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Human Plasma Phospholipid Transfer Protein Increases the Antiatherogenic Potential of High Density Lipoproteins in Transgenic Mice

Rien van Haperen,* Arie van Tol,* Pieter Vermeulen, Matti Jauhiainen, Teus van Gent, Paul van den Berg, Sonja Ehnholm, Frank Grosveld, Arthur van der Kamp, Rini de Crom

Abstract—Plasma phospholipid transfer protein (PLTP) transfers phospholipids between lipoprotein particles and alters high density lipoprotein (HDL) subfraction patterns in vitro, but its physiological function is poorly understood. Transgenic mice that overexpress human PLTP were generated. Compared with wild-type mice, these mice show a 2.5- to 4.5-fold increase in PLTP activity in plasma. This results in a 30% to 40% decrease of plasma levels of HDL cholesterol. Incubation of plasma from transgenic animals at 37°C reveals a 2- to 3-fold increase in the formation of pre- β -HDL compared with plasma from wild-type mice. Although pre- β -HDL is normally a minor subfraction of HDL, it is known to be a very efficient acceptor of peripheral cell cholesterol and a key mediator in reverse cholesterol transport. Further experiments show that plasma from transgenic animals is much more efficient in preventing the accumulation of intracellular cholesterol in macrophages than plasma from wild-type mice, despite lower total HDL concentrations. It is concluded that PLTP can act as an antiatherogenic factor preventing cellular cholesterol overload by generation of pre- β -HDL. (*Arterioscler Thromb Vasc Biol.* 2000;20:1082-1088.)

Key Words: atherosclerosis ■ pre- β -HDL ■ transgenic mice ■ macrophages ■ phospholipid transfer protein

Plasma phospholipid transfer protein (PLTP) is able to promote the net transfer of phospholipids between plasma lipoproteins¹⁻⁴ and mediate the conversion of HDL.^{5,6} Because plasma levels of HDL are among the best indicators for the risk of atherosclerosis in epidemiological studies,^{7,8} PLTP could play a role in the prevention of the development of coronary heart disease and stroke via its effect on HDL.

HDLs are antiatherogenic because they mediate efflux of cholesterol from peripheral cells and transport cholesterol to the liver, for excretion and degradation to bile acids. This process is known as reverse cholesterol transport.⁸⁻¹⁰ It has been postulated that the antiatherogenic effect of HDL can be attributed mainly to a quantitatively minor subclass of HDL, called pre- β -HDL.^{11,12} This assumption is based on in vitro studies showing that pre- β -HDL is a very efficient acceptor of cellular cholesterol.^{13,14} The origin of pre- β -HDL is not well understood, but the available evidence suggests that PLTP participates in its generation, at least in vitro.¹⁵

After the cloning of a human PLTP (HuPLTP) cDNA,¹⁶ 2 groups independently generated transgenic mice for HuPLTP.^{17,18} Unfortunately, these mice showed low levels of

expression of the transgene, and as a result, only small effects on plasma lipoproteins were observed. Changes in HDL levels and subfractions could be demonstrated only in a compound transgenic background with human apoA-I.¹⁸ Recently, the generation of a PLTP knockout mouse was reported by Jiang et al.¹⁹ These mice show a decrease of plasma cholesterol in all lipoprotein classes.

In the present study, we report the generation of transgenic mice that overexpress HuPLTP 2.5- to 4.5-fold. This results in decreased plasma total HDL cholesterol levels, in increased formation of pre- β -HDL, and in a high plasma capability to prevent cholesterol accumulation in macrophages.

Methods

Generation of HuPLTP Transgenic Mice

A human cosmid library was constructed from high-molecular-weight DNA isolated from the blood of a healthy volunteer. This library was screened for cosmids containing the PLTP gene by using HuPLTP cDNA (kindly donated by Drs A-Y. Tu and J.J. Albers, Northwest Lipid Research Laboratories, University of Washington,

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Seattle) as a probe. Isolated cosmids were mapped by using restriction fragments from the cDNA, and a cosmid containing the complete PLTP gene²⁰ was selected. Vector sequences were removed by restriction endonuclease digestion, and DNA was dissolved in microinjection buffer (10 mmol/L Tris-HCl, pH 7.5, and 0.1 mmol/L EDTA) at a concentration of 1 to 2 $\mu\text{g}/\text{mL}$. The DNA was microinjected into fertilized oocytes from FVB mice. These oocytes were transferred into the oviducts of pseudopregnant foster females. All animal experiments were performed in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Erasmus University Rotterdam (No. 132.97.06) and with the European Committee Standards on the care and use of laboratory animals (Ministry of Welfare, Health, and Cultural Affairs, the Netherlands).

