Atherogenic, enlarged, and dysfunctional HDL in human PLTP/apoA-I double transgenic mice

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Abstract **In low density lipoprotein receptor (LDLR)-deficient mice, overexpression of human plasma phospholipid transfer protein (PLTP) results in increased atherosclerosis. PLTP strongly decreases HDL levels and might alter the antiatherogenic properties of HDL particles. To study the potential interaction between human PLTP and apolipoprotein A-I (apoA-I), double transgenic animals (hPLTPtg/hApoAItg) were compared with hApoAItg mice. PLTP activity was increased 4.5-fold. Plasma total cholesterol and phospholipid were decreased. Average HDL size (analyzed by gel filtration) increased strongly, hPLTPtg/hApoAItg mice having very large, LDL-sized, HDL particles. Also, after density gradient ultracentrifugation, a substantial part of the apoA-I-containing lipoproteins in hPLTPtg/hApoAItg mice was found in the LDL density range. In cholesterol efflux studies from macrophages, HDL isolated from hPLTPtg/hApoAItg mice was less efficient than HDL isolated from hApoAItg mice. Furthermore, it was found that the largest subfraction of the HDL particles present in hPLTPtg/hApoAItg mice was markedly inferior as a cholesterol acceptor, as no labeled cholesterol was transferred to this fraction. In an LDLR-deficient background, the human PLTP-expressing mouse line showed a 2.2-fold increased atherosclerotic lesion area.** **These data demonstrate that the action of human PLTP in the presence of human apoA-I results in the formation of a dysfunctional HDL subfraction, which is less efficient in the uptake of cholesterol from cholesterol-laden macrophages.**—Moerland, M., H. Samyn, T. van Gent, M. Jauhiainen, J. Metso, R. van Haperen, F. Grosveld, A. van Tol, and R. de Crom. **Atherogenic, enlarged, and dysfunctional HDL in human PLTP/apoA-I double transgenic mice.** ***J. Lipid Res.*** **2007.** 48: **2622–2631.**

Supplementary key words phospholipid transfer protein • apolipoprotein A-I • cholesterol • high density lipoprotein • low density lipoprotein • atherosclerosis • lipoprotein metabolism

The incidence of coronary heart disease shows a strong inverse relationship with the concentration of plasma HDL (1). Therefore, HDL is believed to have antiatherogenic properties, which are attributed to its effects on endothelial cells, its antioxidant activity, and its role in reverse cholesterol transport, a pathway in which peripheral cellular cholesterol is transported to the liver (2–4). One important determinant of the plasma HDL concentration is phospholipid transfer protein (PLTP) activity. During lipolysis, PLTP transfers phospholipids from apolipoprotein B-containing lipoproteins to HDL (5). Furthermore, PLTP acts as an HDL conversion factor by remodeling HDL to produce large particles and small lipid-poor particles, known as preβ-HDL (6–9). The latter HDL subfraction is a very efficient acceptor of cellular cholesterol. In this way, PLTP plays an important role in the early steps of reverse cholesterol transport (10–12).

Although the involvement of PLTP in the cellular efflux of cholesterol and phospholipids is potentially antiatherogenic, different in vivo studies showed that an increase in plasma PLTP activity is associated with decreased HDL cholesterol levels (9, 13, 14) and increased atherosclerotic lesion development (15–17). The PLTP-dependent decrease in HDL levels was explained by an accelerated HDL catabolism (13). In addition to its effect on HDL cholesterol levels, PLTP may alter the antiatherogenic properties of HDL particles. For example, anti-inflammatory properties of HDL were improved in PLTP-deficient mice (18). To study the action of increased PLTP levels on HDL particles in more detail, we cross-bred human PLTP transgenic mice with human apolipoprotein A-I (apoA-I) transgenic mice, resulting in mice expressing both human PLTP and human apoA-I. Previous studies in transgenic mice showed that introduction of the human apoA-I transgene affected HDL subfraction distribution (19, 20). Expression of human apoA-I resulted in conversion of the

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Abbreviations: AcLDL, acetylated low density lipoprotein; apoA-I, apolipoprotein A-I; CE, cholesteryl ester; CETP, cholesteryl ester transfer protein; FPLC, fast-protein liquid chromatography; LDLR, low density lipoprotein receptor; PLTP, phospholipid transfer protein.

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normal relatively homogeneous mouse HDL population to a more heterogeneous population consisting of different HDL subclasses.

This difference in plasma HDL distribution may be caused by differences in physical properties between mouse and human apoA-I (i.e., molecular properties in solution, incorporation into discoidal complexes, structural stability in HDL particles) (21). PLTP binds directly to apoA-I found on the surface of HDL particles (22). The specificity of the interaction between protein (such as transfer protein or enzyme) and HDL might strongly influence the action of the protein itself. For example, cholesteryl ester transfer protein (CETP) has a much more profound effect on HDL cholesterol levels in transgenic mice when human apoA-I is expressed as well, as a result of an enhanced interaction of CETP with human apoA-I compared with mouse apoA-I (23). In transgenic mice expressing both human LCAT and human apoA-I, human LCAT has a significant preference for HDL containing human apoA-I (24).

In the present study, we investigated whether the action of human PLTP in the presence of HDL containing human apoA-I is altered compared with the situation when only mouse apoA-I is present. In a human apoA-I transgenic mouse model, we studied the effect of the introduction of the human PLTP transgene on different HDL properties: particle size and density and the ability to induce cholesterol efflux from peritoneal macrophages. In addition, the effect of the introduction of the human PLTP gene on atherosclerotic lesion development was determined in low density lipoprotein receptor (LDLR)-deficient mice expressing human apoA-I.

