acyltransferase (ACAT), a significant proportion is released into the culture medium in its unesterified form (*13*). In the present study, we characterized the routes by which endocytosed cholesterol is transported from lysosomes to the extracellular medium and demonstrated that two distinct pathways are involved in this process.

MATERIALS AND METHODS

Materials—[4-^{14}C]Cholesterol (53 mCi/mmol) and [1α, 2α(*n*)-^{3}H]cholesteryl linoleate (48 Ci/mmol) were purchased from Amersham, United Kingdom. Tran^{35}S-Label (1,037 Ci/mmol; a ^{35}S *Escherichia coli* hydrolysate labeling reagent containing 70% [^{35}S]L-methionine) was purchased from ICN Biomedicals. Carrier-free [^{32}P]orthophosphoric acid (8,500–9,120 Ci/mmol) was purchased from Dupont/NEN Research Products. Phospholipids were purchased from Avanti Polar Lipids. Cholesterol and pregnenolone were obtained from Sigma. High-density lipoproteins (HDL; d = 1.063–1.21 g/ml) were isolated from fresh human plasma by preparative ultracentrifugation (*14*). Lipoprotein-deficient serum (LPDS; d > 1.21 g/ml) was prepared as described previously (*15*).

Cells—Mouse peritoneal macrophages were prepared as described previously (*16*). Briefly, peritoneal cells were harvested from unstimulated wild-type mice (ICR) using Hanks' balanced salt solution (HBSS) and then suspended at 2 × 10^{6} cells/ml. Aliquots (0.5 ml) were dispensed into 24-well plastic microplates, and then incubated at 37°C for attachment of the macrophages to the plates. After 2 h, each plate was washed with HBSS. The medium was immediately replaced with 0.5 ml Dulbecco's modified Eagle's medium containing 6.5% LPDS, penicillin (100 units/ml), and streptomycin (100 μg/ml) (hereafter referred to as medium A).

Preparation of Liposomes—Multilamellar liposomes were prepared as described previously (*16*). Briefly, phosphatidylcholine (1 μmol), phosphatidylserine (1 μmol), dicetyl phosphate (0.2 μmol), cholesterol (1.5 μmol), and a trace of [4-^{14}C]cholesterol or [1α,2α(*n*)-^{3}H]cholesteryl linoleate (20 μCi) were dried and then suspended in 1 ml 0.3 M glucose solution.

Metabolism of Liposomes by Cultured Macrophages—To macrophage cultures (0.5 ml), 25 μl liposomes containing 0.5 μCi unesterified [^{14}C]cholesterol ([^{14}C-UC]liposomes) or 0.5 μCi [^{3}H]cholesteryl ester ([^{3}H-CE]liposomes) were added. After incubation for the times indicated, the medium was removed from the cells and spun at 3,000 ×*g* for 5 min to pellet any cells which had become dislodged from the plates. Radioactivity originating from UC released into the medium was determined after separating the lipids on a silica gel plate using a mobile phase of hexane/ethyl ether/acetic acid (70:30:1, by volume). The macrophage monolayers were washed and harvested, and then the radioactivity derived from the intracellular CE and UC was quantified as described previously (*16*).

Efflux of Lysosomal Cholesterol from Macrophages—To determine the metabolism of the cholesterol accumulated within the lysosomes, the macrophages were first incubated with 25 μl [^{14}C-UC]liposomes in the presence of 30 μM pregnenolone for 16 h. Under these conditions, endocytosed cholesterol accumulates in the (phago)lysosomes of macrophages (*13*). The cells were then washed twice with 0.5 ml medium A and incubated for the times indicated in 0.5 ml medium A without liposomes in the absence or presence of pregnenolone. During these incubations, some of the UC accumulated in the (phago)lysosomes were transferred to the ER for esterification and others were released into the medium. The radioactivity originating from CE and UC was determined in both cells and medium after these timed incubations.

Secretion of Phospholipids by Macrophages—Macrophage monolayers were first incubated with 25 μl non-radiolabeled liposomes for 16 h in the presence of 30 μM pregnenolone. For the last 2 h of incubation, the cultures were supplemented with 25 μCi [^{32}P]phosphate. The cells were then incubated for 6 h without liposomes or [^{32}P]phosphate, in the absence or presence of 30 μM pregnenolone. After various chase-incubation periods, the medium was removed from the cells and spun at 3,000 ×*g* for 5 min. The macrophage monolayers were washed with HBSS and then lysed with 0.5 ml 0.1% SDS. The radiolabeled phospholipids were extracted from the medium and cells by the method of Bligh and Dyer. The extracted lipids were subjected to TLC on a Silica Gel G plate. The plate was developed with chloroform/methanol/acetic acid/H_2O (25:15:4:2, by volume) and exposed to X-ray film, and then each spot was scraped off and its radioactivity was determined.

Analysis of Secreted Cholesterol and Phospholipids by Density Gradient Ultracentrifugation—Macrophage monolayers were first incubated for 16 h with 25 μl [^{3}H-CE]liposomes in the presence of 30 μM pregnenolone. For the last 2 h of incubation, the cultures were supplemented with 25 μCi [^{32}P]phosphate. The cells were then incubated for 6 h without [^{3}H-CE]liposomes or [^{32}P]phosphate, in the absence or presence of 30 μM pregnenolone. After various incubation periods, the medium was removed from the cells and spun at 3,000 ×*g* for 5 min. For isopycnic separation, samples of the culture medium (total 3.5 ml) were placed at the top of a 1.3 g/ml density NaBr layer (1.5 ml), and then centrifuged for 12 h at 300,000 ×*g* at 16°C. Following density gradient centrifugation, 15 fractions were collected from the bottom of the gradient by pump-driven aspiration. An aliquot of each fraction was taken for determination of the density and the radioactivity originating from [^{3}H]UC. The phospholipids in the fractions were extracted and subjected to TLC as described above. The plate was developed and exposed to X-ray film.

