Increased Preβ-high Density Lipoprotein, Apolipoprotein AI, and Phospholipid in Mice Expressing the Human Phospholipid Transfer Protein and Human Apolipoprotein AI Transgenes

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Abstract

Human plasma phospholipid transfer protein (PLTP) circulates bound to high density lipoprotein (HDL) and mediates both net transfer and exchange of phospholipids between different lipoproteins. However, its overall function in lipoprotein metabolism is unknown. To assess the effects of increased plasma levels of PLTP, human PLTP transgenic mice were established using the human PLTP gene driven by its natural promoter. One line of PLTP transgenic mice with moderate expression of PLTP mRNA and protein was obtained. The order of human PLTP mRNA expression in tissues was: liver, kidney, brain, small intestine > lung > spleen > heart, adipose tissue. Western blotting using a human PLTP monoclonal antibody revealed authentic human PLTP (M_r 80 kD) in plasma. Plasma PLTP activity was increased by 29% in PLTP transgenic mice. However, plasma lipoprotein analysis, comparing PLTP transgenic mice to control littermates, revealed no significant changes in the plasma lipoprotein lipids or apolipoproteins. Since previous studies have shown that human cholesteryl ester transfer protein and lecithin:cholesterol acyltransferase only function optimally in human apoAI transgenic mice, the human PLTP transgenic mice were cross-bred with human apoAI transgenic mice. In the human apoAI transgenic background, PLTP expression resulted in increased PLTP activity (47%), HDL phospholipid (26%), cholesteryl ester (24%), free cholesterol (37%), and apoAI (22%). There was a major increase of apoAI in preβ-HDL (56%) and a small increase in α-HDL (14%). The size distribution of HDL particles within α- and preβ-migrating species was not changed. The results suggest that PLTP increases the influx of phospholipid and secondarily cholesterol into HDL, leading to an increase in potentially antiatherogenic preβ-HDL particles. (*J. Clin. Invest.* 1996. 96:2373–2380). Key words: transgenic mice • HDL lipoproteins • phospholipids • apolipoproteins A • carrier proteins

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Introduction

Plasma high density lipoproteins (HDL) levels show an inverse relationship with atherosclerosis (1). The metabolism of HDL is regulated by lipases, lecithin:cholesterol acyltransferase (LCAT),[1] and the plasma lipid transfer proteins, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP) (2). Plasma PLTP, a M_r 80 kD glycoprotein, transfers phospholipids among lipoprotein particles (3, 4) and from lipid bilayers to HDL (3). There is accumulating in vitro evidence indicating that the PLTP plays an important role in the remodeling of lipoproteins. During lipolysis of apoB-containing lipoproteins, partially purified PLTP was shown to mediate both the transfer and exchange of phospholipids between these particles and HDL (5). PLTP can also cause conversion of HDL_3 to large (10.9 nm) and smaller (7.8 nm) particles in a time- and concentration-dependent fashion (6, 7). Furthermore, PLTP activity on HDL modulates the activities of LCAT and CETP (2, 4). Both human and mouse PLTP cDNA and gene have been cloned (8–10). The PLTP gene belongs to a family that includes CETP, lipopolysaccharide binding protein, and bactericidal permeability increasing protein genes (8). Mouse plasma PLTP activity is 1.5–2.0 times that of human (10). Murine PLTP activity and mRNA levels are upregulated by a high fat/cholesterol diet and down-regulated by lipopolysaccharide administration (10). Although some information has been learned about the functions of PLTP in vitro, the overall role of the PLTP in lipoprotein metabolism is poorly understood.

Based on in vitro evidence, our working hypothesis is that in vivo PLTP mediates the net transfer of phospholipid from apoB-containing lipoproteins into HDL and perhaps from certain cell types into HDL. Thus the overexpression of human PLTP would result in increased HDL phospholipid and cholesterol. To evaluate this hypothesis, we established lines of transgenic mice expressing the human PLTP gene. Mouse HDL is a relatively homogenous population, while human HDL is more heterogeneous, consisting of discrete HDL_2 and HDL_3 subclasses. The difference appears to be due to the different physical properties of mouse and human apoAI (11, 12). Previous studies demonstrated that both CETP and LCAT show species-specific interaction with HDL, as the phenotypic effects of

1. *Abbreviations used in this paper:* AI, apolipoprotein AI; CETP, cholesteryl ester transfer protein; FPLC, fast protein liquid chromatography; Hu, human; LCAT, lecithin:cholesterol acyltransferase; LPP, lysosomal protective protein; PC, phosphatidylcholine; PLTP, phospholipid transfer protein; Tg, transgenic.

expressing either of these human proteins in transgenic mice were enhanced when human apoAI was also expressed (13, 14). To determine whether human PLTP is also adapted to act specifically on human HDL, human PLTP transgenic mice were cross-bred with human apoAI transgenic mice.