DNA Analysis

Genomic DNA was isolated from tail clips of 10-day-old mice and analyzed for the presence of the HuPLTP transgene by polymerase chain reaction (PCR) analysis: sense primer 5'-GCCACAGCAGGAGCTGATGC-3', antisense primer 5'-GCGGATGGACACACCCTCAGC-3'; 25 to 30 cycles (94°C for 1 minute, 65°C for 1 minute, and 72°C for 1 minute).

Breeding and Treatment of Transgenic Mice

Transgenic founder mice were bred with FVB mice to obtain transgenic mice. FVB transgenic HuPLTP mice were backcrossed with C57Bl/6 mice for 4 generations. These mice were intercrossed to obtain wild-type, hemizygous, and homozygous HuPLTP transgenic mice. Animals were kept on regular chow and fasted overnight before collection of blood from the orbital plexus.

Gene Expression Analysis by RT-PCR

Total RNA was isolated from various tissues obtained freshly from either wild-type, hemizygous, or homozygous HuPLTP transgenic mice. cDNA was obtained by reverse transcription (RT) primed by oligo(dT). This was used in PCR reactions in the presence of [³²P]ATP (20 cycles at 94°C for 1 minute, 62°C for 1 minute, and 72°C for 1 minute). Primers used were as follows: for HuPLTP, sense 5'-CCTGCTGAGCCCAGCAGTG-3', antisense 5'-CTGGACCTCAGGCTGGTCTG-3'; for murine PLTP (MuPLTP), sense 5'-TTGACTCTGCCATGGAGAGC-3', antisense 5'-GCTCCACTTCGGGCAACATG-3'; and for hypoxanthine-guanine phosphoribosyltransferase, sense 5'-CGAAGTGTTGGATACAGGCC-3', antisense 5'-GGCAACATCAACAGGACTCC-3'. PCR products were run on polyacrylamide gels and visualized by using a PhosphorImager (Molecular Dynamics).

Assay of Plasma PLTP Activity

Plasma PLTP activity was assayed by using a phospholipid vesicle-HDL system.^{1,21} EDTA-plasma samples (25 μL of plasma diluted 1:75) were incubated with [³H]dipalmitoylphosphatidylcholine-labeled (Amersham) phosphatidylcholine vesicles and excess pooled normal HDL for 45 minutes at 37°C. After incubation, the vesicles were precipitated as described,²¹ and the radioactivity transferred to HDL was counted in the supernatant. Standard curves based on dilutions of human plasma were included in each run. The measured activity is linear with time for 1 hour. All samples were analyzed in duplicate, and blanks without plasma were subtracted. Duplicates of pooled human plasma, stored at -70°C, were also measured in each series (reference plasma). The between-assay coefficient of variation of reference plasma was 4.1%. Activities are expressed as micromoles of phosphatidylcholine transferred per milliliter of plasma per hour ($\mu\text{mol} \cdot \text{mL}^{-1} \cdot \text{h}^{-1}$).

Anti-HuPLTP Polyclonal Antibodies

A synthetic peptide was made containing amino acids 470 to 493 of HuPLTP (Chiron Mimotopes Peptide Systems). The peptide was chosen to obtain an antibody that recognizes HuPLTP specifically, without cross-reaction with the MuPLTP. Its sequence has a similarity of 69% with the corresponding murine sequence (GenBank accession numbers of the human and murine sequences are L26232 and U37226, respectively). The peptide was coupled to keyhole limpet hemocyanin. New Zealand White rabbits were immunized

with the peptide-hemocyanin complex. Amounts of 0.15 mg of the complex were given in a volume of 0.3 mL each time at intervals of 3 weeks. Blood samples were taken after each period of 3 weeks, and the generation of antibodies was followed by Western blot analysis. Starting after the second booster injection of the antigen complex, serum from immunized rabbits (diluted 1:1000) displayed a strong positive reaction with human plasma and with purified HuPLTP. Furthermore, the reaction with antibody was monospecific, because in both samples only 1 band with an identical molecular mass of 80 kDa was recognized. This molecular mass corresponds to the HuPLTP band obtained with plasma from transgenic animals shown in Figure 2. The antibody also shows a strong reaction with recombinant HuPLTP, which was used as an internal standard in all Western blotting experiments but does not recognize any protein in wild-type mouse plasma (see Figure 2).

Anti-Mouse ApoA-I Polyclonal Antibodies

ApoA-I was purified from mouse plasma HDL (density range 1.063 to 1.21 g/mL) essentially as described²² and used to immunize rabbits by subcutaneous injection with 100 μg of mouse apoA-I; standard procedures were used.

Quantification of Plasma Lipids and ApoA-I

Total cholesterol was enzymatically determined with the F-Chol kit (Boehringer-Mannheim) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Boehringer-Mannheim). Phospholipids were measured enzymatically with a PAPI50 kit (BioMérieux).

