Cholesterol Efflux from Macrophage Foam Cells Is Enhanced by Active Phospholipid Transfer Protein through Generation of Two Types of Acceptor Particles†

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ABSTRACT: Phospholipid transfer protein (PLTP) is expressed by macrophage-derived foam cells in human atherosclerotic lesions, suggesting a regulatory role for PLTP in cellular cholesterol homeostasis. However, the exact role of PLTP in the reverse cholesterol transport pathway is not known. PLTP is present in plasma as two forms, a highly active (HA-PLTP) and a lowly active (LA-PLTP) form. In this study we clarify the role of the two forms of PLTP in cholesterol efflux from [^{3}H]cholesterol oleate−acetyl-LDL-loaded THP-1 macrophages. Incubation of HDL in the presence of HA-PLTP resulted in the formation of two types of acceptor particles, preβ-HDL and large fused HDL. HA-PLTP increased preβ-HDL formation and caused a 42% increase in [^{3}H]cholesterol efflux to HDL, while LA-PLTP neither formed preβ-HDL nor increased cholesterol efflux. Removal of the formed preβ-HDL by immunoprecipitation decreased cholesterol efflux by 47%. Neither HA- nor LA-PLTP enhanced cholesterol efflux to lipid-free apoA-I. Importantly, also the large fused HDL particles formed during incubation of HDL with HA-PLTP acted as efficient cholesterol acceptors. These observations demonstrate that only HA-PLTP increases macrophage cholesterol efflux, via formation of efficient cholesterol acceptors, preβ-HDL and large fused HDL particles.

An inverse relationship between the risk for developing premature coronary heart disease and the serum level of HDL cholesterol has been substantiated in a number of epidemiological and interventional studies. HDL protects against atherosclerosis by several mechanisms. The best known mechanism is the role of HDL in reverse cholesterol transport (RCT).[1] RCT is the pathway by which excess cholesterol is removed from peripheral cells and transported to the liver for excretion (*1*). This route is of special importance for macrophages, which are transformed to cholesterol-loaded foam cells in the presence of excess cholesterol, a key feature of atherosclerosis. HDL and its apolipoproteins facilitate the removal of cholesterol from these macrophage foam cells. This process is mediated by ATP-binding cassette (ABC) transporters A1 (ABCA1) and G1 (ABCG1) (*2*) as well as scavenger receptor class B type I (SR-BI) (*3*). ABCA1 promotes efflux of phospholipids and free cholesterol to lipid-poor apolipoproteins, e.g., apoA-I and apoE, to generate nascent HDL particles (*4*). ABCA1, which is essential for the formation of HDL particles, has been shown to be defective in patients with Tangier's disease, leading to extremely low HDL levels (*5*−*7*). Another ABC transporter, ABCG1, is also expressed in macrophages and endothelial cells and promotes cholesterol removal from macrophages to spherical HDL particles (*2*, *8*).

Circulating HDL is a heterogeneous population of particles with a distinct size, composition, charge, and function. Several plasma factors, such as cholesterol ester transfer protein (CETP), lecithin:cholesterol acyltransferase (LCAT), and various lipases are involved in the continuous remodeling of HDL, causing this heterogeneity (*9*). A central protein in HDL remodeling is the phospholipid transfer protein (PLTP) (*10*, *11*). PLTP has two major functions in HDL metabolism: (i) it facilitates the transfer of phospholipids between lipoproteins (*12*, *13*), and (ii) it mediates HDL conversion, a process generating large fused HDL particles and small preβ-HDL particles (*14*−*16*). The particles with preβ mobility have been demonstrated to act as efficient acceptors of cholesterol from fibroblasts and macrophages (*17*−*19*).

The presence of PLTP in atherosclerotic lesions and its expression in macrophage foam cells (*20*−*22*) suggest that PLTP plays a role in cholesterol retention or removal from foam cells present in the lesions and may thus be either pro- or antiatherogenic (*23*). Studies using bone marrow transplantation have given contradictory results concerning the role of macrophage-derived PLTP in atherogenesis (*24*−*26*). Recent studies in murine macrophages suggest that exogenous and endogenous PLTPs participate in cholesterol removal from these cells (*27*, *28*). However, the exact mechanism by which PLTP improves lipid efflux from macrophage foam cells is not known. Oram et al. (*28*)

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[1] Abbreviations: ABCA1/ABCG1, ATP-binding cassette transporter A1/G1; acLDL, acetylated LDL; apo, apolipoprotein; CETP, cholesterol ester transfer protein; HA-PLTP, highly active form of PLTP; LA-PLTP, lowly active form of PLTP; LPDS, lipoprotein-deficient serum; PC, phosphatidylcholine; PL, phospholipid; PLTP, phospholipid transfer protein; RCT, reverse cholesterol transport; SR-BI, scavenger receptor BI.