Methods

Isolation and characterization of human PLTP genomic clones. Three P1 clones containing the entire human PLTP gene were obtained from Genome Systems Inc. (St. Louis, MO). The presence of the entire PLTP gene within these clones was confirmed by PCR amplification of the coding sequences using two sets of primers (5′-end primers: GGACTAGTCCCGGATCCCCTGAGCTGC and CGCGGATCTTGCAGCCTGGG; 3′-end primers: CCACACCGTCCACAGCAGCT and GGCTCTACAGGCTATGAATG). Digestion of the three P1 clones with several restriction enzymes such as EcoRI, XbaI, SacI, and KpnI indicated that one of the clones (No. 2000) was different from the other two (Nos. 1999 and 2001), although all of them contained the entire PLTP gene. We chose the No. 2000 clone to create transgenic mouse, since this clone contains a larger 5′end flanking region than that of the Nos. 1999 and 2001. PCR analysis revealed that all three P1 clones contain another entire gene, lysosomal protective protein (LPP).

Creation of transgenic mice. To generate transgenic mice, the circular P1 clone (2000) was microinjected into the male pronuclei of fertilized mouse eggs taken from superovulated (C57BL/6J × CBA/J) F1 females. Injected embryos were implanted into the oviducts of surrogate females of the same genetic background (11). Human apoAI transgenic mice (line 427)(12) were crossed with F1 generation of PLTP transgenic mice. All comparisons of transgenic and nontransgenic mice are made between littermates.

DNA and RNA analysis. Tail tip DNA from 3-wk-old mice was used to screen by PCR for integration of human PLTP gene sequences using human-specific primers located at the 3′-untranslated region of PLTP cDNA. The amplification reaction using nontransgenic mouse DNA as a template yielded no amplification products. In some experiments, integration of the PLTP gene was detected by Southern blot hybridization. 10 μg genomic DNA was digested with StuI, electrophoresed in a 1% agarose gel, and transferred to a Hybond-N nylon membrane (Amersham, Arlington Heights, IL). DNA was UV cross-linked to the membranes and hybridized with a ^{32}P-radiolabeled 5′ end-labeled 200-bp PLTP cDNA as a probe.

Total RNA (30 μg) from the tissues of the transgenic and nontransgenic littermates was analyzed for PLTP and LPP mRNA by a solution hybridization-ribonuclease protection assay (15) using riboprobes highly specific for the human PLTP and human LPP mRNA, respectively. The riboprobe (220 nucleotides) for PLTP contains a portion of the vector (Bluescript KS+), and is complementary to 120 nucleotides of the 3′-untranslated region of the human PLTP mRNA.

PLTP monoclonal antibody preparation. PLTP was purified to homogeneity from human plasma using ultracentrifugation and a combination of phenyl-Sepharose, CM-cellulose, DEAE-cellulose, heparin-Sepharose, and hydroxylapatite chromatography as described (4). To generate monoclonal antibodies to PLTP, BALB/c mice were immunized with purified human PLTP and hybridomas were generated by polyethylene glycol–mediated fusion. Hybridoma supernatants were screened by a solid phase radioimmunometric using purified PLTP adsorbed to Immulon II Removawells (Dyntech, Cambridge, MA) as antigen and ^{125}I-rabbit anti–mouse IgG to detect bound antibody. Positive hybridomas were twice recloned at 1 and 0.5 cells/well, respectively. To obtain large amounts of the monoclonal antibody, the hybridoma was injected into the peritoneal cavity of BALB/c mice and the ascitic fluid was harvested about 2 wk later.

PLTP protein and activity analysis. 25 μl of plasma from HuPLTPTg and wild type as well as human was incubated at 37°C for 1 h with 475 μl of 20 mM Tris, pH 7.2, 150 mM NaCl, 1% Triton X-100, 1% deoxycholate, 0.1% SDS and 1% aprotinin. The human PLTP was immunoprecipitated with the anti-PLTP mAb (5D10)-agarose and dissolved in SDS loading buffer. The PLTP was analyzed by Western blotting with ^{125}I-labeled anti-PLTP mAb (5D10), by procedures previously described for plasma CETP detection (16).

PLTP activity assay was performed as follows: 150 μl of fresh plasma was incubated with 150 μl of ^{3}H-PC vesicles (750 nmol PC), which was prepared as described previously (6), at 37°C for 30 min, then the density of the solution was adjusted to 1.063 g/ml and spun at 98,000 rpm for 4 h in an Optima TL ultracentrifuge (Beckman, Brea, CA). The tube was sliced. The top portion (800 μl) which includes VLDL, LDL, and ^{3}H-PC-vesicles was counted, while the radioactivity in the bottom portion (4.2 ml) which contains ^{3}H–PC-HDL, was taken to be the product of PLTP activity.

Plasma lipid and lipoprotein analyses. For small volumes of mouse plasma, HDL was separated from apoB-containing lipoproteins by using HDL cholesterol reagent (Sigma Chemical Co., St. Louis, MO). Using this method, an insignificant amount of mouse apoAI is precipitated (11). The total cholesterol and phospholipids in plasma, HDL, and column fractions were assayed by enzymatic methods (Wako Chemicals, Osaka, Japan). Lipoprotein profiles were obtained by means of fast protein liquid chromatography (FPLC) using a Sepharose 6B column as described previously (17). A 200-μl aliquot of pooled plasma (from five animals) was loaded onto the column, and eluted with TS buffer (50 mM Tris, 0.15 M NaCl, pH 7.5) at a constant flow rate of 0.35 ml/min. An aliquot of 80 μl from each fraction (0.7 ml) was used for the determination of total cholesterol and phospholipid.