Mouse apoA-I was quantified by a sandwich ELISA, with use of a polyclonal rabbit anti-mouse apoA-I IgG, performed in 96-well plates coated with this antibody. Purified mouse apoA-I was used as a primary standard. Plasma samples were diluted in PBS+Tween 20 (0.1%) with BSA (0.5%). Bound apoA-I was detected by the addition of polyclonal rabbit anti-mouse apoA-I, conjugated to horseradish peroxidase. The assay is linear in the range of 6.5 to 420 ng/mL.

Measurement of Plasma Lipoprotein Profiles by Gel Filtration

Lipoprotein cholesterol profiles in mouse EDTA-plasma were obtained by gel permeation chromatography on HR10/30 FPLC columns in tandem that were filled with Superose 6 and Superose 12 (preparation grade, Pharmacia Biotechnology), respectively. Columns were equilibrated and run in 0.9% NaCl containing 0.02% Na₂S₂O₈, 5 mmol/L EDTA, and 2 mmol/L sodium phosphate buffer, pH 7.4. Pooled plasma samples obtained from 5 mice were filtered through 0.45- μm filters (diameter 13 mm, Millipore), and 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 measurement of cholesterol, phospholipids, and PLTP activity.

Quantification of Pre- β -HDL by Crossed Immunoelectrophoresis

Freshly isolated plasma samples from mice were either directly frozen or incubated at 37°C in the presence of iodoacetate to measure the formation of pre- β -HDL in vitro. Iodoacetate (1 mmol/L) was added for complete inhibition of lecithin:cholesterol acyl transferase (LCAT). This prevents maturation of the formed pre- β -HDL into α -HDL by LCAT activity. The LCAT inhibitor had no effect on the plasma pre- β -HDL concentration if added to freshly isolated plasma (not shown).

The crossed immunoelectrophoresis consisted of agarose electrophoresis in the first dimension for separation of lipoproteins with pre- β and α mobility. Electrophoresis in the second dimension (ie, antigen migration from the first gel into an anti-apoA-I-containing gel) was used to quantitatively precipitate apoA-I. Lipoprotein electrophoresis was carried out in 1% (wt/vol) agarose gels in barbital buffer (50 mmol/L, pH 8.6) and run in an LKB 2117 system (4°C for 2 hours, 250 V). Plasma was applied at 5 μL per well. The track of the first agarose gel was excised and annealed with melted agarose to a gel containing 7.5% (vol/vol) rabbit anti-mouse apoA-I

antiserum that was cast on GelBond film (Pharmacia). The plate was run in an LKB 2117 system (4°C for 20 hours, 50 V) in barbital buffer. Unreacted antibody was removed by extensive washing in PBS. The gel was stained with Coomassie brilliant blue R250 and subsequently dried. Areas under the pre- β -HDL and α -HDL peaks were calculated by multiplication of peak height and width at half height. The pre- β -HDL area is expressed as a percentage of the sum of α -HDL and pre- β -HDL areas. Pre- β -HDL concentrations are also given in absolute amounts (micrograms of apoA-I present in pre- β -HDL per milliliter plasma). These values were calculated from the percentage of apoA-I present in pre- β -HDL and the total plasma apoA-I concentrations.

Cholesterol Flux Experiments With Mouse Peritoneal Macrophages

The ability of mouse heparin-plasma to interfere with the intracellular formation by acyl coenzyme A:cholesterol acyltransferase (ACAT) of labeled cholesteryl oleate from [³H]oleate and acetylated LDL (AcLDL)-derived cholesterol was tested by using the assay developed by Brown et al.²³ The formation of labeled cholesteryl esters from [³H]oleate by ACAT is an estimate of intracellular cholesterol concentration.

BSA (fraction V grade) was delipidated by extraction of free fatty acids with activated carbon.²⁴ [9,10(n)³H]Oleic acid (10.0 Ci/mmol, Amersham) was complexed to BSA after evaporating 0.1 mmol of oleic acid (48 Ci/mol) to dryness under a stream of nitrogen. Subsequently, 10 mL of 12% (wt/vol) fatty acid-free BSA in DMEM at 56°C was added to the dried oleate and sterilized by passage through a 0.22- μ m filter.

