Evaluation of phospholipid transfer protein and cholesteryl ester transfer protein as contributors to the generation of pre-β-high-density lipoproteins

Jessica LIE*1, Rini DE CROM†‡1, Matti JAUHAIANEN‡, Teus VAN GENT*, Rien VAN HAPEREN†, Leo SCHEEK*, Hans JANSEN*, Christian EHNHOLM§ and Arie VAN TOL‡‡

*Department of Biochemistry, Cardiovascular Research Institute COEUR, Erasmus University Rotterdam, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands, †MGC-Department of Cell Biology and Genetics, Erasmus University Rotterdam, P. O. Box 1738, 3000 DR Rotterdam, The Netherlands, ‡Department of Vascular Surgery, Academic Hospital “Dijkzigt”, Dr Molewaterplein 40, 3015 GD Rotterdam, The Netherlands, and §Department of Biochemistry, National Public Health Institute, Mannerheimintie 166, FIN-00300 Helsinki, Finland

High-density lipoproteins (HDLs) are considered anti-atherogenic because they mediate peripheral cell cholesterol transport to the liver for excretion and degradation. An important step in this reverse cholesterol-transport pathway is the uptake of cellular cholesterol by a specific subclass of small, lipid-poor apolipoprotein A-I particles designated pre-β-HDL. The two lipid-transfer proteins present in human plasma, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP), have both been implicated in the formation of pre-β-HDL. In order to investigate the relative contribution of each of these proteins, we used transgenic mouse models. Comparisons were made between human CETP transgenic mice (huCETPtg), human PLTP transgenic mice (huPLTPtg) and mice transgenic for both lipid-transfer proteins (huCETPtg/huPLTPtg). These animals showed elevated plasma levels of CETP activity, PLTP activity or both activities, respectively. We evaluated the generation of pre-β-HDL in mouse plasma by immunoblotting and crossed immuno-electrophoresis. Generation of pre-β-HDL was equal in huCETPtg and wild-type mice. In contrast, in huPLTPtg and huCETPtg/huPLTPtg mice, pre-β-HDL generation was 3-fold higher than in plasma from either wild-type or huCETPtg mice. Our findings demonstrate that, of the two plasma lipid-transfer proteins, PLTP rather than CETP is responsible for the generation of pre-β-HDL. These data support the hypothesis of a role for PLTP in the initial stage of reverse cholesterol transport.

Key words: apoA-I, LCAT, lecithin:cholesterol acyltransferase, reverse cholesterol transport, transgenic mice.

INTRODUCTION

Plasma levels of high-density lipoprotein (HDL) cholesterol correlate negatively with the risk of coronary artery disease, and raising HDL cholesterol levels by drug treatment results in prevention of coronary artery disease [1–4]. HDL is considered to protect against atherosclerosis via its role in reverse cholesterol transport [5]. This pathway involves the uptake of cholesterol from peripheral cells and subsequent transport and delivery to the liver for degradation and excretion. Recent data indicate that the ATP-binding cassette transporter A1 (ABCA1) plays a key role in the efflux of cholesterol from cells [6–8]. Patients suffering from Tangier disease, with a mutated form of the ABCA1 gene, show premature atherosclerosis, providing compelling evidence for a substantial protective role of reverse cholesterol transport in the development of atherosclerosis. The initial extracellular acceptor of cellular cholesterol is a specific subclass of HDL, designated pre-β-HDL [9]. Cellular cholesterol taken up by pre-β-HDL is subsequently esterified by the action of lecithin:cholesterol acyltransferase (LCAT). As a consequence, pre-β-HDL matures into α-HDL. Esterified cholesterol from α-HDL can be taken up in the liver either directly by selective uptake or indirectly via low-density lipoproteins (LDLs). The indirect pathway follows transfer of cholesteryl esters to very-low-density lipoproteins (VLDLs) and LDLs by cholesteryl ester transfer protein (CETP) [9,10]. Thus pre-β-HDL is a key factor in reverse cholesterol transport.