MATERIALS AND METHODS

Animals

Human PLTP transgenic mice were described previously (9). In this study, we used mice with a human genomic PLTP construct in which PLTP expression is controlled by its native promoter, resulting in a moderately increased PLTP activity (line P4) (16). Human apoA-I transgenic mice (19) and LDLR-deficient mice were purchased from Jackson Laboratory. All mice were crossed for >15 generations to a C57Bl/6J background. Human PLTP transgenic mice were cross-bred with human apoA-I transgenic mice to obtain hPLTPtg/hApoAItg mice, hemizygous for both transgenes. As a control, hemizygous hApoAItg mice were used. For the atherosclerosis experiments, hApoAItg mice and hPLTPtg/hApoAItg mice were cross-bred with LDLR knockout mice to obtain hApoAItg/LDLR$^{-/-}$ and hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice. For determination of the genotype, genomic DNA was isolated from tail clips of 10 day old mice and analyzed by PCR. Annealing temperatures and primer sequences are available upon request.

Male mice were used in all experiments. After weaning, animals were fed a standard chow diet. Experiments in the hApoAItg and hPLTPtg/hApoAItg mice were performed at an age between 10 and 15 weeks. The development of atherosclerosis was studied in hApoAItg/LDLR$^{-/-}$ and hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice. Animals between 10 and 15 weeks of age were put on a high-fat/high-cholesterol diet [40% (w/w) sucrose, 15% (w/w) fat, and 1% (w/w) cholesterol; Hope Farms] for 9 weeks. Animals had free access to water and food. Blood samples were collected by orbital bleeding after fasting the animals overnight. All procedures in this study were in accordance with national and institutional guidelines.

Plasma PLTP activity

PLTP activity was measured using a phospholipid vesicle-HDL system according to Speijer et al. (25). PLTP activity is expressed as arbitrary units. One arbitrary unit is equal to the level of PLTP activity found in human reference plasma, which was 13.9 μmol/ml/h.

Quantification of plasma lipids and apoA-I

Total cholesterol concentration was determined enzymatically using a free cholesterol C kit (Wako) after hydrolysis of cholesteryl esters (CEs) with cholesterol esterase from *Candida cylindracea* (Boehringer). Triglycerides were measured using a triglyceride kit from Wako. Phospholipids were measured using the PAP150 kit from BioMerieux.

Quantification of preβ-HDL by crossed immunoelectrophoresis

In pooled plasma samples obtained from 8–10 mice, preβ- and α-migrating HDL were separated by agarose gel electrophoresis under nonreducing, nondenaturing conditions as described previously (9). Proteins were transferred to nitrocellulose membranes by capillary blotting, and human apoA-I was detected using an anti-human apoA-I antibody.

Plasma lipoprotein profiles by gel filtration

Lipoprotein profiles in EDTA-plasma were obtained using HR 10/30 fast-protein liquid chromatography (FPLC) columns in tandem that were filled with Superose 6 and Superose 12 (preparation grade; Pharmacia Biotechnology). Columns were equilibrated and run in a 2 mM phosphate buffer containing 0.9% (w/v) NaCl, 0.02% (w/v) NaN_3, and 5 mM EDTA (pH 7.4). Pooled plasma samples obtained from 8–10 mice were filtered through 0.45 μm filters (Millipore). A total of 0.5 ml of plasma was loaded onto the Superose 6 column. The separation was performed at 4°C with a flow rate of 0.1 ml/min. Fractions of 0.8 ml were collected for measurements of cholesterol and phospholipids.

Plasma lipoprotein profiles by density gradient centrifugation

Individual plasma samples freshly isolated from hApoAItg mice and hPLTPtg/hApoAItg mice were pooled (at least 10 mice per pool) and subjected to density gradient ultracentrifugation in a Beckman SW60 Ti rotor, adapted from the method described previously by Redgrave, Roberts, and West (26), at the following densities: $d < 1.006$ (normally corresponding to VLDL), $1.006 < d < 1.019$ (intermediate density lipoprotein), $1.019 < d < 1.063$ (LDL), $1.063 < d < 1.21$ (HDL), and $d > 1.21$ (proteins).

Protein analysis of FPLC fractions

To determine the protein components of lipoprotein fractions, FPLC fractions were subjected to electrophoresis on 4–15% SDS-polyacrylamide gels (Bio-Rad) (27), which were subsequently stained with Coomassie Brilliant Blue R (Sigma) or with the Silver Stain Plus Kit (Bio-Rad).

Cholesterol efflux experiments with mouse peritoneal macrophages

We determined the ability of isolated mouse HDL to induce the efflux of cholesterol from acetylated low density lipoprotein

(AcLDL)-loaded peritoneal macrophages. AcLDL was prepared by isolation of LDL from human plasma by differential centrifugation (density range, 1.019–1.063), followed by acetylation by means of repeated addition of acetic anhydride. C57Bl/6J mice were elicited by intraperitoneal injection of 2 ml of 40.5 g/l Bacto® Brewer Thioglycollate Medium (Sigma) according to the manufacturer's instructions. After 3 days, peritoneal macrophages were obtained as described (28). Cultured macrophage monolayers were incubated with 500 μl of DMEM (Gibco, Invitrogen) containing 50 μg/ml AcLDL, 2% (w/v) BSA, and 1 μCi/ml [^{3}H]cholesterol for 24 h. After lipid loading, cells were washed twice, followed by an overnight equilibration incubation to remove AcLDL from cellular membranes. Subsequently, cells were washed twice again, and cholesterol efflux was initiated by incubating the macrophages for 5 h with efflux medium consisting of DMEM, 2% (w/v) BSA, and mouse HDL (4, 10, 20, or 40 μg total cholesterol/ml).