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suggested, on the basis of data obtained in BHK cells overexpressing human ABCA1, that active recombinant PLTP, but not one inactivated by four point mutations, is in the absence of HDL acceptors able to promote cholesterol removal from cells. Furthermore, the authors suggested that the underlying mechanism involves direct interaction of PLTP with ABCA1. In human plasma, PLTP is present as two distinct forms, one with high (HA-PLTP) and one with low (LA-PLTP) specific activity (*29*, *30*). The two forms exist as different macromolecular assemblies in circulation. The mechanisms by which LA-PLTP are generated and its functions are at present unknown. The data by Oram et al. (*28*) and Curtiss et al. (*20*) leaves open the possibility that also LA-PLTP might act in reverse cholesterol transport. It was therefore of interest to specifically address the role of HA- and LA-PLTP in cholesterol efflux from human THP-1 macrophage foam cells and to reveal the underlying mechanisms. Our results demonstrate that, of the two PLTP forms, only HA-PLTP promotes macrophage cholesterol efflux. The enhancement is due to generation of both preβ-HDL and large fused HDL particles, which act as efficient cholesterol acceptors.

EXPERIMENTAL PROCEDURES

Isolation of Plasma Lipoproteins. LDL and HDL were isolated from fresh normolipidemic human plasma by sequential ultracentrifugation in the density ranges 1.019−1.063 and 1.12−1.21 g/mL using KBr for density adjustments (*31*). Isolated HDL was depleted of apoE by affinity chromatography using a 1 mL HiTrap heparin column (Amersham Biosciences AB, Uppsala, Sweden) with 25 mM Tris, pH 7.4, containing 1 mM EDTA as the running buffer. The nonbound fractions contained apoE-free HDL that was sterile-filtered using Millex-HV filter units (filter diameter 33 mm, filter pore size 0.45 μm, hydrophilic PVDF membrane, Millipore, Billerica, MA) prior to use. LDL was acetylated in the presence of acetic anhydride (*32*), and the acetylated LDL was filtered using sterile plastic syringe filters (filter diameter 26 mm, filter pore size 0.2 μm, SFCA membrane; Corning, Corning, NY). Thereafter, acetyl-LDL (acLDL) was labeled by treatment with [1α,2α(n)-^{3}H]-cholesteryl oleate (Amersham Biosciences, Buckinghamshire, U.K.) dissolved in dimethyl sulfoxide. The amounts of LDL and HDL used in experiments are expressed as their total protein content. Lipoprotein-deficient serum (LPDS; $d > 1.25$ g/mL) was prepared from fetal bovine serum (FBS) by sequential ultracentrifugation and sterile-filtered prior to use.

Purification of the Highly Active (HA) and Lowly Active (LA) Forms of Human Plasma PLTP. HA-PLTP was isolated as described earlier (*33*) except that the Mono Q HR 5/5 column was not used for the purification. After isolation of HDL by ultracentrifugation, it was washed at a density of 1.21 g/mL, and the lowly active form of PLTP was isolated from the bottom fraction of this wash and used for further purification. First, 50 U/mL Trasylol and 5 mM β-mercaptoethanol were added, and the preparation was applied to a Butyl-Toyopearl 650(M) column equilibrated with 10 mM Tris−HCl, pH 7.4, containing 2 M NaCl and 1 mM EDTA, and recycled overnight. After washing of the column with 50 mM Tris−HCl, pH 7.4, containing 1 mM EDTA, PLTP was eluted with 50% (v/v) ethanol. Fractions containing PLTP were combined and dialyzed against phosphate-buffered saline (PBS), pH 7.4, and applied to an anti-PLTP Mab 66 column equilibrated with PBS. The column was washed in three steps: first with PBS, then with PBS containing 0.2% Tween 20, and finally again with PBS. The bound material was eluted with 0.1 M glycine, pH 2.5, into tubes containing 1 M Tris−HCl, pH 8.8, for neutralization. Fractions containing PLTP were combined and dialyzed against PBS, and the concentration of PLTP was determined. The HA-PLTP preparation displayed a single 80 kDa band in silver-stained gels (data not shown). The LA-PLTP was similar to that characterized previously (*30*) but devoid of immunodetectable apoA-I or apoE.

Assay of PLTP Activity and PLTP Concentration. For the radiometric PLTP activity assay, phosphatidylcholine (PC) liposomes were prepared essentially as described by Damen et al. (*34*), and the activity assay was carried out as described (*14*). The PLTP mass was determined using an ELISA method (*35*) based on the procedures reported earlier (*36*, *37*).

Quantification of ApoA-I and ApoE. ApoA-I and apoE were quantified using a noncompetitive ELISA assay (*38*).

Serum Lipid Analysis. Total cholesterol (CHOD-PAP 1489232 kit, Roche Diagnostics GmbH, Mannheim, Germany), choline-containing phospholipids (990-54009, Wako Chemicals GmbH, Neuss, Germany), and triglycerides (GPO-PAP 1488872 kit, Roche Diagnostics) were measured using enzymatic methods.

Cell Culture and Loading of THP-1 Cells with Cholesteryl Esters. Human THP-1 monocytes were purchased from the American Type Culture Collection (ATCC, Manassas, VA, catalog no. TIB-202). The monocytes were grown and maintained in complete RPMI 1640 medium containing 10% (v/v) FBS, 10 mM Hepes, pH 7.4, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C under 5% CO_2 and 95% air, until the experimental treatments. To differentiate the monocytes into macrophages, the cells were plated onto 24-well plates and treated with 100 nM phorbol 12-myristate 13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO) in the growth medium for 72 h prior to the experiment. The macrophages were washed twice with PBS and loaded by incubating them in the presence of 25 μg of protein/well of [1α,2α(n)-^{3}H]cholesteryl oleate−acLDL in RPMI 1640 supplemented with 5% (v/v) LPDS, 10 mM Hepes, pH 7.4, and penicillin/streptomycin for 48 h. The loaded macrophages were washed twice with PBS and incubated for 24 h in serum-free RPMI 1640 medium supplemented with 10 mM Hepes, pH 7.4, and antibiotics to allow equilibration of the radioactive label in free and esterified cholesterol pools (*32*). After equilibration, the cells were washed twice with PBS, and cholesterol acceptors were added to analyze cholesterol removal from the cells.