Although the plasma lipid-transfer proteins phospholipid transfer protein (PLTP) and CETP have non-overlapping functions [11], studies both in vitro and in vivo have demonstrated that CETP and PLTP may both contribute to the formation of pre-β-HDL [12–18]. PLTP is involved in the transfer of phospholipids between lipoproteins and in the remodelling of HDL [19–21]. Its physiological function has been studied in several mouse models [12,22–24]. PLTP-deficient mice show an impaired transfer of post-hepatic VLDL surface remnant phospholipids to HDL and, as a consequence, decreased plasma HDL levels [21]. In PLTP transgenic mice with slightly elevated PLTP activity levels minor effects on plasma lipids and lipoprotein patterns were found [15,23]. However, in double-transgenic mice expressing both PLTP and human apolipoprotein A-I (apoA-I), pre-β-HDL levels were increased [23]. We generated transgenic human PLTP mice that show a moderate, though substantial overexpression of PLTP (2.5–3-fold in hemizygous transgenic mice) [12]. Plasma from these mice has an increased ability to generate pre-β-HDL and prevents cholesterol accumulation in macrophages to a greater extent than plasma from wild-type animals. From these studies, we concluded that PLTP could have anti-atherogenic properties via its ability to generate pre-β-HDL.
Both CETP and PLTP belong to a family of lipid-transfer/lipopolysaccharide-binding proteins[10,25]. Although CETP has profound effects on lipoprotein metabolism in humans, some species, including mice, lack CETP activity[26]. In vitro studies demonstrate that CETP transfers cholesteryl esters from HDL to apoB-containing lipoproteins in exchange for triglycerides, whereas PLTP does not transfer neutral lipids[5]. In addition to PLTP, CETP is able to transfer phospholipids in vitro, but it is unknown whether they share this ability in vivo.

In the present study we evaluated the relative contributions of CETP and PLTP to the formation of preβ-HDL. To this end, we made comparisons between various lines of transgenic mice expressing human CETP, human PLTP or both lipid-transfer proteins.

**MATERIALS AND METHODS**

**Breeding and treatment of transgenic mice**

Human CETP transgenic mice (line 5203; huCETPtg) were kindly donated by Dr A. R. Tall (Columbia University, New York, NY, U.S.A.)[27] and have a C57BL/6 background. Human PLTP transgenic mice (huPLPTg) were generated as described previously[12] and were backcrossed to the C57BL/6 background for at least seven generations. Mice expressing the human CETP and PLTP transgenes (huCETPtg/huPLPTg) were obtained by crossbreeding huCETPtg and huPLPTg mice. Both transgenes had the natural flanking sequences, including the autologous promoters. Animals were housed in a temperature-controlled room operating under a 12 h:12 h light:dark cycle. Animals were fed regular chow and water ad libitum.

Blood samples were collected, from animals that had been fasted overnight, from the orbital plexus by using Vitrex™ sodium-heparinized micro pipettes (50 μl; Modulohm A/S, Copenhagen, Denmark) and were immediately stored on ice. Blood was centrifuged at 1500 gmax for 15 min at 4°C. Plasma was either used directly or stored in small aliquots at −80°C before analysis. All animal experiments were done in compliance with the Guidelines of the Ethical Committee on the Use of Laboratory Animals of the Erasmus University Rotterdam (protocol no. 120.99.05) 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 analysed for the presence of the huCETP transgene and/or huPLPT transgene by PCR analysis. Conditions and primers were as follows: huCETP, sense primer, 5'-CTAGGACCC-AGAGAGGAGGTGC-3'; antisense primer, 5'-CTGAGCC-CAGCCGCACATAC-3'; 28 cycles (94°C, 1 min; 65°C, 1 min; 72°C, 1.5 min); huPLPT, sense primer, 5'-GCCACA-GCAGGAGCTGATGC-3'; antisense primer, 5'-GCCGATGG-ACACGCCCTCCAGC-3'; 28 cycles (94°C, 1 min; 65°C, 1 min; 72°C, 2 min).