HDL of individual hApoAItg and hPLTPtg/hApoAItg mice was isolated by density gradient ultracentrifugation at a density range of 1.019–1.21, and HDL of hApoAItg/LDLR$^{-/-}$ and hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice was isolated at a density range of 1.063–1.21. In every experiment, incubations without HDL were included to measure HDL-independent cholesterol efflux. Efflux medium was collected and radioactivity was determined. Remaining cholesterol was extracted from the intact monolayers with isopropanol. Protein was measured in the cells after the addition of 0.1 M NaOH by the method of Lowry et al. (29) using BSA as a reference. Cholesterol efflux was corrected for HDL-independent efflux and subsequently calculated as cholesterol in the medium/(cellular cholesterol content + cholesterol in the medium). The resulting percentage of the total label, adjusted for HDL-independent efflux, was normalized for small differences in cell protein content between the different wells. In one experiment, individual efflux media were pooled for each group and subjected to FPLC. In the resulting lipoprotein fractions, total cholesterol and radioactivity were measured to determine the efficiency of lipoprotein particles to incorporate cellular cholesterol.

Quantification of atherosclerosis

After 9 weeks of a high-fat/high-cholesterol diet, the animals were anesthetized with isoflurane, the thorax was opened, and the animals were subjected to perfusion fixation through the left ventricle of the heart using 4% phosphate-buffered formalin. The heart was dissected and processed for cryosectioning. Cryosections of the aortic valves (7 μm thick) were stained with Oil Red O and hematoxylin. The sections were photographed with a Sony digital camera. The atherosclerotic area was measured in five sections at intervals of 90 μm according to Paigen et al. (30) using Scion Image image-analysis software (available at www.scioncorp.com).

Statistics

Data are expressed as means ± SEM. Differences were analyzed by two-sample Wilcoxon rank sum tests using Intercooled Stata 8.2/SE software (Stata Corp., College Station, TX).

RESULTS

Effects of human PLTP overexpression on plasma lipids and apoA-I levels

First, we investigated the effect of introduction of the human PLTP gene on PLTP activity and plasma lipids. hPLTPtg/hApoAItg mice had a 4.5-fold increased PLTP activity compared with hApoAItg mice (**Table 1**). Overexpression of human PLTP resulted in a decrease in total cholesterol, caused mainly by a decrease in free cholesterol level. CE levels were similar. Phospholipid levels decreased strongly. Plasma triglyceride levels did not change. Overexpression of human PLTP resulted in a 30% decrease in human apoA-I levels and a 40% decrease in mouse apoA-I levels. Mouse apoA-I levels were very low compared with human apoA-I levels, accounting for only 1–2% of total apoA-I levels (Table 1). Crossed immunoelectrophoresis showed that expression of human PLTP resulted in a decrease of apoA-I in α-HDL, whereas apoA-I in the preβ-HDL range did not decrease (**Fig. 1**). Human apoA-I plasma concentration with preβ mobility was 1.27 mg/ml (28% of total plasma human apoA-I) in hApoAItg mice versus 1.17 mg/ml (37%) in hPLTPtg/hApoAItg mice. Human apoA-I plasma concentration with α mobility was 3.26 mg/ml (72%) in hApoAItg mice versus 1.98 mg/ml (63%) in hPLTPtg/hApoAItg mice.

Effects of human PLTP overexpression on plasma lipoprotein profiles (gel filtration and density gradient centrifugation)

Next, we investigated the effect of an increased PLTP activity on the distribution of lipoprotein subclasses. Analysis of plasma lipoproteins by gel filtration showed that introduction of the human PLTP transgene in hApoAItg mice resulted in the conversion of normal HDL (top at fraction 17) into a much larger particle (top at fraction 11) with a highly increased molar ratio of cholesterol to phospholipid (**Fig. 2**, compare A with B). Next, plasma lipoproteins were separated by density gradient centrifugation to determine the density of the lipoprotein par-

TABLE 1. Plasma parameters in hApoAItg versus hPLTPtg/hApoAItg mice

Mouse	Phospholipid Transfer Protein Activity	Total Cholesterol	Phospholipid	Triglyceride	Free Cholesterol	Cholesteryl Ester	Human ApoA-I	Mouse ApoA-I
	arbitrary units			*mmol/l*			*mg/ml*	
hApoAItg	4.12 ± 0.10	5.68 ± 0.13	5.38 ± 0.12	1.20 ± 0.10	2.17 ± 0.04	3.51 ± 0.09	4.53 ± 0.09	0.10 ± 0.01
hPLTPtg/hApoAItg	18.41 ± 0.67[a]	5.07 ± 0.13[b]	3.53 ± 0.07[a]	1.16 ± 0.12	1.74 ± 0.06[a]	3.34 ± 0.10	3.15 ± 0.15[c]	0.06 ± 0.00[c]

ApoA-I, apolipoprotein A-I. Values shown are means ± SEM.
[a] $P < 0.0001$ versus hApoAItg (n = 16–17).
[b] $P < 0.01$ versus hApoAItg (n = 16–17).
[c] $P < 0.001$ versus hApoAItg (n = 16–17).