Quantitation of HDL subspecies. To determine the proportion of pre-β and α-electrophoretic mobility HDL species in the transgenic and nontransgenic mice, 20 μl plasma samples containing 1.5 mM 5,5′-dithio–bis(2-nitrobenzoic acid) (to inactivate LCAT activity) was electrophoresed in 0.75% (wt/vol) agarose gel in 50 mM barbital buffer on Gelbond (FMC, Rockville, ME) for 3 h and transferred to NitoPlus transfer membranes (Micron Separation Inc., Westboro, MA) as previously described (13). To identify the human apoAI-containing HDL species, nitrocellulose membranes were incubated for 2 h at room temperature with 2% milk in 10 mM phosphate buffer, pH 7.4, and then with a biotinylated goat polyclonal antibody to human apoAI (2 h at room temperature) in 2% milk in 10 mM phosphate buffer. ApoAI containing HDL species were visualized with ^{125}I-Streptavidin (Amersham). Unbound ^{125}I was washed 4 times with 1% milk in phosphate buffer and nitrocellulose membranes were exposed to Fuji XLS film at −70°C. The relative abundance of the human apoAI among the α- or preβ-HDL species was calculated by quantitative scanning using a Phosphorimager (Fuji, Stamford, CT). The distribution of human apoAI among the α-migrating HDL species was determined by two-dimensional gel electrophoresis. The first dimensional gel electrophoresis was run on an agarose gel described above. The agarose strip was placed on a 3-16% polyacrylamide gradient gel (Integrated Separation Systems, Natick, MA) in 25 mM Tris-glycine buffer (pH 8.3). Electrophoresis was carried out for 4.5 h. Plasma proteins were transferred to NitroPlus transfer membranes and then immunoreacted with a goat polyclonal antibody to human apoAI as described above. The proportion of apoAI among the HDL fractions was determined by quantitative scanning using a Phosphorimager. Each subfraction was individually quantitated by a customized box which isolated one fraction from another. Various exposure times were used to optimize the separation of the individual subspecies. Background was uniformly subtracted.

Statistical analysis. Results are expressed as mean±standard deviation. The statistical significance of the differences between the groups was estimated by the Student's *t*-test. A P value less than 0.05 was considered significant.

Results

PLTP-containing P1 clone and PLTP transgenic mice. The PLTP-containing P1 clone (No. 2000) contains intact 5′ and

3′ ends of the PLTP gene as determined by PCR with 5′- and 3′-specific primers, as well as by Southern blotting. Microinjection of supercoiled P1 DNA into fertilized embryos resulted in 34 offsprings, of which 2 were positive for human PLTP sequences by PCR with human-specific primers (see Methods). Southern blot analysis of these two PCR-positive mice showed the presence of multiple copies of the gene when compared with human genomic DNA and one founder had higher copy number (about 10 copies) than the other (about 3 copies). DNA from nontransgenic control mice did not hybridize with the human PLTP probe. The two founders were used to generate two lines of PLTP transgenic mice (HuPLTPTg).

Tissue specificity of human PLTP expression. RNase protection assay using a human-specific probe revealed that human PLTP mRNA was expressed in both transgenic lines. The order of human PLTP mRNA expression in the line with higher expression was as follows: liver, kidney, brain, small intestine > lung > spleen > heart, adipose tissue, skeletal muscle. This is similar to the distribution of PLTP mRNA in human tissues (8, 10). The PLTP mRNA abundance ranged from 10–100 pg/mg total RNA (Fig. 1), whereas the low copy number line only expressed very low levels, 1–5 pg/mg total RNA, in testis, kidney, and liver (data not shown). Mouse PLTP mRNA is expressed in a similar distribution at levels of 10–100 pg/mg total RNA (10). Analysis of murine PLTP mRNA in HuPLTPTg mice showed no alteration in pattern or levels of expression (data not shown). In order to study PLTP on a protein level, we developed a human PLTP monoclonal antibody (mAb, 5D10). This mAb, which is specific to human PLTP, immunoprecipitates 60% of human PLTP activity. Western blot using 5D10 revealed authentic human PLTP (M_r 80 kD) expression at about 1.5–2.0-fold human plasma levels in the high expressor (Fig. 2). No human PLTP was detectable in the low expressor line. All subsequent analyses were done on the high expressor line.

Subsequent to the generation of HuPLTPTg mice we learned in a sequence database search that the 3′ non-coding end of PLTP mRNA is exactly complementary to the 3′ non-coding end of LPP mRNA. This indicates that PLTP and LPP which were previously localized to chromosome 20q12-q13.1 and 20q13.1, respectively (18, 19), are convergently transcribed from opposite DNA strands, with overlapping complementary 3′ ends. The PLTP and LPP gene overlap exists not only in the human but also in the mouse genome. Based on the cDNA sequences, the overlapping region in human is > 57-bp and in mouse is > 64-bp. The P1 clone (#2,000) was found to contain in close proximity to the PLTP gene an intact gene for the human LPP. The HuPLTPTg mice also express LPP mRNA.