**Plasma activity assays**

CETP and PLPT assays were performed according to Speijer et al.[28]. CETP activity was determined by measuring the rate of transfer/exchange of radiolabelled cholesteryl oleate between exogenously added human LDL and HDL. PLPT activity was determined by measuring the transfer of radiolabelled dipalmitoyl phosphatidylcholine (DPPC) from liposomes (composed of egg lecithin (Amersham, Little Chalfont, Bucks., U.K.) and [3H]DPPC as a tracer) to exogenously added human HDL (density, 1.063 g/ml < d < 1.21 g/ml). CETP and PLPT activities are expressed as a percentage of the human reference plasma: 100% is equivalent to the following activities; CETP, 215.6 nmol/ml per h; PLPT, 13.9 μmol/ml per h.

Hepatic lipase activity was measured as described by Jansen et al.[29]. LCAT activity was determined by measuring the formation of radiolabelled cholesteryl ester after addition of 10 or 20 μl of mouse plasma to excess heat-inactivated plasma containing [3H]cholesterol (Amersham) [30]. Hepatic lipase and LCAT activities are expressed as a percentage of plasma from wild-type mice (C57BL/6).

**Quantification of plasma lipids and apolipoproteins**

Plasma lipids were determined enzymically with commercially available kits: total cholesterol with the F-chol kit of Boehringer Mannheim (Mannheim, Germany), and after hydrolysis of cholesterol esters with cholesterol esterase from Candida cylindracea (Boehringer Mannheim). Phospholipids were measured with the PAP150 kit from BioMérieux (Lyon, France). Plasma triglycerides were measured with the Sigma GPO-Trinder kit (procedure no. 337-B; Sigma, St. Louis, MO, U.S.A.). Mouse apoA-I was quantified by sandwich ELISA as reported in[12].

**Gel-filtration chromatography of mouse plasma lipoproteins**

Lipoprotein profiles were determined by gel filtration of freshly isolated plasma on two HR10/30 FPLC columns in tandem (Superose 6 prepgrade and Superdex 200 prepgrade; Pharmacia Biotech, Uppsala, Sweden). The columns were equilibrated with 2 mM NaH2PO4/Na2HPO4, pH 7.4, containing 0.9% NaCl (w/v), 0.02% NaN3 (w/v) and 5 mM EDTA. Combined plasma samples from four to seven mice were passed through 0.45 μm filters from Millipore S. A. (Molsheim, France), and 0.5 ml were subjected to gel filtration. The columns were run at 4°C with a flow rate of 0.1 ml/min. Fractions of 0.8 ml were collected. Recoveries were > 90% for all analyses.

**Quantification of preβ-HDL**

Mouse plasma samples were either frozen directly or incubated in the presence of iodoacetate in order to measure preβ-HDL formation. Incubation conditions were as reported in[12]. Plasma samples were separated by agarose-gel electrophoresis under non-reducing, non-denaturing conditions and low ionic strength according to the manufacturer’s instructions (Paragon Lipo system; Beckman, Fullerton, CA, U.S.A.) to separate preβ- and α-migrating HDL. Proteins were transferred by capillary blotting to nitrocellulose membranes (Schleicher & Schuell NY 13N 6214/95, Dassel, Germany). Membranes were blocked for 1 h in 5% (w/v) skimmed milk in 20 mM Tris/HCl, pH 7.4/0.5 M NaCl. Membranes were subsequently incubated with an affinity-purified rabbit anti-mouse apolipoprotein A-I polyclonal antibody. [125I]-labelled donkey anti-rabbit F(ab’)2 fragment (17 Ci/g; Amersham) was used as the detection antibody. The relative abundance of apoA-I among the α- or preβ-HDL species was calculated by quantitative scanning using a PhosphorImager (GS-363; BioRad). Crossed immuno-electrophoresis was performed as described in[12].