Analysis of Secreted Proteins by Density Gradient Ultracentrifugation—Macrophage monolayers were first incubated for 16 h with 25 μl [^{3}H-CE]liposomes and 50 μCi Tran^{35}S-Label in the presence of 30 μM pregnenolone. The cells were then incubated for 6 h without [^{3}H-CE]liposomes or Tran^{35}S-Label, in the absence or presence of 30 μM pregnenolone. Samples of the medium were periodically processed by NaBr density gradient ultracentrifugation as described above. An aliquot of each fraction was taken for determination of the density and the radioactivity originating from [^{3}H]UC. Each fraction was also subjected to SDS-PAGE, and the resulting gels were dried and autoradiographed.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

Effect of HDL on Pregnenolone-Sensitive and -Insensitive Cholesterol Efflux—Macrophage monolayers were incubated with [^{3}H-CE]liposomes in the presence of 30 μM pregnenolone for 16 h. The monolayers were then washed and incubated in 0.5 ml medium A with or without HDL (0.25 mg protein) in the absence or presence of 30 μM pregnenolone. During the total 6-h incubation period, the amount of [^{3}H]UC in the medium was determined at various times.

RESULTS

Efflux of Unesterified Cholesterol from Macrophages during the Metabolism of Cholesteryl Ester–Containing Liposomes—We previously demonstrated that mouse peritoneal macrophages in culture efficiently take up and metabolize negatively charged liposomes as well as modified LDL, which results in massive accumulation of lipid droplets in their cytoplasm (*16*). Using this system, we found that macrophages release significant amounts of UC into the medium during the metabolism of cholesterol-containing liposomes (*13*). In the current study, we characterized this pathway by utilizing liposomes containing traces of [^{3}H]CE as well as [^{14}C]UC, in addition to non-radioactive UC (see "MATERIALS AND METHODS"). [^{14}C-UC]liposomes and [^{3}H-CE]liposomes were used to measure the synthesis of [^{14}C]-CE within the cells and the efflux of [^{3}H]UC into the medium, respectively. When macrophages take up [^{3}H-CE]liposomes, they hydrolyze the liposomal [^{3}H]CE in their lysosomes and produce [^{3}H]UC, some of which is released into the medium. The cholesterol from the liposomes added to the culture (ester form) can thus be distinguished from that secreted into the medium (unesterified form) during metabolism by macrophages.

When macrophages were incubated with [^{14}C-UC]liposomes, the accumulation of UC reached a plateau within 12 h and CE was also accumulated time-dependently (Fig. 1A). As shown in Fig. 1C, the macrophages progressively released significant amounts of [^{3}H]UC into the extracellular medium during incubation with [^{3}H-CE]liposomes. Incubation of the liposomes themselves with the conditioned medium did not lead to the production of UC (data not shown). It has previously been demonstrated that pregnenolone induces the accumulation of endocytosed cholesterol within the lysosomes of macrophages (*11*), but has little effect on the lysosomal hydrolysis of endocytosed CE (data not shown) or the ACAT reaction in cultured macrophages (*11*). Indeed, pregnenolone (30 μM) completely inhibited the CE synthesis in the cells (Fig. 1B), which resulted in the accumulation of UC in the lysosomes (data not shown, see Ref. *13*). The efflux of UC into the medium was also greatly suppressed by this agent, but an appreciable proportion of the UC was still released into the medium (Fig. 1D). As shown in Fig. 2, the efflux of UC was progressively inhibited by increasing amounts of pregnenolone, but about one-third of the cholesterol was released even in the presence of high doses of pregnenolone (up to 100 μM). This level of cholesterol efflux was also observed in the presence of other steroid inhibitors such as progesterone and U18666A (data not shown). These data suggest that the esterification of lysosomal cholesterol is completely inhibited by steroidal cholesterol transport inhibitors, but that the efflux of UC into the extracellular medium is only par-

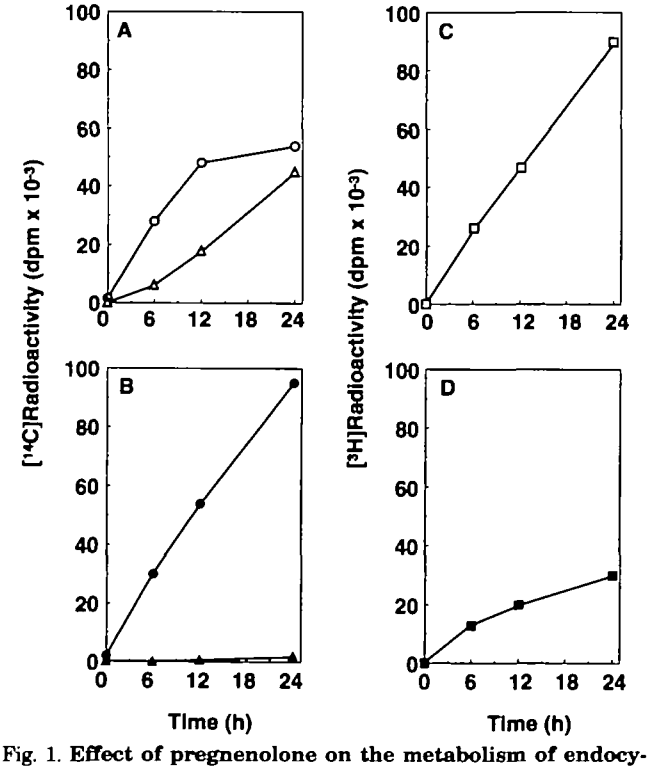

Fig. 1. **Effect of pregnenolone on the metabolism of endocytosed cholesterol in macrophages.** A, B: Macrophage monolayers were incubated with [^{14}C-UC]liposomes in the absence (open symbols) or presence (solid symbols) of 30 μM pregnenolone. After incubation for the times indicated, the amounts of intracellular unesterified (circles) and esterified (triangles) cholesterol were determined as described under "MATERIALS AND METHODS." C, D: Macrophage monolayers were incubated with [^{3}H-CE]liposomes in the absence (open symbols) or presence (solid symbols) of 30 μM pregnenolone. After incubation for the times indicated, the amount of UC (squares) in the medium was determined. Each value is the average of duplicate incubations.