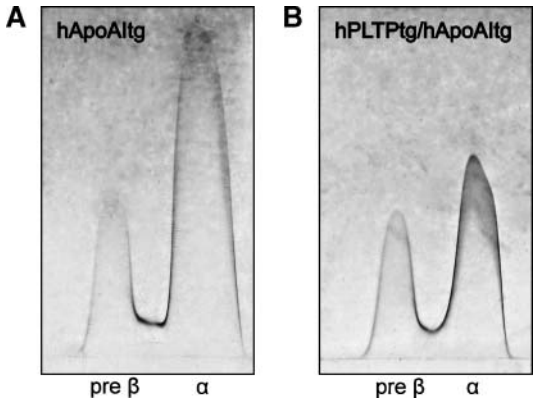

Fig. 1. Quantification of preβ-HDL by crossed immunoelectrophoresis. A: Plasma pool from hApoAItg mice. B: Plasma pool from hPLTPtg/hApoAItg mice (>10 mice per pool). The positions of preβ- and α-migrating human apolipoprotein A-I (apoA-I) are indicated.

ticles (**Table 2**). In hApoAItg mice, almost all lipoproteins were found in the HDL density range from 1.063 to 1.21 g/ml, whereas hPLTPtg/hApoAItg mice displayed lipoproteins in the HDL density range and in addition a very substantial lipoprotein fraction in the density range from 1.006 to 1.063 g/ml, which corresponds with the density of LDL.

Protein analysis of FPLC fractions

hApoAItg mice fed a standard chow diet lack substantial levels of plasma LDL (Fig. 2A, fractions 6–10, Table 2, 1.019 < d < 1.063). Introduction of the human PLTP transgene resulted in the formation of large lipoprotein particles with LDL density, as demonstrated by gel filtration and density gradient centrifugation (Fig. 2B, Table 2). To investigate whether these particles contained apoA-I, FPLC fractions were subjected to electrophoresis on a gradient SDS-polyacrylamide gel, which was subsequently stained with Coomassie blue. In hApoAItg mice, apoA-I was found mainly in fractions 12–18, which is the normal HDL range (**Fig. 3A**). In the FPLC fractions of the hPLTPtg/hApoAItg mice (Fig. 3B), apoA-I was detected not only in fractions in the HDL range but also in fractions with sizes usually corresponding with LDL particles (fractions 7–12). Apolipoprotein B patterns in fractions 7–12 did not differ between hPLTPtg/hApoAItg mice and hApoAItg mice (data not shown). These results show that concomitant expression of human PLTP and human apoA-I results in the formation of a very large, cholesterol-rich HDL particle with LDL density.

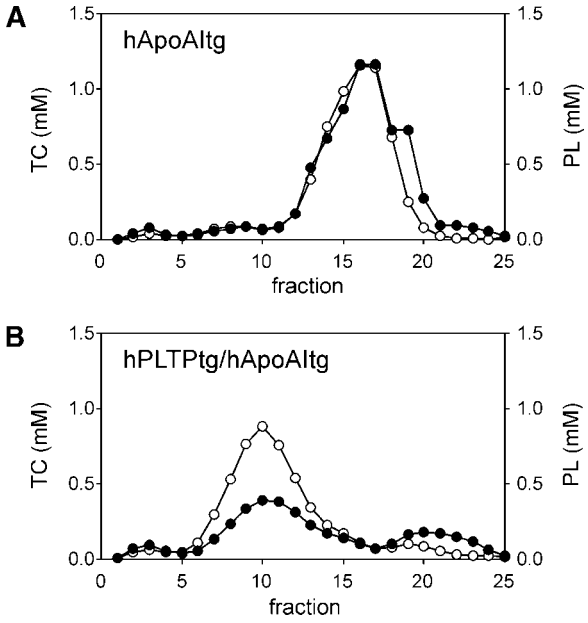

Fig. 2. Total cholesterol (TC) and phospholipid (PL) in fast-protein liquid chromatography (FPLC) fractions of plasma from hApoAItg mice (A) and hPLTPtg/hApoAItg mice (B). Individual plasma samples were pooled (>10 mice per pool) and subjected to FPLC. Total cholesterol (white circles) and phospholipid (black circles) were determined in each fraction (see Materials and Methods). Elution volumes for wild-type mice are as follows: LDL in fractions 5–10, HDL in fractions 11–20.

To check for the presence of apoE in the plasma lipoproteins from hPLTPtg/hApoAItg mice, lipoproteins were isolated by density gradient centrifugation and subsequently subjected to FPLC. The apolipoproteins present in the FLPC fractions were analyzed by silver staining of 4–15% SDS-polyacrylamide gels. As shown in Fig. 3C, apoA-I was present in all FPLC subfractions from the d = 1.019–1.063 g/ml range (corresponding to the normal size of LDL) and from the d = 1.063–1.21 g/ml range (corresponding to the normal size of HDL), whereas apoE could not be detected.

The ability of isolated HDL fractions to induce cholesterol efflux from mouse peritoneal macrophages

To test the functionality of the large HDL particles that are formed in hPLTPtg/hApoAItg mice, we studied the ability of HDL to induce cholesterol efflux from mouse peritoneal macrophages. Therefore, HDL fractions of both mouse lines were isolated from individual mice of both mouse lines by density gradient ultracentrifugation and used as acceptors for ^{3}H-labeled cellular cholesterol. Cholesterol efflux from macrophages induced by the large HDL isolated from hPLTPtg/hApoAItg mice was lower than that induced by HDL isolated from hApoAItg mice (**Fig. 4**), with the largest effect at an HDL cholesterol concentration of 40 μg/ml. We calculated the relative phospholipid and free cholesterol contents of the HDL particles that were used to induce cellular cholesterol efflux and found that this was similar for hApoAItg mice and hPLTPtg/hApoAItg mice. Therefore, it is unlikely that the exchange of labeled cellular cholesterol for unlabeled free HDL cholesterol differed between genotypes.