Isolation of Primary Monocytes from Blood Samples. Human monocyte-derived macrophages were obtained by cell culturing from 60 mL of fasting blood containing citrate as an anticoagulant. The buffy coat was separated by centrifugation at 2800 rpm for 15 min at room temperature. The buffy coat was diluted with PBS, layered over Ficoll-Paque PLUS (Amersham Biosciences AB), and centrifuged at 2300 rpm for 30 min at room temperature. The mononuclear cells were recovered as a cell layer on top of Ficoll-Paque PLUS. Isolated mononuclear cells were washed three times with PBS to remove excess platelets, and finally the cell pellet

was suspended in DMEM (with 4.5 g/L glucose, with L-glutamine, without sodium pyruvate) (Cambex Bio Science Verviers, Belgium) supplemented with penicillin/streptomycin. The cells were counted, plated on 24-well plates (1.5×10^6 cells per well), and allowed to attach to the wells for 1 h. Next, the cells were washed three times with PBS and macrophage SFM medium (Invitrogen Corp., Carlsbad, CA) supplemented with GM-CSF (Nordic Biosite AB, Täby, Sweden), and penicillin/streptomycin was added. The medium was changed every 2−3 days. After 7 days in culture the monocytes were morphologically converted to macrophages which were washed twice with PBS and loaded with 25 μg of protein/well of [1α,2α(n)-^{3}H]cholesteryl oleate−acLDL in DMEM supplemented with penicillin/streptomycin for 48 h. The loaded cells were washed three times with PBS, and cholesterol acceptors were added to measure cholesterol efflux.

Cholesterol Efflux Assay. To measure cholesterol efflux, cholesterol-loaded THP-1 macrophages (see above) were incubated in serum-free RPMI 1640 supplemented with 10 mM Hepes, pH 7.4, and antibiotics with or without indicated acceptor particles. Lipid-free apoA-I (kindly obtained from Dr. Peter Lerch, Swiss Red Cross Laboratory) and apoE-free HDL_3 were used as cholesterol acceptors. The efflux conditions were first optimized for THP-1 macrophage foam cells, and on the basis of these results, an HDL concentration of 15 μg/mL, an apoA-I concentration of 10 μg/mL, and a efflux time of 16 h were chosen for further experiments. Similar conditions have been widely used in efflux experiments. Control wells were incubated in the absence of acceptor particles. After incubation for 16 h at 37 °C in a humidified CO_2 incubator, the medium was collected and centrifuged at 2500 rpm for 5 min to remove detached cells. Radioactivity in the medium was determined by liquid scintillation counting (Wallac WinSpectral 1414, Wallac, Turku, Finland). The cells were washed twice with PBS and lysed with 0.2 M NaOH. The cell lysates were analyzed for radioactivity and for total cell protein. Cholesterol efflux was expressed as the percentage of the medium [^{3}H]cholesterol radioactivity of the total [^{3}H]cholesterol radioactivity measured from the medium and the cells.

For quantification of cholesterol efflux from human monocyte-derived macrophages, loaded macrophages were incubated with cholesterol acceptors in DMEM supplemented with penicillin/streptomycin for 16 h. Radioactivity in the medium and cells and the protein concentration of the cell lysates were analyzed, and cholesterol efflux was expressed as with THP-1 cells.

Incubation of HDL and Apolipoprotein A-I in the Presence of PLTP. The effect of PLTP on cholesterol efflux to HDL_3 was studied by preincubating apoE-free HDL for 24 h at 37 °C with (i) the active form of human plasma PLTP (300 μg of HDL protein, PLTP activity of 6000 nmol/h, PLTP mass of 14.2 μg), (ii) the inactive form of human plasma PLTP (100 μg of HDL protein, PLTP mass of 4.7 μg), or (iii) PBS only serving as a control. To study the effects of different forms of human plasma PLTP on cholesterol efflux to apoA-I, apoA-I (100 μg of apoA-I protein) was incubated for 24 h at 37 °C with (i) the active form of PLTP (PLTP activity of 1000 nmol/h, PLTP mass of 2 μg), (ii) the inactive form of PLTP (PLTP mass of 2 μg), or (iii) PBS only as a control. After 24 h of preincubation at 37 °C, HDL and apoA-I with or without PLTP treatment were diluted in RPMI 1640 supplemented with 10 mM Hepes, pH 7.4, and antibiotics, and 1 mL of these dilutions (15 μg/mL HDL_3 protein or 10 μg/mL apoA-I) was added to the cholesterol-loaded THP-1 macrophage foam cells for efflux measurements (16 h).