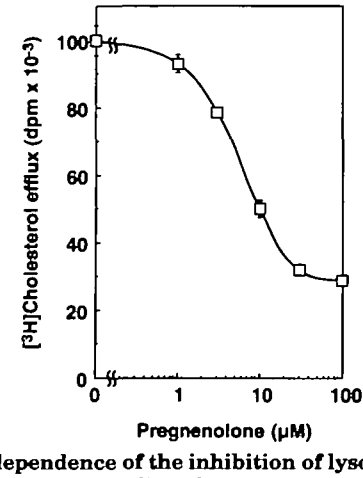

Fig. 2. **Dose dependence of the inhibition of lysosomal cholesterol efflux into the medium by pregnenolone.** Macrophage monolayers were incubated with [^{3}H-CE]liposomes in the absence or presence of the indicated concentrations of pregnenolone. 5 μl of each 100-fold concentrated pregnenolone solution (dissolved in ethanol) was added to macrophage cultures. After incubation for 16 h, the amount of UC in the medium was determined.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

tially inhibited by these agents. In other words, the efflux of endocytosed cholesterol from macrophages may involve two distinct pathways, one of which is steroid-sensitive and the other insensitive.

Efflux of Unesterified Cholesterol Accumulated within Lysosomes—The accumulation of CE within macrophages was not observed in the presence of pregnenolone (Fig. 1B). Regardless of the conditions, the cells released significant amounts of UC during the endocytosis of CE-containing liposomes (Fig. 1D). This indicates that cholesterol efflux resulted from the hydrolysis of liposomal CE and not from the hydrolysis of cytosolic CE droplets. In order to characterize the pathway of cholesterol efflux further, we simplified the experimental system. Macrophage monolayers were pulsed with [^{3}H-CE]liposomes for 16 h in the presence of 30 μM pregnenolone (Fig. 3, A and B). These cells were then incubated for the times indicated without the liposomes, in the absence or presence of pregnenolone. The efflux of UC from the macrophages resumed upon removal of the pregnenolone. Even though cholesterol efflux was also greatly diminished when pregnenolone was present during the chase-incubation period, some was still secreted, suggesting that both the pregnenolone-sensitive and -insensitive pathways were operating during the chase-incubations.

Phospholipid Secretion Associated with Cholesterol Efflux—In this study, we used fetal calf serum depleted of lipoprotein, a possible acceptor for effluxed cholesterol, as a constituent of the medium. Nevertheless, UC was secreted into the culture medium. This suggests that macrophages may produce lipoprotein-like particles, with which the UC is associated. We used a preincubation phase followed by a chase-incubation phase for our experiments, as described above, since the cholesterol released into the medium could have been transferred from its original particles [or binding protein(s)] to the liposomes added to the cultures. We first used this system to determine whether or not macrophages secrete phospholipids, possible components of lipoprotein-like particles, along with the cholesterol released during chase-incubations. The macrophages were preincubated with non-radiolabeled liposomes for 16 h in the presence of 30 μM pregnenolone. For the last 2 h of preincubation, the macrophage cultures were supplemented with inorganic [^{32}P]phosphate in order to label their intracellular phoshlipids (see "MATERIALS AND METHODS"). After a subsequent 6-h chase incubation without liposomes or inorganic [^{32}P]-phosphate, [^{32}P]phospholipids which had appeared in the culture medium were analyzed by TLC. As shown in Fig. 4A, when the macrophages were incubated in the absence of pregnenolone, they secreted significant amounts of phosphatidylcholine (PC) and lysophosphatidylcholine (lysoPC), and a small amount of sphingomyelin (SM). However, the secretion of PC and SM was greatly diminished in the cells incubated in the presence of pregnenolone. Pregnenolone also reduced lysoPC secretion but to a lesser degree. The level of cellular phospholipid synthesis in the cells incubated with pregnenolone was almost identical to that in the cells incubated without pregnenolone. Approximately 1% of the phospholipids synthesized by the macrophages was secreted into the medium after the 6-h chase incubation period in the absence of pregnenolone (data not shown). Secretion of these phospholipids was only stimulated in

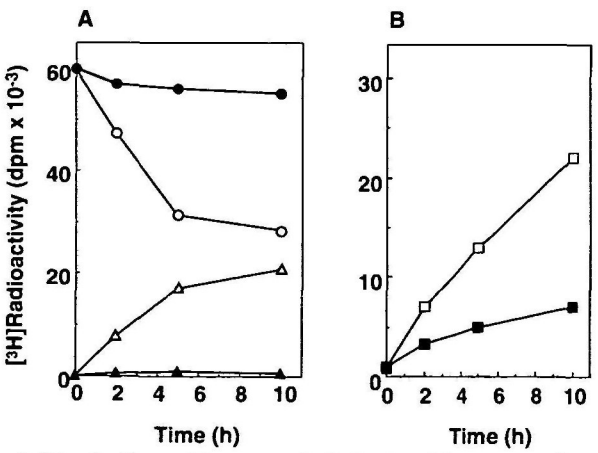

Fig. 3. **Metabolism of lysosomal cholesterol by macrophages.** Macrophage monolayers were preincubated with [^{3}H-CE]liposomes in the presence of 30 μM pregnenolone for 16 h. The monolayers were then washed, refed with 0.5 ml medium A, with (solid symbols) or without (open symbols) 30 μM pregnenolone, and then incubated for the times indicated. The amounts of UC (circles) and CE (triangles) in the cells (A), and the amounts of UC (squares) in the medium (B) were determined. Each value is the average of duplicate incubations.