**Statistical analysis**

Data are expressed as means±S.D. Differences between two genotypes were analysed by two-sample Wilcoxon rank-sum
RESULTS

Plasma activities of CETP and PLTP

CETP activities were measured in plasma from huCETPtg mice and huCETPtg/huPLTPtg mice (Figure 1A). CETP activity in wild-type mice was virtually absent ( < 4 % of human reference

Figure 1  Plasma CETP and PLTP activities in transgenic mice

Plasma activities were determined in individual plasma samples from huCETPtg mice (n = 12) and huCETPtg/huPLTPtg mice (n = 10). Values are expressed as a percentage of human reference plasma (means ± S.D.). (A) CETP activity: 100 % human reference plasma is equivalent to CETP activity of 215.6 nmol/ml per h; *P < 0.01 compared with huCETPtg mice (rank-sum test). (B) PLTP activity: 100 % human reference plasma is equivalent to PLTP activity of 13.9 μmol/ml per h; **P < 0.0001 compared with huCETPtg mice (rank-sum test).

Figure 2  Plasma hepatic lipase and LCAT activities in transgenic mice

Plasma activities were measured in individual plasma samples from wild-type mice (wt; n = 16), huCETPtg mice (n = 12), huPLTPtg mice (n = 8) and huCETPtg/huPLTPtg mice (n = 10). Values are means ± S.D. and are expressed as a percentage of the wild-type value. (A) Hepatic lipase activity: 100 % hepatic lipase activity of the wild-type is equivalent to 56.1 nmol/ml per h; *P < 0.05, significantly different from huCETPtg mice (ANOVA followed by Bonferroni correction). (B) LCAT activity: 100 % LCAT activity of the wild-type is equivalent to 71.3 nmol/ml per h. Transgenic mice showed similar LCAT activities (not significantly different).

Figure 3  Lipoprotein profiles of plasma from transgenic mice

Equal amounts of plasma from either huCETPtg mice (n = 4) or huCETPtg/huPLTPtg mice (n = 7) were pooled and subjected to gel filtration on Superose 6 and Superdex 200 columns connected in tandem as described in the Materials and methods section. Fractions were analysed for (A) cholesterol, (B) phospholipids, (C) CETP activity and (D) PLTP activity. Fractions 1–4 contain VLDL, 5–10 contain LDL, 11–19 contain HDL and 20–25 contain lysophosphatidylcholine bound to albumin.
plasma). HuCETPtg mice had a CETP activity of 144 ± 34 % of human reference plasma whereas CETP activity in huCETPtg/huPLTPtg mice was about 20 % lower (P < 0.01).

PLTP activity levels were measured in plasma samples of huCETPtg and huCETPtg/huPLTPtg mice (Figure 1B). The PLTP activity in plasma of huCETPtg mice was somewhat higher than human PLTP activity levels: 136 ± 16 % of human reference plasma, which is in agreement with measurements in wild-type mice [12]. The PLTP activity in the huCETPtg/huPLTPtg mice was 4-fold higher (Figure 1B). This activity level closely resembles the level reported previously for huPLTPtg mice [12].

Plassma hepatic lipase activity and LCAT activity

Mice express hepatic lipase activity in plasma, even without intravenous injection of heparin [31]. We investigated whether any differences in the activity of hepatic lipase in plasma between huCETPtg mice and huCETPtg/huPLTPtg mice could be observed. As shown in Figure 2(A), the huCETPtg/huPLTPtg mice had lower levels of hepatic lipase activity than huCETPtg mice (P < 0.05). Hepatic lipase activity in the huPLTPtg mice was 91 ± 27 % of wild-type mouse plasma.

In plasma, preβ-HDL matures into α-HDL by the action of LCAT. The levels of LCAT activity were not significantly different between the three tested genotypes (Figure 2B).