To further study the effect of PLTP on cholesterol efflux, cholesterol acceptors for loaded human monocyte-derived macrophages were prepared by preincubating HDL (350 μg of HDL protein) for 24 h at 37 °C with (i) the active form of human plasma PLTP (PLTP activity of 2000 nmol/h, PLTP mass of 3.2 μg), (ii) the inactive form of human plasma PLTP (PLTP mass of 3.2 μg), or (iii) PBS only serving as a control. The cells were incubated with cholesterol acceptors (25 μg of HDL/well) in DMEM supplemented with penicillin/streptomycin for 16 h. To analyze cholesterol efflux from the loaded macrophages to PLTP protein alone, the corresponding activities and concentrations of HA-PLTP and LA-PLTP as in diluted preincubation mixtures were incubated with loaded THP-1 macrophages and human monocyte-derived macrophages for 16 h.

HDL Conversion and Quantification of Preβ-HDL. The amount of preβ-HDL formed during 24 h of preincubation of HDL_3 in the presence or the absence of PLTP was quantified by resolving the samples (1−2 μg of protein) by two-dimensional crossed immunoelectrophoresis (*39*). The amount of preβ-HDL was expressed as a percentage of the area of the preβ-mobile peak from the sum of the preβ- and α-mobile areas.

Isolation of Preβ-HDL and Large Fused HDL Particles. To analyze cholesterol efflux to particles generated during incubation of HDL with PLTP, preβ-HDL and large fused HDL particles were isolated. First, HDL was preincubated in the presence or the absence of the active form of human plasma PLTP for 24 h at 37 °C. The incubation mixtures contained 4 mg of HDL and a PLTP activity of 8000 nmol/h (PLTP mass of 9.0 μg). The control reaction contained the corresponding amount of HDL incubated in PBS. The HDL conversion products, small preβ-HDL and large fused HDL particles, were isolated by size-exclusion chromatography using two Superose 6 HR 10/30 gel filtration columns (Amersham Biosciences AB) in tandem. Chromatography was performed at a flow rate of 0.5 mL/min, and 0.5 mL fractions were collected. Fractions containing preβ-HDL and large fused HDL particles were combined, and Nanosep 10K Omega-tubes (Pall Life Sciences, Michigan) were used to concentrate the pooled fractions. HDL incubated in the presence or the absence of HA-PLTP as well as isolated preβ-HDL and large fused HDL particles were analyzed with native gradient gel electrophoresis (GGE) (*40*). The protein concentration of the preparations was determined, after which these preparations were diluted in serum-free RPMI 1640 supplemented with 10 mM Hepes, pH 7.4, and antibiotics and used as cholesterol acceptors (preβ-HDL, 5 and 10 μg/well; large fused HDL particles, 5, 10, and 20 μg/well) for cholesterol-loaded THP-1 macrophages.

Immunoprecipitation of Preβ-HDL. We utilized anti-preβ_1-HDL monoclonal antibody 55201 (*41*) (Daiichi Pure Chemicals Co., Tokyo, Japan) to immunoprecipitate preβ-HDL particles formed in the HDL preparations incubated in the presence or the absence of active PLTP (300 μg of HDL_3 protein, PLTP activity of 6000 nmol/h, PLTP mass of 14.2 μg). Anti-preβ_1-HDL antibody (100 μg) was coupled to

Protein G-Sepharose beads (Amersham Biosciences AB) and incubated with the HDL_3 preparations (100 μg of HDL_3 protein) for 2 h at 4 °C. After incubation, the Protein G-Sepharose beads were pelleted by centrifugation at 3500 rpm for 5 min, and the nonprecipitated HDL fractions were used as cholesterol acceptors. Control reactions were carried out from samples incubated with PBS only. The performance of the immunoprecipitation was verified by crossed immunoelectrophoresis (*39*).

Other Methods. The protein concentration was determined by the Lowry method using bovine serum albumin as a standard (*42*). Lipids from the efflux media were extracted by the addition of hexane−2-propanol (3:2, v/v), lipid extracts separated by high-performance TLC, and finally their quantities determined with an automatic plate scanner (CAMAG TLC) (*27*).

Statistical Analyses. The results are expressed as the mean ± standard deviation (SD). Efflux results were analyzed by the Student's *t* test for paired samples, and a *p* value of less than 0.05 was considered statistically significant.

RESULTS

HA-PLTP Facilitates Cholesterol Efflux from THP-1 Macrophages to HDL. We (*17*, *27*) and others (*28*) have reported that PLTP affects cholesterol efflux from macrophage foam cells. As PLTP circulates in plasma in two forms (*29*, *30*), we studied the role of these two forms in cholesterol efflux from macrophage foam cells. Our results demonstrate that preincubation of HDL in the presence of HA-PLTP increases cholesterol efflux from macrophages to HDL by 42% as compared to that of HDL preincubated in the absence of HA-PLTP ($p < 0.001$) (Figure 1a). LA-PLTP was not capable of promoting cholesterol efflux to HDL (Figure 1a). This observation demonstrates that PL transfer activity of PLTP is a prerequisite for enhanced cholesterol efflux from macrophages to HDL. When lipid-free apoA-I was used as a cholesterol acceptor, no increase in cholesterol efflux was observed independent of whether apoA-I was preincubated with HA- or LA-PLTP (Figure 1b). Furthermore, incubation of either HA-PLTP or LA-PLTP with the loaded macrophages alone, in the absence of acceptors, did not induce cholesterol efflux over background levels (data not shown).