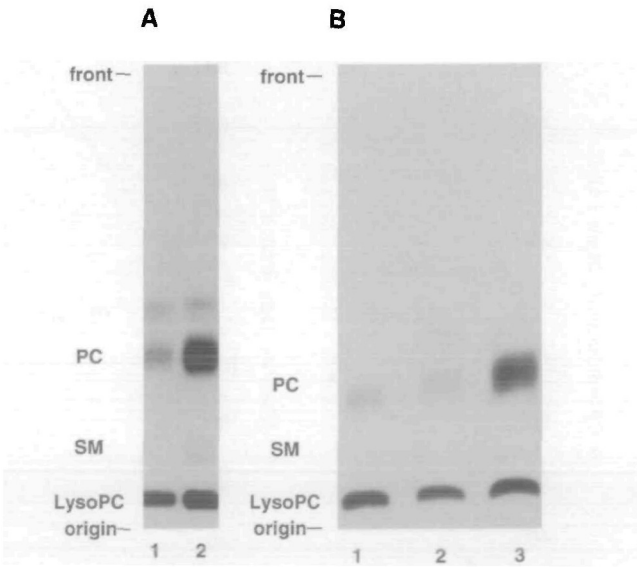

Fig. 4. **Efflux of phospholipids from cholesterol-loaded macrophages.** A: Macrophage monolayers were preincubated with cholesterol-containing liposomes in the presence of 30 μM pregnenolone for 16 h. The monolayers were supplemented with [^{32}P]phosphate for the last 2 h of preincubation. The cells were then washed and incubated without liposomes or [^{32}P]phosphate for 6 h in the presence (lane 1) or absence (lane 2) of pregnenolone. The [^{32}P]phospholipids secreted into the medium during the 6-h chase incubation period were analyzed by thin layer chromatography. B: Macrophage monolayers were preincubated alone (lane 1), with unesterified cholesterol–depleted liposomes (lane 2), or with unesterified cholesterol-containing liposomes (lane 3) in the presence of 30 μM pregnenolone for 16 h. The monolayers were supplemented with [^{32}P]phosphate for the last 2 h of preincubation. The cells were then washed and incubated without liposomes or [^{32}P]phosphate for 6 h in the absence of pregnenolone. The [^{32}P]phospholipids secreted were analyzed by thin layer chromatography and subsequent autoradiography. PC, phosphatidylcholine; SM, sphingomyelin; lysoPC, lysophosphatidylcholine.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

macrophages which accumulated UC in their lysosomes, since control macrophages or macrophages preincubated with cholesterol-depleted liposomes did not secrete these phospholipids in such large amounts as cholesterol-loaded cells even after pregnenolone washout (Fig. 4B). All these data suggest that, when macrophages release lysosomal cholesterol *via* the pregnenolone-sensitive pathway, they also secrete both PC and SM into the medium. Consistent with these observations, it was previously shown that free cholesterol-loading of fibroblasts leads to an increase in the biosynthesis of phospholipid, most notably PC and SM (*17*).

Density Gradient Analysis of the Released Cholesterol and Phospholipids—Since it was highly possible that the secreted cholesterol and phospholipids were associated with each other in the culture medium, we next analyzed the medium by NaBr density gradient ultracentrifugation. The macrophages were preincubated with [^{3}H-CE]liposomes and inorganic [^{32}P]phosphate in the presence of 30 μM pregnenolone, as described above, and then chase-incubated in the absence or presence of pregnenolone. The resulting culture medium, containing secreted [^{3}H]UC and [^{32}P]phospholipids, was subjected to density gradient analysis. As shown in Fig. 5A, [^{3}H]UC was distributed in two different density peaks (bottom and d = ~1.1). When pregnenolone was present during the chase-incubation, the large peak at d = ~1.1 was lost, whereas the peak for the bottom fraction was not significantly affected (Fig. 5, A and B), suggesting that the cholesterol in the bottom fraction was secreted *via* the pregnenolone-insensitive pathway, while that detected at d = ~1.1 was released *via* the pregnenolone-sensitive route.

Next, the phospholipids in the medium were analyzed by density gradient fractionation. As shown in Fig. 5A, PC and SM were detected in both the peak at d = ~1.1 as well as in the bottom fraction. In contrast, other secreted phospholipids, such as lysoPC and phosphatidylserine and/or phosphatidylinositol (PS/PI), were only detected in the bottom fraction. Pregnenolone treatment caused loss of the peak at d = ~1.1 but had little effect on the bottom region (Fig. 5B). These data indicate that PC and SM are the phospholipid components of HDL-like particles that carry secreted UC, and that cholesterol efflux *via* the pregnenolone-sensitive pathway utilizes these HDL-like particles. Although phospholipids such as PS/PI, PC, and lysoPC were observed in the bottom fraction of the density gradient, it is not clear at present whether or not they are associated with the secreted cholesterol.

Protein Components Associated with the HDL-Like Particles—We next examined whether or not these HDL-like particles contain specific proteins by adding [^{35}S]methionine and [^{35}S]cysteine to the cultures during preincubation to label the cellular proteins. As shown in Fig. 6A, only a band corresponding to a molecular weight of ~35,000 comigrated with the HDL-like particles, while most of the other proteins were observed in the bottom fraction. Upon chase-incubation in the presence of pregnenolone, the 35,000-dalton protein band disappeared, as well as the cholesterol peak at d = ~1.1 (Fig. 6B). These data indicate that this 35,000-dalton protein was associated with the HDL-like particles that carry the secreted cholesterol. This protein was later found to be apo E on Western blot analysis (data not shown).