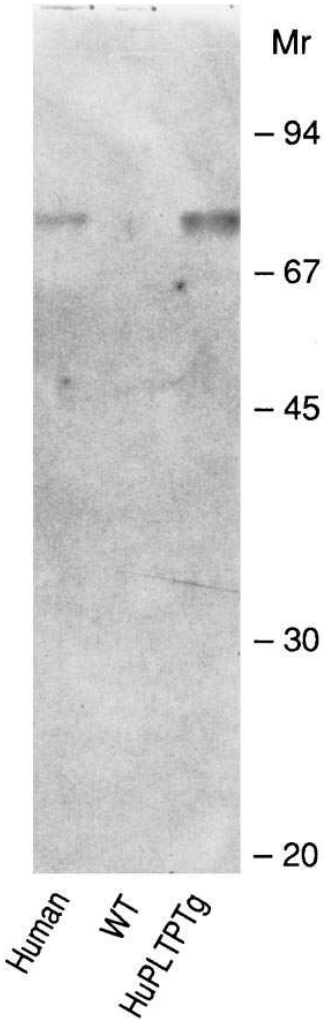

Figure 2. Detection of human PLTP in transgenic mouse plasma. The human PLTP was precipited from HuPLTPTg, wild type mouse and human plasma by incubation with anti-PLTP mAb (5D10)-agarose and disolved in SDS-loading buffer. The PLTP was analyzed by Western blotting with ^{125}I-labeled anti-PLTP mAb (5D10). Mobility of molecular weight standards are indicated on the right side of the figure. Tg, transgenic.

RNase protection assay using a human-specific probe for LPP revealed a different pattern of LPP mRNA distribution in this animal compared to PLTP mRNA (Fig. 3). Furthermore, the low expressor HuPLTPTg line expressed the same level of LPP as the high expressor. Thus, despite their close proximity, these two genes are unlikely to be coordinately regulated.

PLTP activity plasma lipids and lipoprotein analysis. To measure PLTP activity, we incubated PC vesicles in whole plasma and measured transfer of PC radioactivity into HDL. PLTP activity was increased in HuPLTPTg mice at all time

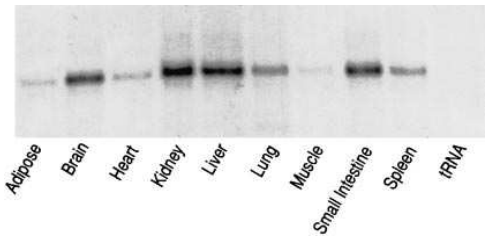

Figure 1. Sites of PLTP transgene expression. Total RNA (30 μg) from the tissues of the HuPLTPTg and wild type mice was analyzed for human PLTP mRNA by RNase protection assay (15) using riboprobe highly specific for the human PLTP mRNA that yields a protected fragment of 120 nucleotides.

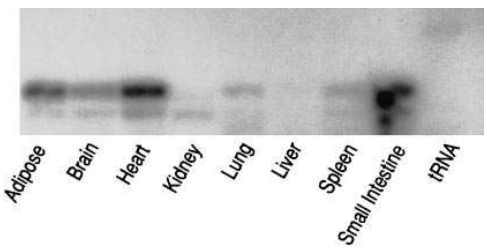

Figure 3. Sites of human LPP expression in HuPLTPTg mice. Total RNA (30 μg) from the tissues of the HuPLTPTg and wild type mice was analyzed for human LPP mRNA by RNase protection assay (15) using a riboprobe highly specific for the human LPP mRNA that yields a 110 nucleotide protected fragment.

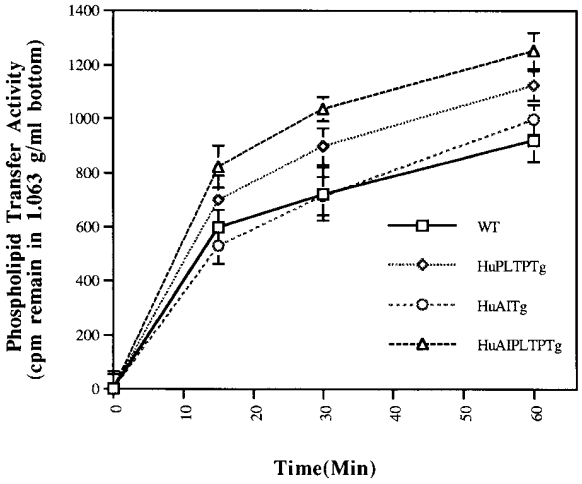

Figure 4. The plasma PLTP activity assay in wild type, HuPLTPTg, HuAITg and HuAIPLTPTg mice. Fresh plasma (150 μl) was incubated with 150 μl of ^{3}H-PC vesicles (750 nmol PC), at 37°C for 30 min, then the density of the solution was adjusted to 1.063 g/ml and spun in an ultracentrifuge. Then the tube was sliced. The top fraction (containing VLDL, LDL and ^{3}H-PC–vesicles) was counted for the remaining radioactivity, and the radioactivity in the bottom fraction (containing ^{3}H-HDL) was taken to be the product of PLTP activity. Each time point is mean±SD (n = 3).

points (Fig. 4). For example the PLTP activity in the high expressor line was 29% higher than that of the wild type animals at the 30 min timepoint ($P < 0.02$) (Fig. 4). After crossing the HuPLTPTg to human apoAI transgenic mice (HuAITg), the PLTP activity in the compound transgenic mice was 47% higher than that of the HuAITg in a 30-min period of incubation ($P < 0.01$) (Fig. 4). The difference in PLTP activity between HuPLTPTg and HuAIPLTPTg mice was significant ($P < 0.05$) at all time points. This increase in PLTP activity was not due to the human apoAI overexpression, since mouse PLTP activity was similar in HuAITg and wild type mice (Fig. 4).