HA-PLTP Generates Efficient Acceptors for Cholesterol Efflux. The above results suggest that the acceptor properties of HDL are changed during incubation in the presence of HA-PLTP. We therefore incubated HDL in the presence and absence of HA-PLTP and characterized the subpopulations of HDL formed. The incubation of HDL in the presence of HA-PLTP resulted in an increase of the preβ-HDL fraction from 5.2 ± 2.6% to 32.4 ± 9.4% ($n = 8$) of the total HDL apoA-I. To obtain further insight into the mechanism by which the active form of PLTP increases cholesterol efflux, preβ-HDL particles as well as large fused HDL particles generated by the action of HA-PLTP were separated using size-exclusion chromatography (Figure 2). During conversion, large fused HDL particles with an apparent size of 250 kDa were formed (D, fractions 53−57), while the apparent size of HDL particles before conversion was 140 kDa (C, fractions 55−59). LA-PLTP had no effect on the size of HDL particles (data not shown). Conversion was further verified using GGE analysis (Figure 2). In the preβ-HDL fraction

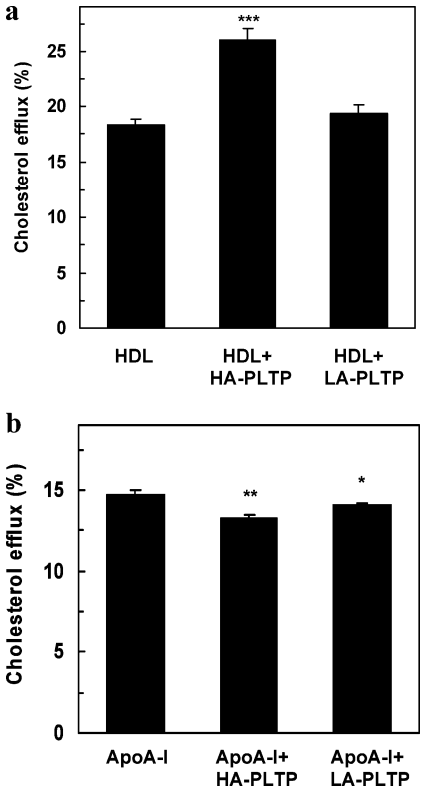

FIGURE 1: Effects of HA- and LA-PLTP on cholesterol efflux from THP-1 macrophage foam cells to HDL and apoA-I. To differentiate monocytic THP-1 cells into macrophages, the cells were treated with 100 nM PMA for 72 h prior to the experiment. The macrophages were incubated with [^{3}H]cholesteryl oleate−acLDL for 48 h, followed by incubation without cholesterol for 24 h. To study the effect of plasma PLTP on cholesterol efflux, HDL (a) and lipid-free apoA-I (b) were first incubated in the presence or the absence of HA-PLTP or LA-PLTP for 24 h at 37 °C. Cholesterol efflux was studied by incubating the loaded macrophages for 16 h in efflux media containing no acceptors (blank), 15 μg/mL HDL protein, or 10 μg/mL apoA-I protein. The figure is representative of 3−7 independent experiments, and each value is the mean (±SD) of triplicate incubations expressed as a percentage of [^{3}H]cholesterol released into the medium from the total [^{3}H]cholesterol in the medium and the cells. Values of the blank incubations are subtracted. Key: *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

(E, fractions 64−68) isolated by gel filtration, more than 94% of HDL migrated in the preβ-position (data not shown). Isolated preβ-HDL was a functional cholesterol acceptor in the efflux assay (Figure 3).

The development of monoclonal antibodies against preβ-HDL has made it possible to selectively study these particles. To immunoprecipitate preβ-HDL, we used an anti-preβ_1-HDL monoclonal antibody that has been used to immunoprecipitate pre-HDL (*41*). Immunoprecipitation of preβ-HDL was verified by crossed immunoelectrophoresis, which demonstrated that the antibody against preβ-HDL effectively removed preβ-HDL from the sample (preβ-HDL content 48.9% and 2.7%, before and after immunoprecipitation, respectively) (Figure 4a,b). The removal of preβ-HDL from the HDL preparation incubated with HA-PLTP caused a 47% decrease in cholesterol efflux as compared to that of the nonimmunoprecipitated control ($p < 0.001$) (Figure 4c). These results demonstrate that the effect of active PLTP on cholesterol efflux is mediated in part by its ability to enhance preβ-HDL formation.