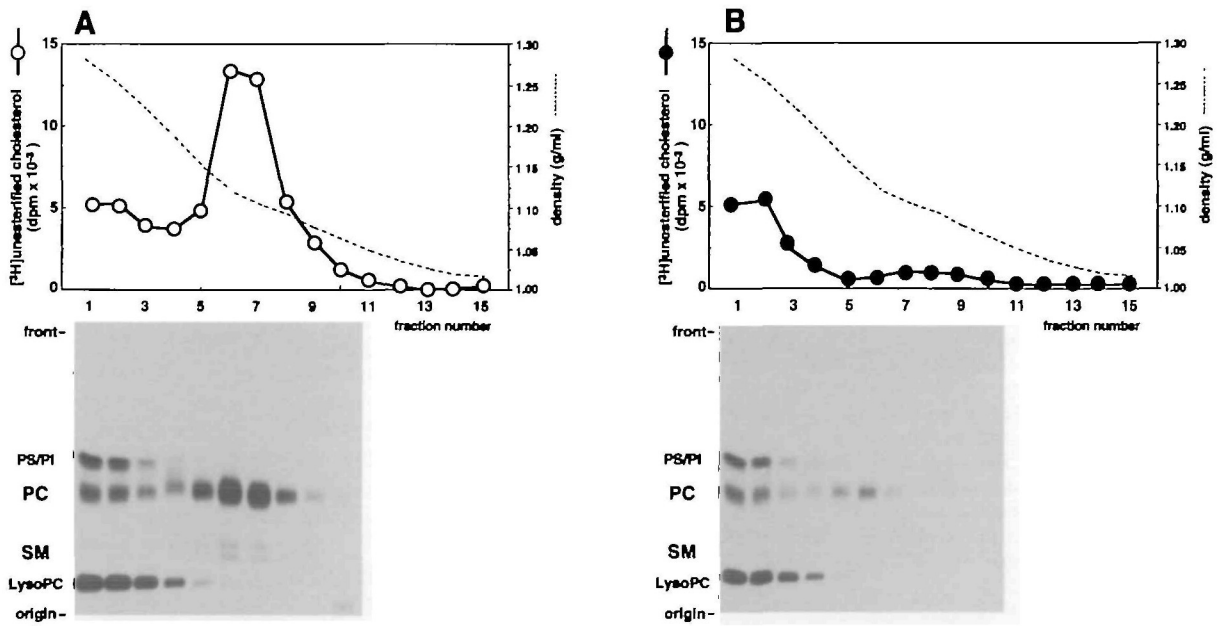

Fig. 5. **Analysis of cholesterol and phospholipids secreted into the medium by density gradient ultracentrifugation.** Macrophage monolayers were preincubated with [^{3}H-CE]liposomes in the presence of 30 μM pregnenolone for 16 h. The monolayers were supplemented with [^{32}P]phosphate for the last 2 h of preincubation. The cells were then washed and incubated without liposomes or [^{32}P]phosphate for 6 h in the absence (A, open symbols) or presence (B, solid symbols) of pregnenolone. The medium obtained after these chase incubations was fractionated by NaBr density gradient ultracentrifugation. The radioactivity derived from UC (circles) and the density (dashed line) were determined for each fraction. Also, the [^{32}P]phospholipids in each fraction were extracted with chloroform and methanol and then subjected to thin layer chromatography. The resulting plate was autoradiographed. PS/PI, phosphatidylserine and/or phosphatidylinositol; PC, phosphatidylcholine; SM, sphingomyelin; lysoPC, lysophosphatidylcholine.

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

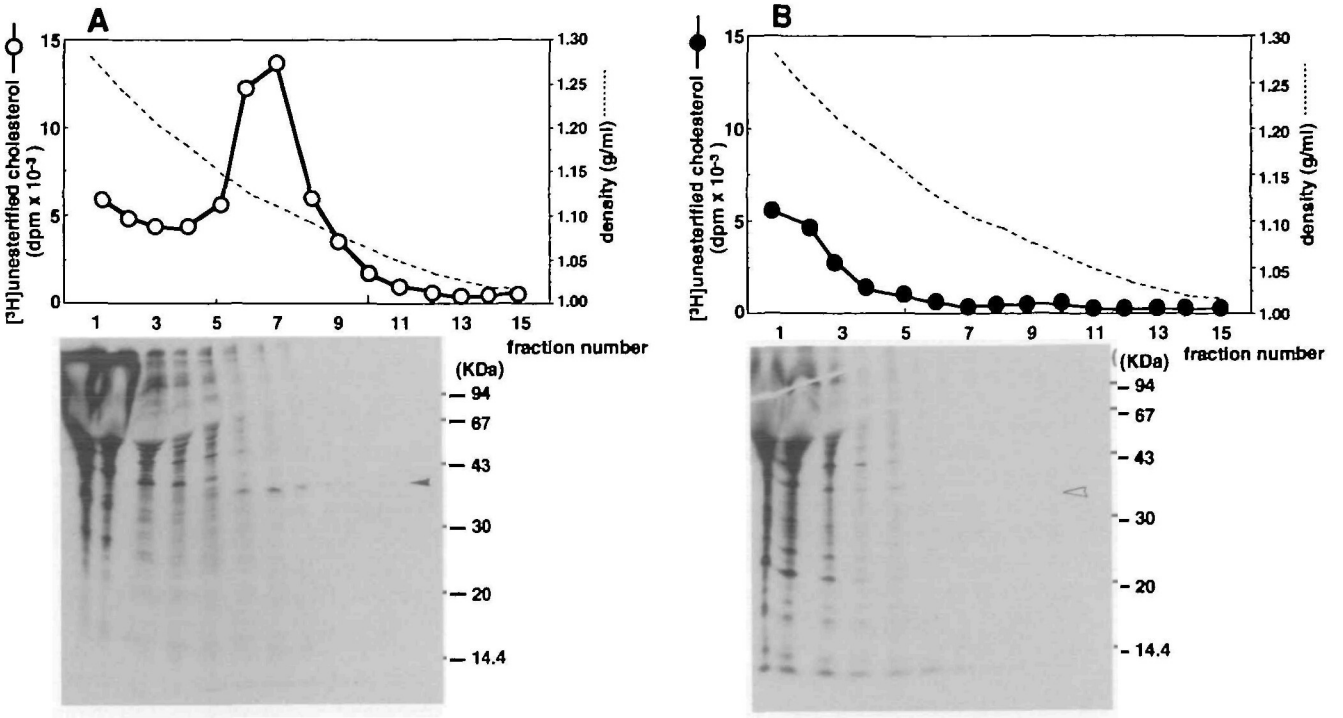

Fig. 6. **Association of a 35 kDa protein with the HDL-like particles secreted into the medium.** Macrophage monolayers were preincubated with [³H-CE]liposomes and 50 μCi Tran³⁵S-Label in the presence of 30 μM pregnenolone for 16 h. The cells were then washed and chase-incubated without liposomes or Tran³⁵S-Label for 6 h in the absence (A, open symbols) or presence (B, solid symbols) of pregnenolone. The medium obtained after chase-incubation was fractionated by NaBr density ultracentrifugation. The radioactivity derived from UC (circles) and the density (dashed line) were determined for each fraction. Also, the [³⁵S]proteins secreted into the medium during the chase-incubation were analyzed by SDS-PAGE and autoradiography.