AcLDL was prepared from LDL (density range 1.019 to 1.063 g/mL), isolated from human plasma by differential centrifugation, and subsequently acetylated by repeated additions of acetic anhydride.²⁵ Increased electrophoretic mobility of the AcLDL was confirmed by agarose electrophoresis at pH 8.6.¹²

C57Bl/6 mice were elicited by intraperitoneal injection of 0.8 mL of eliciting agent, prepared from Baker's thioglycollate (DIFCO) according to the manufacturer's instructions. After 4 days, macrophages were obtained as described.²⁶ The macrophage monolayers were washed with DMEM and incubated with 500 μ L aliquots of DMEM that contained 3 μ g/mL AcLDL, 0.1 mmol/L BSA-[³H]oleate (48 Ci/mol), and plasma that was diluted (12 times) from fasted mice. After 18 hours, medium was removed, and the cells were washed twice with PBS. Cholesteryl esters were extracted from the intact monolayers with 1 mL hexane-isopropanol (3:2 [vol/vol]) and purified by thin-layer chromatography as previously described.²⁷ Labeled cholesteryl ester bands were excised from the silica, and radioactivity was determined. Protein was extracted from the cell remnants with 0.1 mol/L NaOH and quantified by the method of Lowry et al.²⁸ using BSA as a standard. Duplicate assays were performed for each plasma sample.

Statistics

Data are given as mean \pm SD. Differences between groups were analyzed by 1-way ANOVA. The Bonferroni correction was used for multiple pairwise comparisons when the ANOVA indicated a significant effect.

Results

Generation of Transgenic Mice

A cosmid clone containing the complete HuPLTP gene was isolated and analyzed by Southern blotting. Approximately

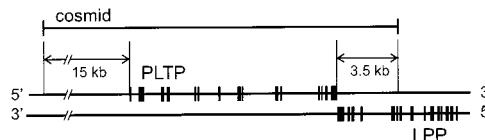


Figure 1. Schematic drawing of PLTP and lysosomal protective protein (LPP) genes, which are on opposite DNA strands.²⁰ The sequences included in the cosmid used to generate transgenic mice are indicated.

15 kb 5' to the first exon and \approx 3.5 kb 3' to the last exon of the PLTP gene were present in the cosmid as well. The 3' end, but not the 5' end, of the lysosomal protective protein gene was included (Figure 1). The purified 35-kb cosmid was microinjected in fertilized oocytes. This resulted in 27 newborn mice, 2 of which harbored the transgene, as determined by PCR analysis and Southern blotting (data not shown). Both founder mice were bred into 2 independent lines of HuPLTP transgenic mice.

Expression of the Transgene

PLTP activity was measured in plasma samples of mice from 2 HuPLTP transgenic lines. In line 1, PLTP activity in the plasma was increased by 281% compared with the activity in the plasma from wild-type mice (Table 1), whereas in line 2, activity was increased by 262% (not shown). Subsequent analyses were performed with line 1 only. The use of either human HDL or mouse HDL as acceptor in the PLTP activity assay showed the same differences between wild-type, hemizygous, and homozygous transgenic animals, demonstrating that HuPLTP interacts with human HDL and mouse HDL (not shown).

The expression of the transgene was confirmed by Western blotting (Figure 2). An antibody that reacts specifically with human PLTP was used. Compared with hemizygous transgenic mice, the mice homozygous for the transgene showed a higher plasma protein level of HuPLTP. No immunoreactive PLTP was detected in wild-type mice, because the antibody used does not cross-react with MuPLTP. Human recombinant PLTP was used as a positive control and gave 1 immunoreactive band at a relatively low molecular weight because of the lower extent of glycosylation in the baculovirus expression system.

The transgene was found to be expressed in all tissues analyzed (Figure 3), with relatively high mRNA levels in adrenal, testis, and (to a lesser extent) lung. Moderate mRNA levels were present in liver, kidney, intestine, brain, and spleen. The mRNA expression of the endogenous PLTP gene is highest in adrenal, testis, and lung. The prominent expression in lung tissue is found for MuPLTP but not for the (human) transgene. The expression of endogenous PLTP was

TABLE 1. PLTP Activity, ApoA-I, and Lipid Concentrations in Plasma of HuPLTP Transgenic Mice

	PLTP Activity, $\mu\text{mol} \cdot \text{mL}^{-1} \cdot \text{h}^{-1}$	Cholesterol, mmol/L	Phospholipids, mmol/L	ApoA-I, mg/mL
Wild-type (n=11)	32.6 \pm 6.2	1.65 \pm 0.25	2.11 \pm 0.26	1.09 \pm 0.33
Hemizygotes (n=19)	91.1 \pm 9.3*† (280%)	1.28 \pm 0.17‡§ (77%)	1.78 \pm 0.22‡ (84%)	0.86 \pm 0.23 (79%)
Homozygotes (n=7)	142.5 \pm 25.2* (437%)	1.01 \pm 0.27* (62%)	1.53 \pm 0.29* (72%)	0.75 \pm 0.24‡ (69%)

Values are mean \pm SD. Values between brackets are percentage of the wild-type values. For further details see Methods.

* P <0.001 vs wild-type mice; † P <0.001 vs homozygous mice; ‡ P <0.05 vs wild-type mice; and § P <0.05 vs homozygous mice.