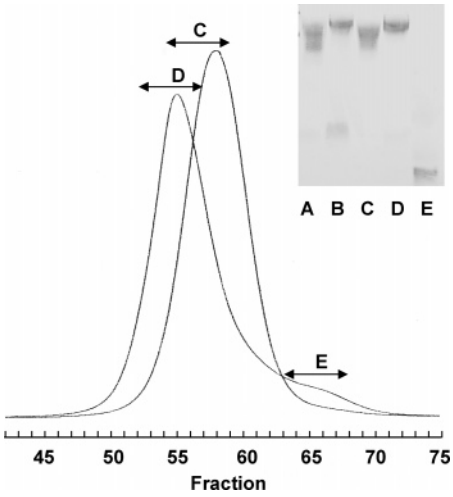

FIGURE 2: Isolation of preβ-HDL and large fused HDL particles generated by HA-PLTP. HDL was preincubated in the presence or the absence of HA-PLTP for 24 h at 37 °C. The HDL conversion products, preβ-HDL and large fused HDL particles, were isolated by size-exclusion chromatography using two Superose 6 HR 10/30 gel filtration columns in tandem. Fractions from HDL incubation in the presence of HA-PLTP containing large fused HDL particles (D, fractions 53−57) and preβ-HDL (E, fractions 64−68) and fractions from HDL incubation in the absence of HA-PLTP (C, fractions 55−59) were concentrated and used as cholesterol acceptors for cholesterol-loaded THP-1 macrophages. HDL incubated in the absence (inset, lane A) or the presence (inset, lane B) of HA-PLTP, large fused HDL particles (inset, lanes C in the absence of HA-PLTP and D in the presence of HA-PLTP), and preβ-HDL particles (inset, lane E) were analyzed using native GGE.

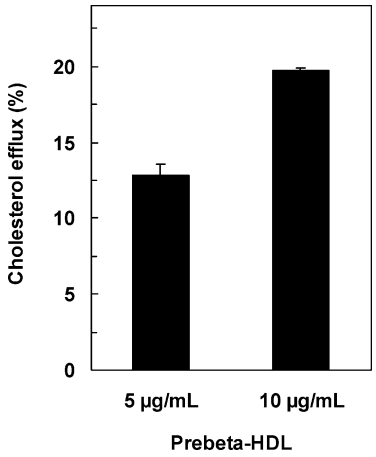

FIGURE 3: Cholesterol efflux from THP-1 macrophage foam cells to isolated preβ-HDL particles. Preβ-HDL particles formed following the incubation of HDL with HA-PLTP for 24 h at 37 °C were isolated using size-exclusion chromatography. The purity of the isolated preβ-HDL particles was determined by crossed immunoelectrophoresis, and the particles were used as cholesterol acceptors in the efflux experiments. THP-1 cells were treated as described in the caption for Figure 1. Cholesterol efflux was studied by incubating the loaded macrophages for 16 h in efflux media containing either 5 or 10 μg/mL preβ-HDL as a protein. The figure is representative of three independent experiments, and each value represents the mean (±SD) of triplicate incubations. The values of the blank incubations (mean 6.1%) containing no cholesterol acceptors were subtracted from the data.

As it has been shown that also spherical HDL particles can act as acceptors in cholesterol efflux (*2*), it was of interest to study cholesterol efflux to the large fused HDL particles generated by HA-PLTP. The large fused HDL particles isolated from PLTP-treated HDL preparations (see Figure 4) were more efficient acceptors of cholesterol as compared to the original HDL particles not treated with PLTP ($p < 0.01$) (Figure 5). To exclude the possibility of bare lipid exchange being a modifying factor, we also analyzed net transport of cholesterol by determining cholesterol in efflux media. The large fused HDL particles generated by PLTP released more cholesterol from macrophages as compared to the original HDL particles (+PLTP, 6.74 ± 0.33 μg, vs −PLTP, 5.38 ± 0.19 μg, $p = 0.004$). Compositional analyses revealed that the phospholipid (+PLTP, 21.3 ± 4.4%, vs −PLTP, 18.6 ± 4.9%, $n = 4$) and the triglyceride (+PLTP, 13.0 ± 1.9%, vs −PLTP, 11.7 ± 1.4%, $n = 4$) contents of these particles were slightly increased and the protein (+PLTP, 50.3 ± 4.1%, vs −PLTP, 55.3 ± 4.6%, $n = 4$) content was lower as compared to those of the particles not treated with HA-PLTP. The total cholesterol content of the