To determine whether human PLTP has any impact on the mouse lipoprotein pattern, we next compared the lipoprotein profiles and apoAI concentration of PLTP transgenic mice and nontransgenic littermates under both fed and fasted conditions. There was no significant difference in plasma lipoprotein lipid concentrations between human PLTP transgenic (HuPLTPTg) and nontransgenic mice (Tables I and II). However, we observed several differences between HuAIPLTPTg and HuAITg mice. The phospholipid and cholesterol levels in plasma of HuAIPLTPTg mice were increased significantly under both fed (phospholipid, 22%, $P < 0.02$; cholesterol, 23%, $P < 0.05$) and, fasted (phospholipid, 17%, $P < 0.05$: cholesterol, 20%, $P < 0.05$) conditions compared to those found in HuAITg mice (Table I). These changes were due to increases in HDL-phospholipid (26%, $P < 0.02$, and 20%, $P < 0.05$, for fed and fasted conditions, respectively), HDL-cholesteryl ester (24%, $P < 0.01$, and 22%, $P < 0.05$, for fed and fasted conditions, respectively), HDL–free-cholesterol (37%, $P < 0.01$, and 33%, $P < 0.02$, for fed and fasted conditions, respectively) (Table II). There were no obvious changes in VLDL and LDL lipids (Table II). FPLC gel filtration analysis using pooled plasma confirmed that there were increases of plasma HDL-phospholipid and HDL-cholesterol in HuAIPLTPTg com-

Table I. Plasma Lipid Composition in Wild Type, HuPLTPTg, HuAITg, and HuAI/PLTPTg Mice

Mice	PL	TC	CE	TG
		mg/dl		
Fed				
Non-Tg	174±21	73±5	67±7	186±39
HuPLTpTg	182±15	70±3	71±5	162±41
HuAITg	280±26	135±19	126±19	282±69
HuAIPLTPTg	342±32‡	166±21*	133±31	263±41
Fasted				
Non-Tg	185±31	75±7	65±8	58±10
HuPLTPTg	178±23	77±6	66±10	57±9
HuAITg	266±30	130±14	121±22	85±12
HuAIPLTPTg	312±26*	156±18*	130±24	89±15

5 ml of plasma was used for total cholesterol, free cholesterol, phospholipid, and triglyceride measurement using enzymatic methods kits (Wako Chemicals). *$P < 0.05$, HuAITg vs HuAIPLTPTg; ‡$P < 0.02$, HuAITg vs HuAIPLTPTg. Values are mean±SD of 8–9 animals/group.

Table II. Lipoprotein Lipid Concentration in Wild Type, HuPLTPTg, HuAITg, and HuAIPLTPTg Mice

Mice	HDL-PL	HDL-CE	HDL-FC	V+LDL-PL	V+LDL-CE	V+LDL-C
			mg/dl			
Fed						
Non-Tg	154±21	48±4	6±2	57±8	15±3	6±2
HuPLTPTg	157±19	45±3	5±3	63±12	15±2	5±1
HuAITg	240±22	97±12	8±3	41±8	24±5	6±2
HuAI/PLTPTg	302±19‡	120±18‡	11±2§	39±7	26±4	8±4
Fasted						
Non-Tg	165±17	49±9	6±2	32±11	14±5	6±3
HuPLTPTg	158±14	54±8	6±1	36±8	17±6	7±2
HuAITg	226±25	95±9	9±2	39±5	20±3	8±4
HuAI/PLTPTg	272±14*	116±17*	12±2‡	41±7	22±4	8±3

HDL was separated from V+LDL by using HDL cholesterol reagent (Sigma). The total cholesterol, free cholesterol, and phospholipid concentrations were determined by enzymatic methods kits (Wako Chemicals). *$P < 0.05$, HuAITg vs HuAIPLTPTg, ‡$P < 0.02$, HuAITg vs HuAIPLPTg; §$P < 0.01$, HuAITg vs HuAIPLTPTg. Values are mean±SD of 8–9 animals/group.

pared with HuAITg mice (Fig. 5). These increases were coincident with the main HDL peak.

We also measured human apoAI levels in HuAITg and HuAIPLTPTg mice as well as mouse apoAI in wild type and HuPLTPTg mice. The mouse apoAI concentration was similar in wild type and HuPLTPTg mice (Table III). However, HuAIPLTPTg mice had significantly higher human apoAI concentration than HuAITg mice (Table III). As the increases in HDL phospholipid, cholesterol, cholesteryl ester, and apoAI were proportional to each other (Tables I–III), these changes indicated that the HDL particle numbers were increased in HuAIPLTPTg mice relative to HuAITg mice. SDS polyacrylamide gel electrophoresis confirmed the increase of human apoAI in HuAIPLTPTg mice compared with HuAITg mice and there were no obvious changes in the levels of other apolipoproteins (data not shown).