Subsequently, efflux media were collected and subjected to FPLC. In the FPLC fractions, both total cholesterol and ^{3}H counts were measured. When "normal-sized" HDL isolated from hApoAItg mice was used as an acceptor for cellular cholesterol, we found an equal distribution of cholesterol and ^{3}H counts in the FPLC fractions (**Fig. 5A**). In contrast, the profiles of labeled and unlabeled cholesterol did not completely overlap when HDL from hPLTPtg/

TABLE 2. Components of lipoprotein subclasses of pooled plasma samples, separated by density gradient ultracentrifugation

Component	d < 1.006	1.006 < d < 1.019	1.019 < d < 1.063	1.063 < d < 1.21	d > 1.21
hApoAItg					
Protein	0.05	0.02	0.12	2.72	NA
Phospholipid	0.12	0.03	0.17	2.34	0.02
Free cholesterol	0.03	0.01	0.05	0.42	0.02
Esterified cholesterol	0.04	0.02	0.14	1.53	0.05
Triglycerides	0.58	0.04	0.03	0.03	0.01
hPLTPtg/hApoAItg					
Protein	0.07	0.08	0.44	0.72	NA
Phospholipid	0.15	0.06	0.87	0.76	0.03
Free cholesterol	0.05	0.03	0.28	0.15	0.04
Esterified cholesterol	0.19	0.08	1.24	0.54	0.07
Triglycerides	0.79	0.06	0.08	0.02	0.02

Values shown are mg/ml.

hApoAItg mice was used (Fig. 5B). In this case, the largest HDL particles did not accept labeled cholesterol from the macrophages, whereas the smaller particles did (Fig. 5B, fractions 5–11 vs. fractions 11–15). To confirm this observation, another cholesterol efflux experiment was performed with HDL of hPLTPtg/hApoAItg mice that was separated by FPLC into three different subfractions (**Fig. 6A**): fractions 6–9 (very large HDL), fractions 10–14 (large HDL), and fractions 6–14 (total HDL). The ability of these subfractions to induce cholesterol efflux from ^{3}H-labeled AcLDL-loaded peritoneal macrophages was studied (Fig. 6B). Indeed, large HDL induced cholesterol efflux in a more efficient way than very large HDL. It is clear that the HDL subfraction with a very large size is an inferior acceptor in the cholesterol efflux experiments.

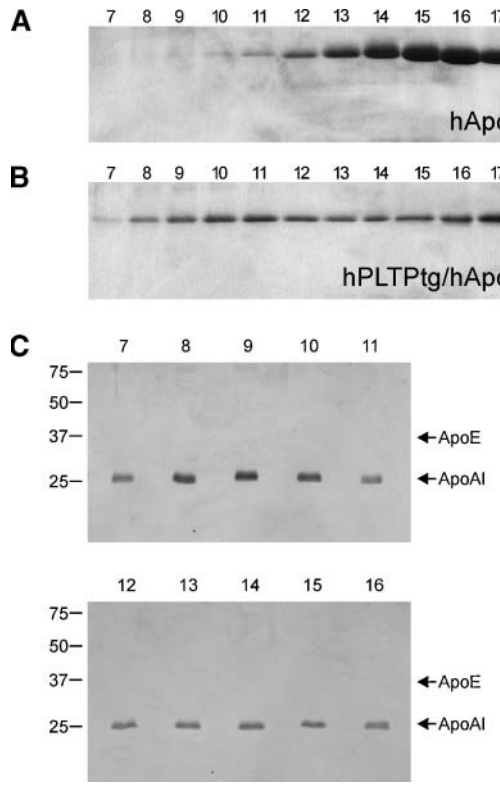

Fig. 3. ApoA-I and apoE protein levels. A, B: FPLC fractions 7–18 of hApoAItg mice (A) and hPLTPtg/hApoAItg mice (B) were subjected to electrophoresis on 10–20% SDS-polyacrylamide gels and subsequently stained with Coomassie blue. C: Plasma lipoproteins from hPLTPtg/hApoAItg mice were isolated by density gradient ultracentrifugation (see Materials and Methods). The isolated fractions with densities of 1.019–1.063 g/ml (upper gel) and 1.063–1.21 g/ml (lower gel) were subfractionated by FPLC, followed by silver staining of 4–15% SDS-polyacrylamide gels. The positions of molecular weight markers are indicated at left, and those of apoA-I and apoE are indicated at right.

Atherosclerosis experiments

To determine the effect of the expression of human PLTP on the development of atherosclerosis, hApoAItg mice and hPLTPtg/hApoAItg mice were crossed into an LDLR-deficient background. Animals were fed a high-fat/high-cholesterol diet for 9 weeks to induce the development of atherosclerosis. hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice had a 3-fold increased PLTP activity compared with hApoAItg/LDLR$^{-/-}$ mice (**Table 3**). No differences in plasma cholesterol or phospholipids between groups

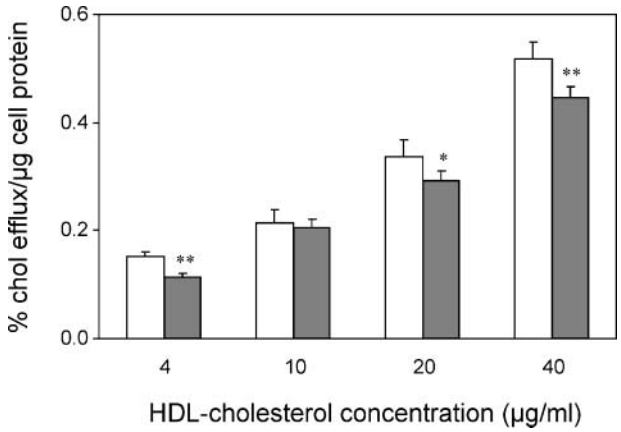

Fig. 4. Percentage cholesterol efflux per microgram of cell protein. The ability of HDL isolated from hApoAItg mice (white bars) and hPLTPtg/hApoAItg mice (gray bars) to induce cholesterol efflux from labeled acetylated low density lipoprotein (AcLDL)-loaded peritoneal macrophages was determined (see Materials and Methods). Values shown are means ± SEM obtained from >10 mice per group. * $P < 0.05$, ** $P < 0.01$ versus hApoAItg.