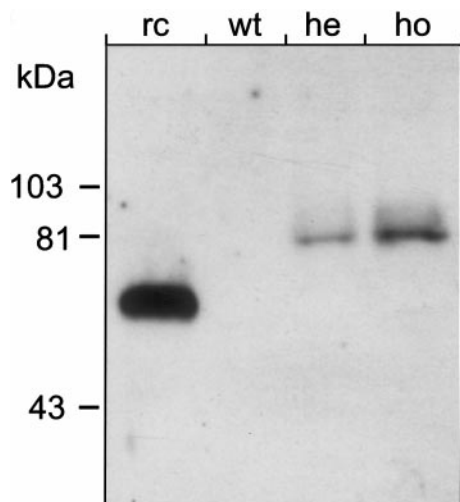


Figure 2. Western blot analysis of plasma levels of HuPLTP in transgenic mice. Five microliters of mouse plasma (diluted 10 times) was loaded per lane. SDS-PAGE was carried out in 12.5% (wt/vol) gels under reducing conditions, and proteins were electrophoretically transferred to polyvinylidene difluoride membranes and visualized with a rabbit polyclonal antibody raised against a synthetic peptide with identity to amino acids 470 to 493 of HuPLTP. Goat anti-rabbit IgG conjugated to peroxidase was used as a secondary antibody. Antigen-antibody complexes were visualized by chemiluminescence with the ECL system (Amersham). Blots were exposed for 20 seconds to a Kodak XAR-5 film. The abbreviation rc refers to human recombinant PLTP from a baculovirus expression system. Human recombinant PLTP displays a relatively lower molecular mass because of differences in glycosylation when produced in the baculovirus expression system. Plasma from wild-type, hemizygous, and homozygous HuPLTP mice is indicated by wt, he, and ho, respectively.

not affected by HuPLTP expression in any of the tissues tested (Figure 3).

Effects of HuPLTP Overexpression on Plasma Lipids and Lipoproteins

The overexpression of PLTP resulted in a decrease in plasma cholesterol levels in the HuPLTP hemizygous transgenic mice and a further decrease in levels in the homozygous transgenic mice (Table 1). Plasma levels of cholesterol, phospholipids, and apoA-I were decreased by about the same extent, indicating that the decrease reflects a lowering of HDL, which is the major lipoprotein in mouse plasma. Separation of plasma lipoproteins by gel filtration confirmed

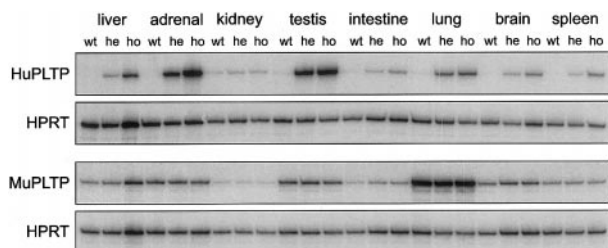


Figure 3. Expression of PLTP in various tissues. Total RNA was isolated from various tissues of wt, he, or ho HuPLTP transgenic mice. RNA levels of either the transgene (HuPLTP) or the endogenous murine gene (MuPLTP) were examined by gel electrophoresis of RT-PCR products. RT-PCR on hypoxanthine-guanine phosphoribosyltransferase was used as a loading control.

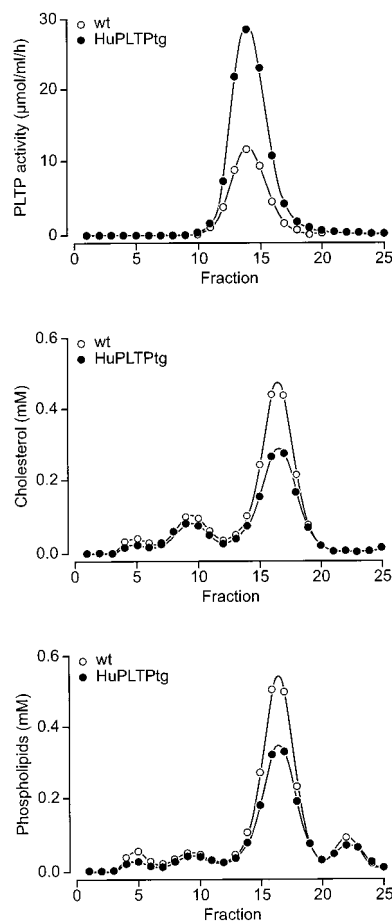


Figure 4. Gel filtration of plasma from wt mice and hemizygous HuPLTP transgenic (HuPLTPtg) mice. Pooled plasma samples (0.5 mL) were run on a Superose 6 and Superose 12 column in tandem. For details see Methods.

that the decrease in plasma lipids can be largely attributed to a decreased level of HDL (fractions 15 to 18), because only minor changes were found in VLDL (fraction 4 to 6) and LDL (fraction 8 to 11), as seen in Figure 4. The peak of PLTP activity was found in fractions 13 to 15 on filtration of plasma from wild-type mice, as well as plasma from HuPLTP transgenic mice (Figure 4).