The effect of PLTP on HDL subclass distribution was studied by 2-dimensional non-denaturing gradient gel electrophoresis. Preβ-HDL was markedly increased (56%, $P < 0.01$) in plasma from HuAIPLTPTg mice compared to HuAITg mice plasma and α-HDL levels were moderately increased (14%, $P < 0.05$) as well (Fig. 6 and Table III). Preβ-HDL are distributed into three different sized populations located in the preβ_1 electrophoretic mobility range described for human plasma (20). The expression of the PLTP transgene did not affect the size of the preβ-HDL species.

HuAITg mice α-HDL consists of four distinct populations of particles with sizes corresponding to the human HDL_{2a}, HDL_{2b}, HDL_{3a}, and HDL_{3c} (12, 14). Although total α-HDL was increased in HuAIPLTPTg mice by 14% compared with HuAITg mice (Table III), there were no significant changes in the proportion of its different subclasses (Table IV).

In order to eliminate the possibility that the overexpression human LPP rather than PLTP caused the phenotype observed in HuAIPLTPTg mice, we crossed PLTP low-expressor mice, which expressed the same level of LPP as high-expressor mice, with HuAITg mice. There were no differences between HuAITg and HuAIPLTP (low expressor) Tg mice comparing plasma phospholipid, cholesterol, cholesteryl ester, HDL-phospholipid, and HDL-cholesterol, indicating that human LPP overexpression did not influence plasma HDL lipid levels.

Table III. Distribution of Human apoAI Among Preβ and α-migrating HDL in HuAITg and HuAIPLPTg Mice

Mice	ApoAI	Preβ-HDL	α-HDL	HDL-PL/ApoAI
		mg/dl		
Mouse				
Non-Tg	107±13	2.1±0.6	105±12	1.45±0.12
HuPLTPTg	101±9	1.8±0.6	99±8	1.51±0.23
Human				
HuAITg	288±30	59±17	229±20	0.78±0.16
HuAIPLTPTg	352±43*	93±21*	260±36‡	0.77±0.21

Lipoproteins fractions from fasted HuAITg and HuAIPLTPTg mice were separated by agarose gel electrophoresis and transferred to nitrocellulose membranes as described (see Methods). Mouse apoAI was visualized with rabbit polyclonal antibody to mouse apoAI. Areas containing preβ and α-migrating HDL were visualized and quantitated using a Phosphorimager. Values shown are mean±SD, n = 8–9. *$P <$ 0.01. ‡$P < 0.05$.

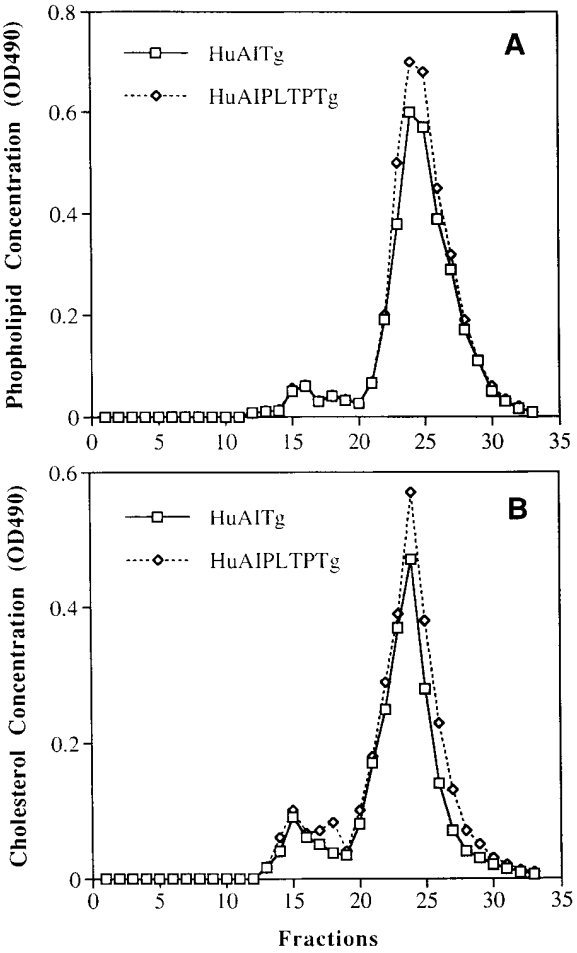

Figure 5. Concentration of phospholipid and cholesterol in plasma lipoproteins of HuAITg and HuAIPLTPTg mice as determined by FPLC. A 200-μl aliquot of pooled plasma (from five animals) was loaded onto the column, and eluted with TS buffer (50 mM Tris, 0.15M NaCl, pH 7.5) at a constant flow rate of 0.35 ml/min. An aliquot of 80 μl from each fraction (0.7 ml) was used for the determination of total cholesterol and phospholipid. (*A*) Phospholipid distribution; (*B*) Cholesterol distribution.

Discussion

In this study, we have utilized mice expressing human PLTP and apoAI transgenes to show that, in vivo, expression of human PLTP caused moderate increases in HDL phospholipid, free cholesterol, cholesteryl ester and apoAI levels and markedly promoted the accumulation of small HDL with pre-β electrophoretic mobility. Since preβ-HDL has been shown to be the optimal mediator of initial cellular cholesterol efflux (20), these results suggest that the activity of PLTP may enhance the initial steps of the reverse cholesterol transport pathway.