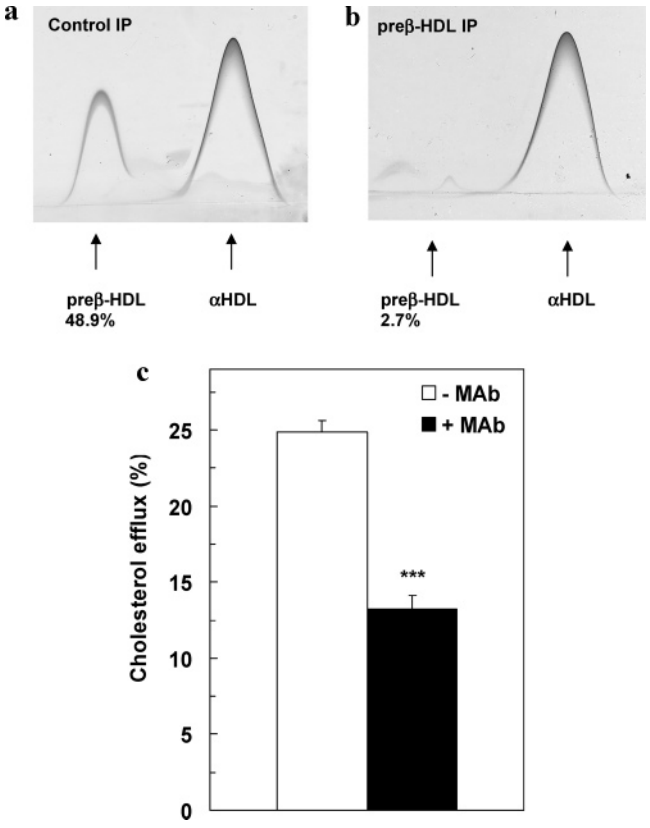

FIGURE 4: Effect of preβ-HDL removal by immunoprecipitation on cholesterol efflux from THP-1 macrophage foam cells. HDL was first incubated with HA-PLTP for 24 h at 37 °C, and thereafter, anti-preβ_1-HDL monoclonal antibody was used to immunoprecipitate preβ-HDL. The amounts of preβ-HDL left in PLTP-treated HDL preparations after control immunoprecipitation (a) and after immunoprecipitation with anti-preβ_1-HDL MAb (b) were quantified by two-dimensional crossed immunoelectrophoresis. The amounts of preβ-HDL particles are expressed as percentages of the total amounts of α-mobile and preβ-mobile HDL particles. Cholesterol efflux was measured by incubating the loaded macrophages for 16 h in efflux media containing either 15 μg/mL PLTP-treated HDL preparation (control immunoprecipitation) or the corresponding volume of PLTP-treated HDL immunoprecipitated with anti-preβ_1-HDL MAb (c). THP-1 cells were treated as described in the caption for Figure 1. The figure is representative of four independent experiments, and each value represents the mean (±SD) of triplicate incubations. The values of the blank incubations containing no cholesterol acceptors are subtracted from the values representing cholesterol efflux to PLTP-treated HDL preparations. Key: ***, $p < 0.001$.

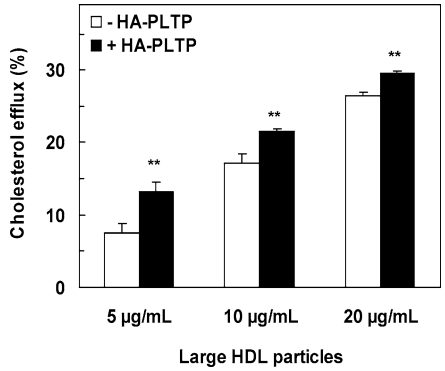

FIGURE 5: Cholesterol efflux from THP-1 macrophage foam cells to isolated large fused HDL particles. Large fused HDL particles formed during the incubation of HDL with HA-PLTP for 24 h at 37 °C were isolated using size-exclusion chromatography, and these particles were used as cholesterol acceptors. THP-1 cells were treated as described in the caption for Figure 1. Cholesterol efflux was studied by incubating the loaded macrophages for 16 h in efflux media containing 5, 10, or 20 μg/mL (protein) large fused HDL particles. The figure is representative of four independent experiments, and each value represents the mean (±SD) of triplicate incubations. The values of the blank incubations containing no cholesterol acceptors were subtracted from the data. The open bars represent incubations with the size-exclusion chromatography fractions for HDL incubated in the absence of HA-PLTP. Key: **, $p < 0.01$.

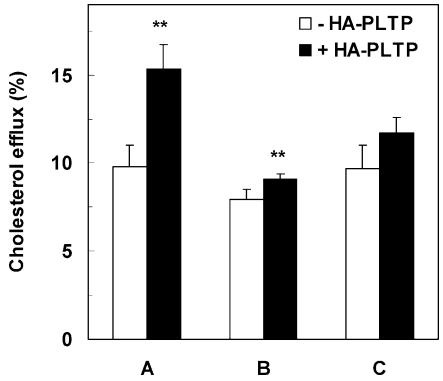

FIGURE 6: Effect of plasma HA-PLTP on cholesterol efflux from primary monocyte-derived macrophage foam cells to HDL. Human monocyte-derived macrophages from three subjects (A, B, C) were transformed to foam cells by incubation with [^{3}H]cholesteryl oleate−acLDL for 48 h. To study the effect of HA-PLTP on cholesterol efflux to HDL, HDL was first incubated in the presence or the absence of HA-PLTP for 24 h at 37 °C. Cholesterol efflux was studied by incubating the cells for 16 h in efflux media containing either no acceptors (blank) or 25 μg/mL HDL_3 protein. Each value represents the mean (±SD) of triplicate incubations. The values of the blank incubations were subtracted from the values representing cholesterol efflux to HDL. Key: **, $p < 0.01$.

particles did not differ (+PLTP, 15.3 ± 1.0%, vs −PLTP, 14.3 ± 0.9%). In addition, the relative apoE content of these large fused HDL particles was about 40% higher than that in the HDL particles without PLTP treatment.

HA-PLTP Enhances Cholesterol Efflux from Primary Human Macrophages. Since THP-1 is a modified cell line, we also studied the effect of HA- and LA-PLTP on cholesterol efflux from primary monocyte-derived macrophages isolated from human blood. Increased cholesterol efflux to HDL preincubated with HA-PLTP was observed (average increase in cholesterol efflux 31%, range 15−56%, $n = 3$) ($p < 0.01$) (Figure 6). Preincubation of HDL with LA-PLTP failed to enhance the cholesterol efflux (average increase in cholesterol efflux 3%, $n = 3$), confirming the observation from THP-1 cells that the phospholipid transfer activity of PLTP is necessary for facilitation of cholesterol efflux from macrophages.