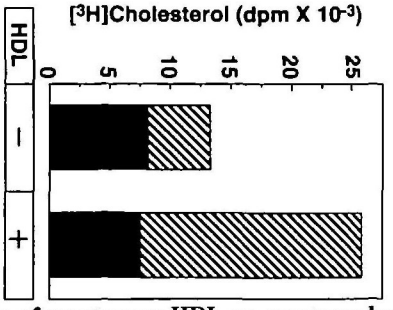

Fig. 7. **Effects of exogenous HDL on pregnenolone-sensitive and -insensitive cholesterol efflux.** Macrophage monolayers were preincubated with [³H-CE]liposomes in the presence of 30 μM pregnenolone for 16 h. The monolayers were then washed and refed with 0.5 ml medium A, with or without HDL (0.5 mg protein/ml), in the absence or presence of pregnenolone. After a 6 h chase-incubation period, the amount of [³H]UC in the medium was determined. Hatched bars, radioactivity derived from the secreted cholesterol in the presence of pregnenolone (*i.e.* pregnenolone-insensitive secretion). Solid bars, radioactivity in the absence of pregnenolone minus that in the presence of pregnenolone (*i.e.* pregnenolone-sensitive pathway).

Effect of Exogenous HDL on Cholesterol Efflux—It is widely recognized that the efflux of cellular cholesterol into the culture medium is effectively stimulated by exogenous HDL. Here, we examined the effect of HDL added to the culture on cholesterol efflux *via* the pregnenolone-sensitive and -insensitive pathways. As shown in Fig. 7, the efflux of lysosomal UC occurring in the presence of pregnenolone (*i.e. via* the pregnenolone-insensitive pathway) was markedly stimulated by the extracellular HDL, with which the secreted cholesterol was associated (data not shown), whereas efflux *via* the pregnenolone-sensitive pathway (cholesterol released in the absence of pregnenolone minus that released in the presence of pregnenolone) was not appreciably affected by the exogenous HDL.

DISCUSSION

Macrophages are known to release both UC produced through neutral cholesterol esterase–mediated hydrolysis of cytosolic CE droplets and that produced through lysosomal cholesterol esterase–mediated hydrolysis of endocytosed lipoproteins. A recent study (*18*) also revealed that a lysosomal component is involved in the hydrolysis of stored CE in foam cells. In order to simplify the experimental system, we followed the efflux of UC that had accumulated in the lysosomes by utilizing pregnenolone, an inhibitor of lysosomal cholesterol transport. Under these conditions, the macrophages did not accumulate CE in their cytosol even after incubation with cholesterol-containing liposomes (see Fig. 3A). Therefore, the efflux of CE droplet-derived cholesterol appeared to be almost negligible under our conditions.

Using this system, we have demonstrated that UC that had accumulated within the lysosomes of cultured mouse peritoneal macrophages is released into the medium through at least two distinct pathways. The first pathway is completely suppressed by pregnenolone treatment. Inter-

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

estingly, most of the cholesterol secreted through this pregnenolone-sensitive pathway was associated with the HDL-like particles which were synthesized and secreted by the macrophages. These HDL-like particles were unique in that they contained PC, SM, and UC as their lipid components but no other neutral lipids such as CE, and their sole protein component was apo E. Cholesterol efflux *via* the pregnenolone-sensitive pathway was not appreciably affected by the exogenous HDL. The second pathway was pregnenolone-insensitive. Cholesterol efflux occurred through this route irrespective of the production of HDL-like particles, but was greatly stimulated when exogenous HDL were added to the medium. Indeed, the secreted cholesterol was associated with these exogenously added HDL (data not shown).

It was previously demonstrated that macrophages release their accumulated cholesterol even when they are incubated in medium without any added cholesterol acceptors such as HDL, and that apo E-containing lipoprotein particles produced by macrophages may mediate the efflux of accumulated cholesterol (*19*). Kruth *et al.* (*20*, *21*) also demonstrated that human monocyte-derived macrophages produce two types of lipid particles, discoidal and vesicular lipoprotein particles. The discoidal particles contain apo E, whereas the vesicles contain a protein with a molecular mass of 22,000 daltons. The HDL-like particles that we detected on NaBr-density gradient analysis are similar to those that Kruth *et al.* observed using human monocyte-derived macrophages, except that they only contained apo E as a protein constituent (Fig. 6A). The difference between the results of these two studies may be due to differences in the cell types used or the experimental protocols. According to our data, the production of HDL-like particles was not a constitutive process. The accumulation of UC within the lysosomal compartment is not a stimulant of HDL-like particle production, but their production is greatly stimulated when this cholesterol is released from the lysosomes (Figs. 4, 5, and 6). It should be noted that HDL-like particles were formed only when lysosomal cholesterol was released *via* the pregnenolone-sensitive pathway.

The transport of lysosomal cholesterol to other intracellular sites is the subject of much debate. Lange *et al.* (*22*) used FU5AH rat hepatoma cells to demonstrate that all LDL-derived cholesterol is transported to the plasma membrane before moving to the ER, and that the inhibition of LDL-cholesterol transport to ACAT caused by progesterone and hydrophobic amines is due solely to a block in plasma membrane-to-ER movement. If this is also true for mouse peritoneal macrophages, lysosomal cholesterol transported *via* the pregnenolone-sensitive pathway may primarily enter the plasma membrane, from where UC and some phospholipids are released into the medium by associating with the exogenously secreted apo E. However, Underwood *et al.* (*23*) presented evidence of the presence of an alternative LDL-cholesterol transport pathway from lysosomes to the ER that is independent of the plasma membrane. This pathway is more sensitive to U18666A, another inhibitor of lysosomal cholesterol transport, than is cholesterol movement from lysosomes to the plasma membrane and from the plasma membrane to the ER. We hypothesize at present that macrophages may utilize both the lysosome-to-plasma membrane and lysosome-to-ER pathways, the latter of which is much more sensitive to pregnenolone, for the metabolism and secretion of lysosomal cholesterol. We also suppose that the former pathway may have high affinity but low capacity for lysosomal cholesterol, whereas the latter pathway may have low affinity but high capacity for it. In our experiments, we analyzed the secretion of lysosomal cholesterol using macrophages that had accumulated UC in their lysosomes. Under these conditions, the lysosome-to-ER pathway may dominate the pregnenolone-sensitive secretion of lysosomal cholesterol, since the budding off of the bulk of phospholipids and UC from the ER into the lumenal spaces might explain why extracellular HDL did not stimulate the efflux of cholesterol derived *via* the pregnenolone-sensitive pathway. The complete inhibition by pregnenolone of CE formation, which occurs in the ER membrane (Fig. 1B), may also support this idea.