To investigate changes in HDL subclass distribution, mouse plasma was analyzed by crossed immunoelectrophoresis (Figure 5). Plasma samples from wild-type, hemizygous, and homozygous HuPLTP transgenic mice were collected and incubated in the presence of an inhibitor of LCAT to prevent maturation of the formed pre- β -HDL into α -HDL.¹⁴ The formation of pre- β -HDL particles is clearly increased in plasma from transgenic mice compared with wild-type mice. Table 2 gives the pre- β -HDL values, before and after incubation in the presence of the LCAT inhibitor. No significant differences were found in freshly frozen plasma between the different genotypes. Before incubation, the percentage of pre- β -HDL tends to be highest in the homozygous HuPLTP transgenic animals, but the differences between genotypes are not significant. However, in incubated samples, clear differences arise with the highest relative and absolute concentrations of pre- β -HDL in the transgenic animals.

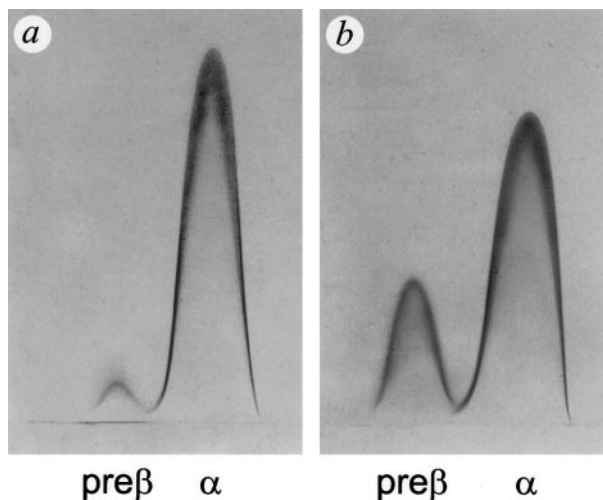


Figure 5. Apo-I immunoprecipitation patterns of plasma from wild-type or HuPLTP transgenic mice obtained after crossed immunoelectrophoresis. The figure shows a representative display of the pre- β -HDL and α -HDL bands obtained by analysis of mouse plasma, incubated for 3 hours at 37°C in the presence of iodoacetic acid (1 mmol/L), an inhibitor of LCAT. Samples were then analyzed by crossed immunoelectrophoresis as described in Methods. To confirm monospecificity of the anti-apoA-I antibody toward α -HDL and pre- β -HDL fractions, we performed immunoelectrophoresis as described.²⁹ Two single precipitation arches appeared without the formation of spurs, indicating that the antibody recognizes immunologically identical determinants in both HDL subfractions.³⁰ Left, Plasma from a wild-type mouse. Right, Plasma from a homozygous HuPLTP transgenic mouse. The positions of pre- β -HDL and α -HDL migration are indicated.

Effect of Wild-Type and Transgenic Plasma on Accumulation of AcLDL-Derived Cholesterol in Mouse Peritoneal Macrophages

We investigated whether differences in the HDL-subfraction pattern affect the ability of wild-type and transgenic plasma to interfere with the esterification of intracellular cholesterol by ACAT. ACAT activity determined in this manner is a measure of the intracellular cholesterol concentration. Mouse peritoneal macrophages were incubated in the presence of [³H]oleate, AcLDL, and diluted mouse plasma containing the acceptor HDL particles (see Methods). Figure 6 shows that the formation of labeled cholesteryl oleate by ACAT was 25.7±9.7% lower in the presence of hemizygous transgenic plasma compared with wild-type plasma, indicating less accumulation of cellular cholesterol in the presence of plasma from transgenic animals. Thus, in spite of a lower HDL

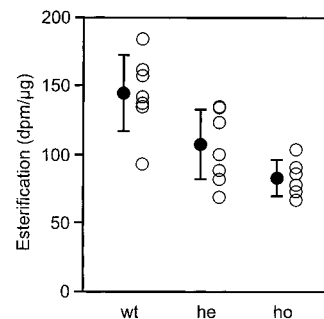


Figure 6. Esterification of cholesterol by ACAT in peritoneal macrophages incubated simultaneously with AcLDL, [³H]oleate, and plasma of wt, he, or ho HuPLTP transgenic mice. Plasma was obtained from age-matched wt, he, and ho HuPLTP transgenic mice. Peritoneal macrophages were incubated for 18 hours in the presence of 3 μg/mL AcLDL, 0.1 mmol/L [³H]oleate-BSA (48 Ci/mol), and diluted mouse plasma. Esterification by ACAT is expressed as disintegrations per minute (dpm) in cholesteryl oleate per microgram cell protein. A decreased esterification indicates a decreased cellular cholesterol concentration. For further details, see Methods. Values for individual plasma samples are shown (open circles), as well as mean values (closed circles) with standard deviations (vertical bars). In the absence of plasma, ACAT activity was 1770±90 dpm/μg. ACAT activity was very low in the absence of AcLDL: 16.0±0.4 dpm/μg with wt plasma. Values for he and ho mice differed at the *P*=0.021 (*n*=8) and *P*=0.001 (*n*=6) level from those for wt mice (*n*=7), respectively. The data presented show a typical example of 4 independent experiments performed.