Previous in vitro experiments showed that partially purified PLTP mediates both the transfer and exchange of phospholipid between triglyceride-rich lipoproteins and HDL during lipolysis (5). The hypothesis to be tested in this transgenic approach was that human PLTP overexpression would promote net phospholipid movement into HDL, and as a result,

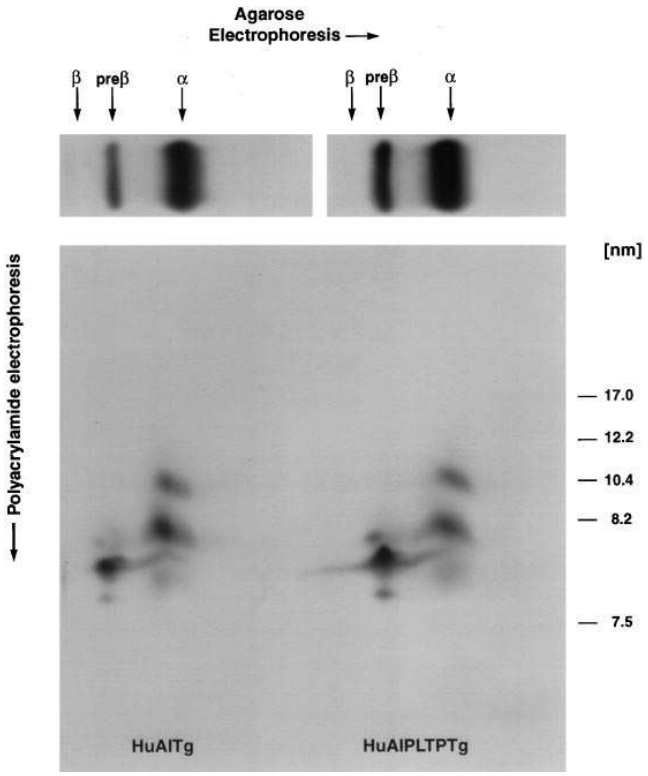

Figure 6. Distribution of human apoAI in HuAITg and HuAIPLTPTg mice plasma determined by nondenaturing two-dimensional gradient gel electrophoresis. HuAITg and HuAIPLTPTg mice plasma (20 μl) was electrophoresed on a 0.75% agarose gel (first dimension) and then placed on a 3–16% polyacrylamide gradient gel. Electrophoresis in the second dimension was carried out for 4.5 h. Plasma proteins were transferred to nitrocellulose membranes and the human apoAI was visualized with goat anti-human apoAI antibody. Stokes diameter standards from top to bottom: thyroglobulin (17 nm), apoferritin (12.2 nm), catalase (10.4 nm), lactate dehydrogenase (8.2 nm), bovine albumin (7.5 nm).

HDL-phospholipid and free cholesterol would be higher. Even though the HuPLTPTg mice had no phenotype, the results in HuAIPLTPTg mice support this hypothesis. Unexpectedly other components of HDL (cholesteryl ester, apoAI) were also increased in HuAIPLTPTg and there was a marked increase in preβ-HDL. It is curious that there was no decrease in VLDL and LDL-phospholipid in HuAIPLTPTg mice compared to HuAITg mice. It is possible that some "surface remnants" are cleared by liver during lipolysis and PLTP overexpression results in a more efficient transfer of this pool of phospholipid to HDL. Alternatively, PLTP may promote the phospholipid transfer from certain cell membranes to HDL.

Table IV. Proportion of Human apoAI in the α-migrating HDL Species

Mice	HDL_{2b}	HDL_{2a}	HDL_{3a}	HDL_{3c}
HuAITg ($n = 5$)	26.7±5.8	5.4±3.9	43.7±7.7	24.1±5.3
HuAIPLTPTg ($n = 5$)	28.2±3.0	6.9±0.9	38.7±2.9	26.2±3.9

HDL species from HuAITg and HuAIPLTPTg mice were separated by two dimensional gradient gel electrophoresis. Human apoAI on nitrocellulose membranes was visualized with a goat polyclonal antibody to human apoAI and quantitated using a Phosphorimager. Values shown are mean±SD, $n = 8–9$.

Crossing HuAITg mice with either HuCETPTg or HuLCATTg mice, the phenotypic effect of CETP and LCAT becomes much more pronounced, indicating that human CETP and LCAT work much better when they use human-like HDL as their substrate in mice (13, 14). This preference is also shared by PLTP. The production of HuAIPLTPTg mice allows us to examine the authentic function of PLTP in vivo. It has been proposed based on in vitro observations that during lipolysis of TG-rich lipoproteins there is a net transfer of phospholipid by PLTP into HDL (5), so we expected that in HuAIPLTPTg mice there would be an increase in HDL-phospholipid relative to HuAITg mice. In agreement with our hypothesis, we observed not only a phospholipid enrichment of HDL in the HuAIPLTPTg mice, but also increases of HDL-free cholesterol, cholesteryl ester, and apoAI levels, without a change in HDL size distribution. This indicates that HDL particle numbers are increased. Preβ-HDL shows the largest increase but α-HDL levels also show a small increase. These findings indicate that PLTP has an important role in the metabolism of preβ-HDL in vivo.