We are uncertain as to where apo E associates with secreted phospholipids and cholesterol. Phospholipid vesicles carrying UC may be budded off from the ER membrane, associate with newly synthesized apo E in the lumenal space, and then be secreted into the medium. There is, however, evidence that apo E associates with lipid particles after their secretion rather than within the cells based on the observation that adding anti-apo E to the culture medium inhibited cholesterol and phospholipid efflux from human monocyte-derived macrophages (*24*). Phospholipid vesicles formed in the lumenal spaces could possibly be secreted into the medium *via* the ER-Golgi pathway and then associate with apo E in the extracellular medium. At present, we do not have any data refuting this. Differentiated human monocyte-derived macrophages are known to secrete significant amounts of apo E irrespective of cholesterol enrichment (*24*), which indicates that apo E synthesis and secretion occur independently of cholesterol secretion. In contrast, the synthesis and secretion of apo E in mouse peritoneal macrophages appears to be coupled to cellular cholesterol enrichment (*25*, *26*). Therefore, the mechanism for the production of apo E-containing HDL-like particles may differ with the cell type. It is certain that further studies are needed to delineate the mechanisms involved in this process in macrophages. In addition, identification of a target(s) of lysosomal cholesterol transport inhibitors will increase our understanding of the mechanisms underlying intracellular cholesterol transport and the production of HDL-like particles (*27*). The product of a causative gene for Niemann-Pick type C disease could be the target of cholesterol transport inhibitors (*28*, *29*), since fibroblasts obtained from patients exhibit a defect in the movement of free cholesterol out of the lysosomes (*30*).

In contrast to the pregnenolone-sensitive pathway, the pregnenolone-insensitive pathway is greatly stimulated by exogenous HDL. This may indicate that cholesterol transported *via* the pregnenolone-insensitive pathway primarily reaches the plasma membrane, where it is effluxed into the extracellular medium. Most of the cholesterol released through this pathway was recovered in the bottom fraction ($d > 1.25$) on NaBr density gradient centrifugation, when lipoprotein-depleted fetal calf serum was used as a constituent of the culture medium. This fraction also contained most of the serum proteins. Upon separation of this fraction by gel filtration chromatography, the secreted cholesterol was eluted at a position corresponding to a molecular weight of 70,000–100,000 (data not shown). This indicates that the cholesterol in this fraction may be associated with

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

a certain serum protein(s). Although extracellular lipoproteins may be the most efficient acceptors for cholesterol, in the absence of such lipoproteins, macrophages can still release cholesterol using serum protein(s) as an acceptor. This will be the case in the subendotherial spaces of arterial walls. Under these circumstances, the pregnenolone-sensitive pathway seems to control the secretion of lysosomal cholesterol, compared with the pregnenolone-insensitive pathway, based on the observation that pregnenolone treatment causes the accumulation of UC in the lysosomes of macrophages incubated in a medium depleted of plasma lipoproteins.

In conclusion, we have demonstrated that macrophages release lysosomal cholesterol *via* both pregnenolone-sensitive and -insensitive pathways, and that only the cholesterol secreted through the pregnenolone-sensitive pathway is associated with endogenously synthesized HDL-like particles. The lipid components of these particles are PC and SM, while the sole protein component is apo E. Moreover, we found that the pregnenolone-sensitive pathway operated independently of the presence or absence of exogenous HDL, whereas secretion *via* the pregnenolone-insensitive pathway was greatly stimulated by exogenously added HDL.