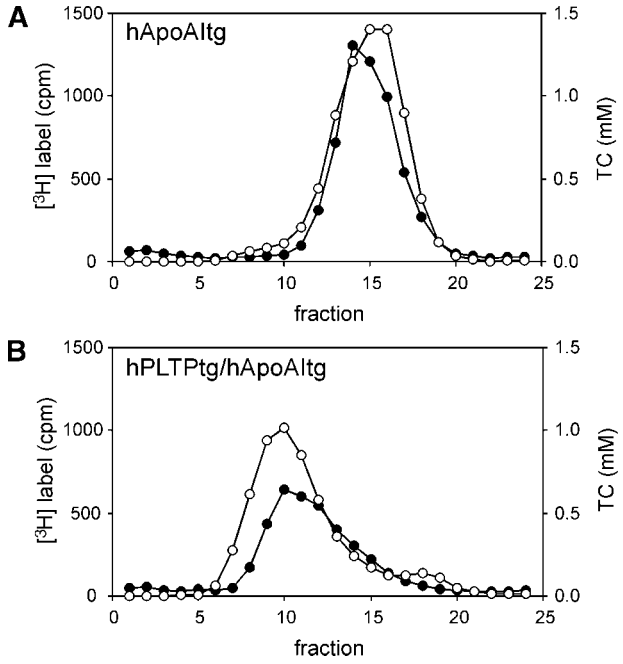

Fig. 5. Total cholesterol (TC) and ^{3}H label (counts per minute) in FPLC fractions of pooled efflux media obtained with HDL from hApoAItg mice (A) and hPLTPtg/hApoAItg mice (B). After cholesterol efflux, efflux media of individual mice were collected. Pools of efflux media (at least 10 mice per group) were subjected to FPLC. Total cholesterol (white circles) and ^{3}H label (black circles) were determined in each fraction. Elution volumes for wild-type mice are as follows: LDL in fractions 5–10, HDL in fractions 11–20.

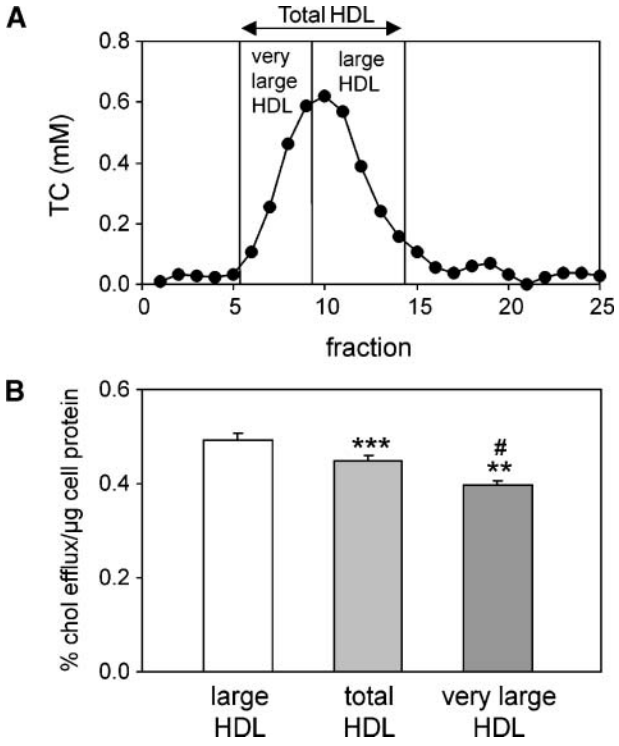

Fig. 6. Percentage cholesterol efflux per microgram of cell protein for very large HDL versus large HDL. A: A plasma pool isolated from a group of 12 hPLTPtg/hApoAItg mice was subjected to FPLC and divided into three pools: very large HDL (fractions 6–9), large HDL (fractions 10–14), and total HDL (fractions 6–14). B: The ability of the three HDL pools to induce cholesterol efflux from labeled AcLDL-loaded peritoneal macrophages was determined (see Materials and Methods). Values shown are means ± SEM of four determinations. ** $P < 0.05$, *** $P < 0.01$ versus large HDL; # $P < 0.05$ versus total HDL. TC, total cholesterol.

were observed. Overexpression of human PLTP resulted in a 60% decrease in human apoA-I levels and a 20% decrease in mouse apoA-I levels. Mouse apoA-I levels were very low compared with human apoA-I levels, accounting for only 1–3% of total apoA-I levels (Table 3). Crossed immunoelectrophoresis showed that expression of human PLTP resulted in a decrease in both α-HDL and preβ-HDL. Human apoA-I plasma concentration with preβ mobility was 0.63 mg/ml (19% of total plasma human apoA-I) in hApoAItg/LDLR$^{-/-}$ mice versus 0.29 mg/ml (26%) in hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice. Human apoA-I plasma concentration with α mobility was 2.69 mg/ml (81%) in hApoAItg/LDLR$^{-/-}$ mice versus 0.82 mg/ml (74%) in hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice.

We again studied the ability of isolated HDL to induce cholesterol efflux from mouse peritoneal macrophages. After 9 weeks of a high-fat/high-cholesterol diet, HDL from hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice and hApoAItg/LDLR$^{-/-}$ mice was isolated individually by density gradient ultracentrifugation and used as an acceptor for ^{3}H-labeled cellular cholesterol. In agreement with the results in hPLTPtg/hApoAItg mice and hApoAItg mice, HDL isolated from hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice proved to induce cholesterol efflux in a less efficient way than did HDL isolated from hApoAItg/LDLR$^{-/-}$ mice (**Fig. 7**). After 9 weeks of the high-fat/high-cholesterol diet, animals were euthanized and atherosclerotic lesion area was determined in sections of the aortic valves. Mice overexpressing human PLTP showed a 2.2-fold increased atherosclerotic lesion area (**Fig. 8**).