cholesterol concentration, transgenic mouse plasma has the ability to prevent cholesterol accumulation to a greater extent. With plasma from homozygous HuPLTP transgenic mice, a 42.4±13.2% decrease in ACAT activity (compared with wild-type plasma; see Figure 6) is measured, showing even less accumulation, indicative of a dose-response effect of PLTP on the prevention of cholesterol accumulation.

Discussion

We generated transgenic mice for HuPLTP that have up to a 4.5-fold elevation of plasma PLTP activity levels. The total plasma concentrations of cholesterol, phospholipids, and apoA-I are decreased because of a 30% to 40% reduction in total HDL. Gel filtration, which is used to measure the plasma lipoprotein profile, confirmed the decrease in HDL cholesterol and showed little variation in LDL and VLDL. No change in HDL size was detected.

Plasma pre- β -HDL concentrations are unchanged in HuPLTP transgenic mice (Table 2). This does not preclude the possibility of increased turnover of pre- β -HDL in the transgenic animals. Pre- β -HDL is the substrate of plasma LCAT that converts it into mature α -HDL, normally carrying most of the circulating plasma HDL cholesterol.¹⁴ The formation of pre- β -HDL during plasma incubation at 37°C is therefore masked by the LCAT reaction, and the maximal capacity for pre- β -HDL formation can be measured only when LCAT is inactive. For this reason, plasma was incubated in the presence of an LCAT inhibitor. These experiments revealed that the relative and absolute plasma levels of pre- β -HDL are clearly increased in plasma from transgenic animals incubated under these conditions (Table 2). These data indicate that rapid pre- β -HDL conversion to α -HDL, driven by LCAT, may obscure the effects of the HuPLTP gene on plasma pre- β -HDL levels in vivo. Our data imply that HuPLTP transgenic plasma has a much greater ability to

TABLE 2. Pre- β -HDL Levels in Plasma of HuPLTP Transgenic Mice Before and After Incubation at 37°C

	Relative Pre- β -HDL, % Total Plasma ApoA-I		Absolute Pre- β -HDL, μg/mL ApoA-I in Pre- β	
	Plasma Incubation		Plasma Incubation	
	No	Yes	No	Yes
Wild-type (<i>n</i> =9)	3.7±1.8	7.8±2.9	45±31	93±57
Hemizygotes (<i>n</i> =10)	3.0±2.0	21.0±3.0*†	28±23	181±58‡
Homozygotes (<i>n</i> =10)	5.1±2.2	27.5±3.4*	39±23	207±62*

Values are mean±SD. For details see Methods.
**P*<0.001 vs wild-type mice; †*P*<0.001 vs homozygous mice; and ‡*P*<0.01 vs wild-type mice.

generate pre- β -HDL than does wild-type plasma, in spite of only marginal differences in pre- β -HDL levels.

HuPLTP transgenic mice have been described previously by 2 other groups, but these models did not show an appreciable overexpression of the transgene. The mice described by Albers et al¹⁷ showed little expression of the transgene and only small changes in plasma lipoproteins. Jiang et al¹⁸ reported a 29% increase in PLTP activity, but significant effects on total plasma lipids or lipoproteins were not observed. After their HuPLTP transgenic mice were crossbred with mice transgenic for human apoA-I, they detected minimal effects on plasma lipids and lipoproteins (including an increase in pre- β -HDL levels), together with a 47% elevation in plasma PLTP activity. It must be noted that compared with wild-type mice, human apoA-I transgenic mice already have elevated levels of total and pre- β -HDL cholesterol.¹⁸ The HuPLTP transgenic mice described in the present study have altered HDL metabolism, without the complication of the additional human apoA-I gene, which by itself has substantial effects on HDL. Moreover, our mice are not transgenic for lysosomal protective protein. Jiang et al¹⁸ used a DNA construct containing both genes that have a 3' overlap on opposite DNA strands. Although unlikely, it is difficult to exclude the possibility that this condition interferes with lipoprotein metabolism.