DISCUSSION

Foam cell formation due to excessive accumulation of cholesterol in arterial macrophages is a pathological sign of progressing atherosclerosis. As macrophages are not able to limit their uptake of cholesterol, they depend on cholesterol efflux to prevent their transformation into lipid-laden foam cells. The initial step in the RCT is efflux of unesterified cholesterol from peripheral cells to plasma acceptors. Several ABC transporters, among them ABCA1 and ABCG1, facilitate the efflux of cholesterol. In addition, cholesterol can be removed from foam cells via SR-BI and by aqueous diffusion (*3*, *43*). It has been suggested that ABCA1 and ABCG1 act in a synergistic fashion in peripheral tissues: ABCA1 lipidates lipid-poor apoA-I, generating preβ-HDL particles, and these particles in turn facilitate ABCG1-mediated cholesterol efflux (*44*, *45*). The observation that ABCA1 and G1 transporters are able to mediate cholesterol efflux to apoA-I and to HDL particles, respectively, has important implications regarding the understanding of the antiatherogenic properties of HDL and may partly explain the inverse relationship between HDL levels and atherogenesis. The ABC transporters have been intensively investigated. However, the present knowledge about the initial acceptor particles and their formation is far from complete. Plasma factors involved in the formation of lipid acceptors include the lipid transfer proteins CETP and PLTP, lipases, and LCAT (*46*). It has previously been demonstrated that, of the two lipid transfer proteins, PLTP rather than CETP is responsible for the generation of preβ-HDL particles (*47*).

PLTP is expressed in macrophages, and its expression is further increased in cholesterol-laden macrophage foam cells (*20*−*22*). We recently reported that the absence of endogenous PLTP impairs ABCA1-dependent efflux of cholesterol from acLDL-loaded macrophage foam cells (*27*), suggesting that endogenous PLTP might play a role in ABCA1 function or phospholipidation of intracellular apoA-I. Also exogenous PLTP has been implicated (*17*, *18*, *28*) in lipid efflux although the detailed mechanism(s) is not known. The present study focuses on the role of PLTP in the formation of lipid acceptors. Active HA-PLTP causes conversion of HDL, which results in the formation of preβ-HDL particles and large fused HDL particles (*48*). We here report that preincubation of HDL in the presence of active PLTP but not LA-PLTP results in an approximately 40% increase in cholesterol efflux from THP-1 and human monocyte-derived macrophage foam cells. This increase is in part due to the formation of preβ-HDL during incubation of HDL in the presence of active PLTP as the efflux increment is inhibited upon depletion of preβ-HDL by immunoprecipitation. However, after immunoprecipitation of the preβ-HDL fraction, still approximately 50% of the lipid efflux remained, suggesting the presence of additional lipid acceptors. We therefore isolated the distinct HDL conversion products formed by HA-PLTP, preβ-HDL and the large fused HDL particles. Both isolated HDL fractions induced efficient cholesterol efflux from macrophage foam cells. Of note,

incubation of PLTP alone, in the absence of HDL acceptors, with foam cells did not induce cholesterol efflux, even though these cells display elevated ABCA1 levels due to acLDL loading (*49*). Our results are thus discrepant with the observation by Oram et al. (*28*) that PLTP alone is able to mediate cholesterol efflux. The reason for this discrepancy is currently not known, but it may have to do with the fact they used a nonmacrophage hamster fibroblast cell line (BHK) overexpressing human ABCA1.

It was recently reported that, following cholesterol loading of macrophages, the intracellular trafficking of LDL and acLDL cholesterol used for loading is different. The acLDL delivered cholesterol is preferentially transported to late endosomes and lysosomes, and this pool is readily accessible to apoA-I-facilitated efflux by the ABCA1-dependent pathway (*49*). Our results obtained using acLDL for macrophage loading are in accordance with this and suggest that in our experimental setting a proportion of the efflux occurred through the ABCA1 pathway where preβ-HDL particles generated by PLTP served as acceptors. Our novel observation that also the large fused HDL particles formed upon incubation of HDL with HA-PLTP are efficient acceptors of macrophage cholesterol suggests that the ABCG1-promoted efflux pathway may also be involved. This observation agrees with previous results demonstrating that large spherical as well as large reconstituted HDL particles are more potent cholesterol acceptors than small-sized HDL particles (*44*, *50*). Analysis of the large fused HDL particles demonstrated that, as compared to nontreated HDL, their phospholipid and apoE contents were increased. This observation is in line with previous reports providing evidence that HDL size and phospholipid content can be used to predict the capacity of HDL particles to accept cholesterol (*44*, *50*−*52*). The importance of apoE in accepting lipids from foam cells was recently addressed (*53*). It was shown that HDL with apoE can accept free cholesterol from macrophages and that this cholesterol is esterified by LCAT. This resulted in particle size expansion, and the formed apoE-containing HDL could deliver cholesterol to the liver for excretion. The large fused HDL particles generated in the present study by HA-PLTP represent similar apoE-enriched large HDL particles.

To conclude, our data demonstrate that only the active form of PLTP can increase the efflux of cholesterol from macrophage foam cells and that the underlying mechanism involves PLTP-mediated HDL conversion into preβ-HDL and large fused HDL particles, both of which are efficient acceptors for cellular cholesterol.

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