Western blotting with a PLTP mAb showed authentic human PLTP at a level about 1.5–2.0-fold human plasma. By contrast, PLTP activity was increased by about 30% and 47% in HuPLTPTg and HuAIPLTPTg mice. Given that the Western blotting is semiquantitative, and mouse plasma has about two times the PLTP activity of human plasma (10), the increase in PLTP activity is similar to that expected.

The genomic clone used for the generation of HuPLTPTg mice has the gene for the LPP in very close proximity to the 3′ end of the PLTP gene. LPP is a multifunctional protein associated with beta-galactosidase and neuraminidase in lysosomes, protecting them from degradation (21); it also has serine esterase activities (22, 23). As LPP is primarily an intracellular protein, it is unlikely to interact with plasma lipoproteins. It is also unlikely to interact intracellularly with PLTP as it does with beta-galactosidase or neuraminidase because the tissue distribution of LPP and PLTP mRNAs are dissimilar (Figs 1 and 3). We consider a close coordinate regulation of the two genes to be unlikely based on the dissimilar tissue distribution of their mRNAs and on our observation that in the low-expressor HuPLTPTg mice, the level of LPP expression is similar to that in the high-expressor HuPLTP mice. Furthermore, we determined the PLTP activity in galactosialidosis patients due to LPP deficiency (24) and did not find the alteration of PLTP activity in those patients compared with controls (Jiang, X.-C., and Tall, A., unpublished observation) indicating that LPP expression is not needed for PLTP activity or expression. Although a definite conclusion regarding the relevance of LPP to lipid metabolism will have to await the generation of PLTP transgenic mice not expressing LPP, the available evidence suggests that the findings reported here are PLTP specific.

Preβ-HDL are small, phospholipid rich HDL particles containing apoAI only (25) that have been observed in different species (26, 27). In human, the average concentration of preβ-HDL is about 5–10% of the plasma apoAI (28). Higher concentrations are found in lymph (29), aortic intima, (30) and plasma from hypertriglyceridemic subjects (31). Although several studies (20, 32, 33) have proposed a key role of this HDL fraction in the initial steps of cell-derived cholesterol transport,

the origin and molecular mechanisms responsible for their formation remain poorly understood.

The increase in preβ_1 particle numbers can arise from either an increase in formation or a decrease in catabolism. It has been suggested that nascent, discoidal HDL could correspond to the pool of particles that is rapidly cleared from plasma, and a portion of this apoprotein then reappears in a slowly turning-over pool that constitutes the major mass of apoAI (34). One possibility is that free apoAI or minimally lipidated apoAI, which may be either synthesized and secreted by cells, or released during the processing of HDL (35, 36), is rapidly catabolized, but that addition of phospholipids to it by PLTP leads to a longer half life for the resulting preβ_1 particles.

Another possibility for the increase of apoAI in HuAIPLTPTg mouse plasma may be due to to changes in the preβ-HDL/α-HDL cycle which is catalyzed by hepatic lipase and LCAT. Hepatic lipase reduces the lipid core of triglyceride-rich HDL and promotes the dissociation of apoAI and possibly phospholipid, inducing the formation of preβ-HDL (37). LCAT provides the driving force for the formation cholesteryl ester which enters the core of preβ-HDL and produces the mature form of HDL, α-HDL (38). Thus, PLTP expression in the human apoAI background may enhance the phospholipid enrichment in HDL, perhaps stimulate hepatic lipase activity and increase conversion of α-HDL to preβ-HDL. By increasing the influx of phospholipid, PLTP expression may also prevent the decrease of phospholipid content due to LCAT action on α-HDL, thus preventing the putative fusion of preβ- with α-particles (39), leading to an increase in preβ-HDL particles.

Recent studies (6, 7, 40) have demonstrated that PLTP can promote conversion of an apparently homogenous population of HDL_3 particles into a new population with an increased average size, apparently as a result of fusion between HDL particles and a concomitant release of free apoAI (40). Preβ-HDL was not measured in these studies. We did not observe a difference in HDL size between either HuPLTPTg and wild type mice or between HuAIPLTPTg and HuAITg mice, suggesting that in vivo the conversion process is counteracted by other activities.

Previous studies indicated that preβ-HDL participates in the initial steps of reverse cholesterol transport pathway, i.e., the centripetal movement of cholesterol from peripheral tissues or from macrophage foam cells in the arterial wall to the liver via the plasma compartment (38). Excess cholesterol in peripheral tissues may be removed by preβ-HDL which in conjunction with LCAT action, provides the driving force for net cholesterol movement. However, preβ particles may also be formed by cellular interactions of lipid-free apoAI that dissociates from HDL particles (32). If this is the case, the amount of preβ particles in plasma may reflect an active reverse cholesterol transport pathway rather than drive it. Our results support the involvement of PLTP in the reverse cholesterol pathway. Further studies on cholesterol efflux and cholesterol esterification in HuAIPLTPTg mice will be needed to ascertain the role of PLTP in reverse cholesterol transport and atherogenesis.

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