HuPLTP has also been overexpressed in mice via adenovirus-mediated transfer.^{31,32} These mice showed a 13- to 40-fold elevation of PLTP activity in plasma several days after treatment. This resulted in a dramatic decrease (by 91%) in total HDL cholesterol levels, whereas pre- β -HDL levels were substantially elevated.³¹ These data are in line with our present observations: high PLTP activity results in a decrease in total HDL, whereas pre- β -HDL levels are increased. It is clear that the effects are transient and greatly exaggerated in the adenovirus-treated mice, because of the extremely high plasma levels of PLTP. Four-fold-elevated PLTP activity levels, measured at late time points after adenovirus transfection, did not result in altered plasma HDL cholesterol concentrations, whereas in our homozygous HuPLTP transgenic mice, a 4-fold elevation in PLTP activity resulted in decreased HDL concentrations. Another notable difference is that in the adenovirus-treated mice, PLTP expression is restricted to the liver. The tissue pattern of expression of the HuPLTP gene in transgenic mice more closely resembles the pattern of expression of the endogenous PLTP gene (Figure 3).³³

Compared with plasma of wild-type mice, the plasma of HuPLTP transgenic mice was found to be much more efficient in partially preventing the AcLDL-induced accumulation of intracellular cholesterol in cultured macrophages, in spite of lower levels of total HDL.

The most likely explanation for this observation is the increased plasma concentration of pre- β -HDL, which has been identified previously as a very efficient cholesterol acceptor.^{13,14} It is well known that an operative cholesteryl ester cycle, as present in macrophages,^{34,35} is important for cholesterol efflux. Miyazaki et al³⁶ show that dimyristoylphosphatidylcholine/apoA-I complexes, but not HDL, may modify AcLDL in a concentration-dependent manner, resulting in decreased cellular uptake. However, the plasma concentration of pre- β -HDL is not increased in our HuPLTP transgenic mice. Therefore, it is unlikely that the prevention of cellular cholesterol accumulation by transgenic mouse plasma is due to decreased uptake of

AcLDL by the macrophages. The present results imply that the distribution of HDL subclasses is of major importance in the efficacy of HDL-mediated reverse cholesterol transport; it is even more important than total plasma HDL levels. Compared with plasma from hemizygous mice, plasma from homozygous HuPLTP transgenic animals appeared to be more efficient in preventing cellular cholesterol accumulation, indicating a dose response for the effect of PLTP on cellular cholesterol. In addition to the proposed mechanism of action of PLTP on cellular cholesterol efflux via the generation of pre- β -HDL, PLTP could also contribute directly to cellular cholesterol efflux via its proposed cholesterol transfer activity.³⁷

Plasma levels of total HDL cholesterol are inversely correlated with the incidence of coronary artery disease in humans.^{7,8} However, only a few studies have looked at the relation between total HDL and pre- β -HDL in human plasma. Recently, O'Connor et al¹⁴ analyzed the steady-state levels of pre- β -HDL in 136 normolipidemic individuals by using an isotope-dilution technique. Their relative values for pre- β -HDL in human plasma are quite comparable to the values measured in mouse plasma (see Table 2). The percentage of pre- β -HDL (percentage of total plasma apoA-I) was negatively correlated with total HDL cholesterol concentrations, in line with our observations in mice. Plasma PLTP activity was not measured in their study.

Reconstituted HDL particles enriched in triglycerides, which are model particles for HDL prevalent during alimentary lipemia, are more rapidly converted by PLTP to pre- β -HDL than are triglyceride-poor HDL particles.³⁸ This observation suggests that hypertriglyceridemia may be associated with increased generation of pre- β -HDL by PLTP. In addition, Syväne et al³⁹ reported a positive correlation between PLTP activity and the capability of plasma from patients with diabetes mellitus and coronary heart disease to induce cholesterol efflux from Fu5AH rat hepatoma cells.

Taken together, the combination of increased PLTP activity, increased pre- β -HDL formation, and less accumulation of cellular cholesterol (as seen in our mouse model) is likely to exist also in the human situation. The rate of formation of pre- β -HDL may very well be more important than its steady-state concentration. The present work shows for the first time that PLTP is important in the ongoing generation of plasma pre- β -HDL and that the ability of plasma to prevent cholesterol accumulation in macrophages is increased at high PLTP activity levels. Our findings show that PLTP is a potential antiatherogenic factor because of its ability to generate pre- β -HDL. To test this hypothesis directly in vivo, we will perform a comparative study in which the susceptibility to diet-induced atherosclerosis will be evaluated. We are currently expanding the colony of HuPLTP transgenic mice to this end. Because the PLTP-expressing mice are expected to be even more resistant to diet-induced atherosclerosis than are wild-type mice, these experiments will be very time-consuming. Another approach will be to measure (diet-induced) atherogenesis in mouse models susceptible to the development of atherosclerosis (eg, that due to LDL receptor deficiency⁴⁰ or apoE deficiency^{41,42}), with and without overexpression of HuPLTP.

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