DISCUSSION

In the present study, overexpression of the human PLTP gene in a human apoA-I transgenic mouse line resulted in decreased total plasma cholesterol and phospholipid levels, which were caused by decreased HDL levels. In human apoA-I transgenic mice, the relatively high percentage of apoA-I in the preβ range can be attributed to the presence of the human apoA-I transgene (20, 31, 32). Overexpression of human PLTP did result in an increase in the percentage of human apoA-I in the preβ-HDL range, in accordance with our previous data in mice without human apoA-I (8, 9). However, the absolute plasma level of human apoA-I with preβ mobility did not increase in human PLTP-expressing mice, whereas the plasma level of human apoA-I with α mobility was strongly decreased. This could be explained by the fact that overexpression of PLTP increases HDL catabolism (13). In addition, PLTP has been reported to cause proteolytic cleavage of apoA-I (33). We

TABLE 3. Plasma parameters in hApoAItg/LDLR$^{-/-}$ mice versus hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice measured chow-fed (0 weeks) and after 9 weeks of a Western diet (9 weeks)

	Phospholipid Transfer Protein Activity		Total Cholesterol		Phospholipid		Human ApoA-I	Mouse ApoA-I
Mouse	0 Weeks	9 Weeks	0 Weeks	9 Weeks	0 Weeks	9 Weeks	9 Weeks	9 Weeks
	arbitrary units		*mmol/l*		*mmol/l*		*mg/ml*	
hApoAItg/LDLR$^{-/-}$	3.63 ± 0.26	7.27 ± 1.19	4.09 ± 0.35	15.95 ± 0.87	3.60 ± 0.13	9.19 ± 0.35	3.32 ± 0.24	0.06 ± 0.01
hPLTPtg/hApoAItg/LDLR$^{-/-}$	11.86 ± 0.36[a]	19.32 ± 2.00[b]	3.96 ± 0.85	16.56 ± 0.70	2.97 ± 0.40	8.70 ± 0.33	1.11 ± 0.10[a]	0.04 ± 0.00[a]

Values shown are means ± SEM.
[a] $P < 0.001$ versus hApoAItg/LDLR$^{-/-}$ (n = 7–11).
[b] $P < 0.01$ versus hApoAItg/LDLR$^{-/-}$ (n = 7–11).

found that the action of human PLTP together with human apoA-I resulted in the formation of a large CE-rich lipoprotein particle with LDL density.

We determined protein components in this lipoprotein particle and found that the main apolipoprotein was apoA-I. Therefore, we conclude that the large lipoprotein particle found in hPLTPtg/hApoAItg mice is a very large, cholesterol-enriched subspecies of HDL displaying LDL density. In vitro HDL conversion studies demonstrated that human PLTP is able to promote the conversion of a homogenous population of human HDL_3 particles into a new population with an increased average size via particle fusion (34–36). Earlier, a small increase in HDL size was observed in a human PLTP/human apoA-I double transgenic mouse model displaying a slightly increased PLTP activity (37). Ehnholm et al. (14) also showed that adenovirus-mediated overexpression of human PLTP in human apoA-I transgenic mice resulted in an increase in HDL size and a decrease in HDL density. Furthermore, human apoA-I transgenic mice treated with fenofibrate showed an increase in the expression of murine PLTP in the liver through stimulation of the transcription factor peroxisome proliferator-activated receptor α. The resulting increase in PLTP activity accounted for the HDL size enlargement in plasma from treated animals (38, 39).

Enlarged HDL species have been described in a variety of other mouse models, such as scavenger receptor class B type I knockout mice, hepatic lipase knockout mice, human LCAT transgenic mice, and hepatocyte nuclear factor-1α knockout mice (40–43). In these mouse models, HDL levels were increased and lipoprotein particles were enriched with apoE and CEs. The only mouse model that displays, like our hPLTPtg/hApoAItg mouse model, an increase in HDL size concomitant with a decrease in HDL levels is a model in which apoM is selectively silenced by small interfering RNA (44). It is unknown whether PLTP plays a role in the molecular mechanisms by which apoM affects HDL particle size.

Not only HDL concentration but also HDL composition may be important for its antiatherogenic properties. Scavenger receptor class B type I-deficient mice and mice overexpressing human LCAT, displaying enlarged HDL subspecies, showed an increase in atherosclerotic lesion development despite highly increased levels of HDL cholesterol (40, 42). Human studies confirm the hypothesis

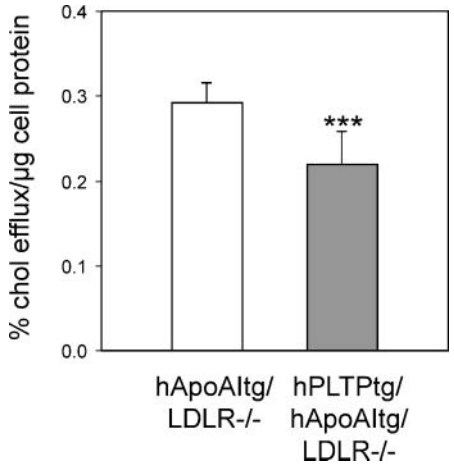

Fig. 7. Percentage cholesterol efflux per microgram of cell protein. The ability of HDL isolated from hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice versus hApoAItg/LDLR$^{-/-}$ mice (fed a Western diet) to induce cholesterol efflux from labeled AcLDL-loaded peritoneal macrophages was determined (see Materials and Methods). Values shown are means ± SEM obtained from >10 mice per group. *** $P < 0.001$ versus hApoAItg/LDLR$^{-/-}$ mice.