REFERENCES

1. Goldstein, J.L., Ho, Y.K., Basu, S.K., and Brown, M.S. (1979) Binding site on macrophages that mediates uptake and degradation of acetylated low density lipoprotein, producing massive cholesterol deposition. *Proc. Natl. Acad. Sci. USA* **76**, 333–337
2. Brown, M.S., Goldstein, J.L., Krieger, M., Ho, Y.K., and Anderson, R.G. (1979) Reversible accumulation of cholesteryl esters in macrophages incubated with acetylated lipoproteins. *J. Cell Biol.* **82**, 597–613
3. Schaffner, T., Taylor, K., Bartucci, E.J., Fischer-Dzoga, K., Beeson, J.H., Glagov, S., and Wissler, R.W. (1980) Arterial foam cells with distinctive immunomorphologic and histochemical features of macrophages. *Am. J. Pathol.* **100**, 57–73
4. Gerrity, R.G. (1981) The role of the monocyte in atherogenesis: I. Transition of blood-borne monocytes into foam cells in fatty lesions. *Am. J. Pathol.* **103**, 181–190
5. Brown, M.S., Ho, Y.K., and Goldstein, J.L. (1980) The cholesteryl ester cycle in macrophage foam cells. Continual hydrolysis and re-esterification of cytoplasmic cholesteryl esters. *J. Biol. Chem.* **255**, 9344–9352
6. Glick, J.M., Adelman, S.J., and Rothblat, G.H. (1987) Cholesteryl ester cycle in cultured hepatoma cells. *Atherosclerosis* **64**, 223–230
7. Brown, M.S. and Goldstein, J.L. (1983) Lipoprotein metabolism in the macrophage: implications for cholesterol deposition in atherosclerosis. *Annu. Rev. Biochem.* **52**, 223–261
8. Hakamata, H., Miyazaki, A., Sakai, M., Suginohara, Y., Sakamoto, Y.I. and Horiuchi, S. (1994) Species difference in cholesteryl ester cycle and HDL-induced cholesterol efflux from macrophage foam cells. *Arterioscler. Thromb.* **14**, 1860–1865
9. Johnson, W.J., Chacko, G.K., Phillips, M.C., and Rothblat, G.H. (1990) The efflux of lysosomal cholesterol from cells. *J. Biol. Chem.* **265**, 5546–5553
10. Butler, J.D., Blanchette-Mackie, J., Goldin, E., O'Neil, R.R., Carstea, G., Roff, C.F., Patterson, M.C., Patel, S., Comly, M.E., Cooney, A., Vanier, M.T., Brady, R.O., and Pentchev, P.G. (1992) Progesterone blocks cholesterol translocation from lysosomes. *J. Biol. Chem.* **267**, 23797–23805
11. Aikawa, K., Furuchi, T., Fujimoto, Y., Arai, H., and Inoue, K. (1994) Structure-specific inhibition of lysosomal cholesterol transport in macrophages by various steroids. *Biochim. Biophys. Acta* **1213**, 127–134
12. Liscum, L. and Faust, J.R. (1989) The intracellular transport of low density lipoprotein-derived cholesterol is inhibited in Chinese hamster ovary cells cultured with 3-β-[2-(diethylamino)-ethoxy]androst-5-en-17-one. *J. Biol. Chem.* **264**, 11796–11806
13. Furuchi, T., Aikawa, K., Arai, H., and Inoue, K. (1993) Bafilomycin A1, a specific inhibitor of vacuolar-type H^+-ATPase, blocks lysosomal cholesterol trafficking in macrophages. *J. Biol. Chem.* **268**, 27345–27348
14. Basu, S.K., Goldstein, J.L., Anderson, R.G.W., and Brown, M.S. (1976) Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. *Proc. Natl. Acad. Sci. USA* **73**, 3178–3182
15. Goldstein, J.L. and Brown, M.S. (1974) Binding and degradation of low density lipoproteins by cultured human fibroblasts. Comparison of cells from a normal subject and from a patient with homozygous familial hypercholesterolemia. *J. Biol. Chem.* **249**, 5153–5162
16. Nishikawa, K., Arai, H., and Inoue, K. (1990) Scavenger receptor-mediated uptake and metabolism of lipid vesicles containing acidic phospholipids by mouse peritoneal macrophages. *J. Biol. Chem.* **265**, 5226–5231
17. Bielicki, J.K., Johnson, W.J., Glick, J.M., and Rothblat, G.H. (1991) Efflux of phospholipid from fibroblasts with normal and elevated levels of cholesterol. *Biochim. Biophys. Acta* **1084**, 7–14
18. Avart, S.J., Bernard, D.W., Jerome, W.G., and Glick, J.M. (1999) Cholesteryl ester hydrolysis in J774 macrophages occurs in the cytoplasm and lysosomes. *J. Lipid Res.* **40**, 405–414
19. Huang, Y., von Eckardstein, A., Wu, S., Maeda, N., and Assmann, G. (1994) A plasma lipoprotein containing only apolipoprotein E and with gamma mobility on electrophoresis releases cholesterol from cells. *Proc. Natl. Acad. Sci. USA* **91**, 1834–1838
20. Kruth, H.S., Skarlatos, S.I., Gaynor, P.M., and Gamble, W. (1994) Production of cholesterol-enriched nascent high density lipoproteins by human monocyte-derived macrophages is a mechanism that contributes to macrophage cholesterol efflux. *J. Biol. Chem.* **269**, 24511–24518
21. Kruth, H.S. (1997) The fate of lipoprotein cholesterol entering the arterial wall. *Curr. Opin. Lipidol.* **8**, 246–252
22. Lange, Y., Ye, J., and Chin, J. (1997) The fate of cholesterol exiting lysosomes. *J. Biol. Chem.* **272**, 17018–17022
23. Underwood, K.W., Jacobs, N.L., Howley, A., and Liscum, L. (1998) Evidence for a cholesterol transport pathway from lysosomes to endoplasmic reticulum that is independent of the plasma membrane. *J. Biol. Chem.* **273**, 4266–4274
24. Zhang, W.Y., Gaynor, P.M., and Kruth, H.S. (1996) Apolipoprotein E produced by human monocyte-derived macrophages mediates cholesterol efflux that occurs in the absence of added cholesterol acceptors. *J. Biol. Chem.* **271**, 28641–28646
25. Mazzone, T., Gump, H., Diller, P., and Getz, G.S. (1987) Macrophage free cholesterol content regulates apolipoprotein E synthesis. *J. Biol. Chem.* **262**, 11657–11662
26. Mazzone, T., Basheeruddin, K., and Poulos, C. (1989) Regulation of macrophage apolipoprotein E gene expression by cholesterol. *J. Lipid Res.* **30**, 1055–1064
27. Underwood, K.W., Andemariam, B., McWilliams, G.L., and Liscum, L. (1996) Quantitative analysis of hydrophobic amine inhibition of intracellular cholesterol transport. *J. Lipid Res.* **37**, 1556–1568
28. Carstea, E.D., Morris, J.A., Coleman, K.G., Loftus, S.K., Zhang, D., Cummings, C., Gu, J., Rosenfeld, M.A., Pavan, W.J., Krizman, D.B., Nagle, J., Polymeropoulos, M.H., Sturley, S.L., Ioannou, Y.A., Higgins, M.E., Comly, M., Cooney, A., Brown, A., Kaneski, C.R., Blanchette-Mackie, E.J., Dwyer, N.K., Neufeld, E.B., Chang, T.Y., Strauss, J.F., Ohno, K., Zeigler, M., Carmi, R., Sokol, J., Markie, D., O'Neil, R.R., van Diggelen, O.P., Elleder, M., Patterson, M.C., Brady, R.O., Vanier, M., Pentchev, P.G., and Tagle, D.A. (1997) Niemann-Pick C1 disease gene: homology to mediators of cholesterol homeostasis. *Science* **277**, 228–231
29. Loftus, S.K., Morris, J.A., Carstea, E.D., Gu, J.Z., Cummings, C., Brown, A., Ellison, J., Ohno, K., Rosenfeld, M.A., Tagle,

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012

D.A., Pentchev, P.G., and Pavan, W.J. (1997) Murine model of Niemann-Pick C disease: mutation in a cholesterol homeostasis gene. *Science* **277**, 232–235

30. Pentchev, P.G., Brady, R.O., Blanchette-Mackie, E.J., Vanier, M.T., Carstea, E.D., Parker, C.C., Goldin, E., and Roff, C.F. (1994) The Niemann-Pick C lesion and its relationship to the intracellular distribution and utilization of LDL cholesterol. *Biochim. Biophys. Acta* **1225**, 235–243

Downloaded from http://jb.oxfordjournals.org/ at Peking University on October 1, 2012