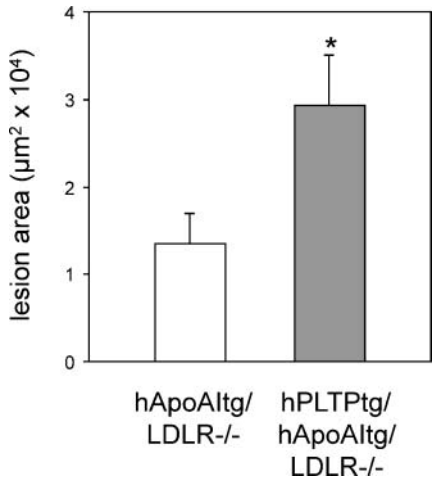

Fig. 8. Atherosclerotic lesion development in hApoAItg/LDLR$^{-/-}$ mice versus hPLTPtg/hApoAItg/LDLR$^{-/-}$ mice. Plaque area was measured in sections from the aortic root (see Materials and Methods). Values shown are means ± SEM obtained from at least 12 mice per group. * $P < 0.05$ versus hApoAItg/LDLR$^{-/-}$ mice.

that HDL size and composition are the main determinants of susceptibility to atherosclerosis development. The inhibitory effect of small dense HDL subfractions isolated from healthy human subjects on the expression of cell adhesion proteins is superior to that of larger and less dense HDL particles (45). This might be of physiopathological relevance in the initial steps of atherogenesis, in which monocytes are arrested on the surface of endothelial cells.

Recently, it was shown that hyperalphalipoproteinemia in cardiac transplant recipients is associated with the formation of partially dysfunctional HDL (46). Other studies showed that average HDL size and CE content increase as a result of low CETP activity (47–50). Also, the large HDL particles that are formed in our double transgenic mice are rich in CEs. In the absence of CETP, CEs cannot be transferred to apoB-containing lipoproteins. Possibly, the expression of CETP in our hPLTPtg/hApoAItg mice would prevent the accumulation of esterified cholesterol in the core of the HDL particle, thereby reducing HDL particle size. Interestingly, expression of CETP corrected dysfunctional HDL in LCAT transgenic mice, thereby reducing the development of atherosclerosis (51). In CETP-deficient subjects, it was reported that these large CE-rich HDL particles have reduced acceptor capacity for cholesterol from lipid-laden macrophages (52, 53). These findings indicate that measuring HDL functionality might be as important as measuring HDL concentration for assessing cardiovascular risk.

In our study, cholesterol efflux capacity of the enlarged HDL isolated from hPLTPtg/hApoAItg mice was decreased compared with that of HDL from hApoAItg mice. The cholesterol efflux experiment in which the efflux of cellular lipids to lipoprotein particles in fractions 6–9 was compared directly with the efflux of lipids to lipoprotein particles in fractions 10–14 confirmed the hypothesis that the very large HDL particles are inefficient at inducing cellular cholesterol efflux. These results show that PLTP may act proatherogenically not only by decreasing plasma HDL levels but also by changing HDL particle composition. Interestingly, Tall and colleagues (54) recently discovered that the enlarged HDL (HDL_2) that is found in patients with CETP deficiency has an increased capacity to promote cellular cholesterol efflux, but only when apoE content of the HDL particle was high. When apoE was removed from the particle, HDL lost this ability. We checked the apoE content of the enlarged HDL particles in hPLTPtg/hApoAItg mice by silver staining of apolipoproteins on SDS-polyacrylamide gels, but no apoE was detected.

Lipoprotein enrichment with apoE is not essential for the formation of large HDL, as there are reports of enlarged HDL in apoE-deficient mice (41). In our mouse model, the absence of an increased apoE content of the large HDL might be caused by the overexpression of human apoA-I. Probably, the large excess of human apoA-I in the HDL particles, including the large HDL in the double transgenic mice, leads to a repression of apoE incorporation. Also, the expression of apoA-II is strongly repressed in our human apoA-I transgenic mouse line, as has been reported previously (19). We analyzed the distribution of apoA-II in FPLC fractions of both genotypes (Coomassie blue staining of SDS-polyacrylamide gels) and found that apoA-II levels were negligible and that there were no differences between genotypes (data not shown). Furthermore, the substantial reduction in the ratio of surface to core lipoprotein components, which may occur in hPLTPtg/hApoAItg mice, might contribute to the dissociation of apolipoproteins from the HDL particles (55).

In hPLTPtg/hApoAItg mice, the size increase renders the apoE-poor HDL particle less efficient at accepting cellular cholesterol. Also, when both mouse lines were crossed into an LDLR-deficient background, HDL isolated from the human PLTP-expressing mice was less efficient at promoting cellular cholesterol efflux. Unfortunately, it is not feasible to perform the same set of analyses for the HDL of animals with an LDLR-deficient background as we did for the HDL of hApoAItg and hPLTPtg/hApoAItg mice. The reason is that LDLR-deficient animals have predominant levels of LDL, which precludes the isolation of large HDL, which is in the same density range. However, the reduced capacity of the HDL to induce cellular cholesterol efflux may explain, at least in part, the observation that human apoA-I transgenic mice overexpressing human PLTP have an increased susceptibility to the development of atherosclerotic lesions.

In conclusion, our data show that in the presence of human apoA-I, human PLTP not only decreases plasma HDL levels but also unfavorably affects the functionality of the remaining HDL particles. When crossed into an atherosclerosis-prone background, mice expressing human PLTP show a strong increase in susceptibility to the development of atherosclerosis. Our findings strengthen the opinion that high plasma PLTP activity levels might be harmful for cardiovascular health.

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