Lipid and lipoprotein analysis

Plasma samples from either huCETPtg or huCETPtg/huPLTPtg animals were analysed by gel-filtration chromatography to examine their lipoprotein profiles. Cholesterol and phospholipid contents as well as CETP and PLTP activities were determined in all fractions (Figure 3). In unfractionated plasma, huCETPtg/huPLTPtg mice had lower levels of plasma cholesterol than huCETPtg mice (1.09 ± 0.15 mM, n = 9, versus 1.43 ± 0.16 mM, n = 11; P < 0.01), whereas triglyceride levels were similar (huCETPtg mice, 0.38 ± 0.14, n = 5; huCETPtg/huPLTPtg mice, 0.48 ± 0.25, n = 5, not statistically significant). As evident from Figure 3(A), which shows the cholesterol profile, this is due to a decrease in HDL cholesterol. The phospholipids have a similar profile, showing a decrease in HDL phospholipids due to PLTP overexpression (Figure 3B). The phospholipids present in fractions 20–25 represent lysophosphatidylcholine bound to albumin. In both groups of mice CETP activity eluted in fractions 14–18, corresponding to the position of HDL particles (Figure 3C). PLTP activity eluted in fractions 11–15, the position of relatively large HDLs (Figure 3D), a situation also found in humans.

Effects of huPLTP and huCETP expression on preβ-HDL formation

To investigate the formation of preβ-HDL, incubated plasma samples from wild-type, huCETPtg, huPLTPtg and huCETPtg/huPLTPtg mice were subjected to agarose-gel electrophoresis followed by immunoblotting. Upon incubation, the levels of preβ-HDL increased in mice expressing human PLTP, whereas neither wild-type nor huCETPtg mice showed appreciable levels of preβ-HDL formation (Figure 4). In order to quantify preβ-HDL formation, crossed immuno-electrophoresis experiments were performed (Figure 5). The results corroborated the findings from the immunoblotting experiments, since incubated samples from mice expressing human PLTP showed relatively
Plasma samples from wild-type, huCETPtg, huPLTPtg and huCETPtg/huPLTPtg mice were incubated as described in the legend of Figure 4. Values represent the differences in the relative and absolute amounts of preβ-HDL in plasma before and after incubation at 37 °C for 3 h. These were calculated, as described in the legend of Figure 6, from the percentage of apoA-I present in preβ-HDL and the total plasma apoA-I concentrations respectively. Values are means ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Total plasma apoA-I (mg/ml)</th>
<th>Relative preβ-HDL (% of plasma apoA-I)</th>
<th>Absolute preβ-HDL (µg of apoA-I/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-incubation</td>
<td>Post-incubation</td>
<td>Pre-incubation</td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.0 ± 0.07</td>
<td>3.7 ± 0.9</td>
<td>7.3 ± 0.5</td>
</tr>
<tr>
<td>huCETPtg</td>
<td>0.88 ± 0.05</td>
<td>5.0 ± 0.8</td>
<td>7.3 ± 0.5</td>
</tr>
<tr>
<td>huPLTPtg</td>
<td>0.73 ± 0.24</td>
<td>6.0 ± 0.8</td>
<td>16.7 ± 0.9*</td>
</tr>
<tr>
<td>huCETPtg/huPLTPtg</td>
<td>0.7 ± 0.09</td>
<td>7.3 ± 1.7</td>
<td>17.3 ± 1.3*</td>
</tr>
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* Significantly different from wild-type mice, P < 0.05 (rank-sum test).
activities or the formation of preβ-HDL, we extended our studies with double-transgenic animals, huCETPtg/huPLPTg mice. This model resembles the situation in humans since, in contrast to mice, humans express both plasma transfer proteins.

huCETPtg and huCETPtg/huPLPTg mice showed a small difference in plasma CETP activity levels. The expression of the human PLTP gene is associated with a decrease in CETP activity of about 20%. This decrease is confirmed on analysis of the CETP activity profiles after gel filtration (Figure 3C). This effect could be the result of a direct effect of overexpression of human PLTP on the level of expression of the CETP transgene. An alternative explanation is that PLTP expression causes a lower plasma HDL concentration. Since HDL is the carrier of CETP, this could eventually result in a decrease in CETP levels. The profiles show that CETP activity elutes at a position corresponding to that of HDL particles.

PLTP activity measured in huCETPtg/huPLPTg mice equals the PLTP activity in the huPLPTg mice described previously [12]. PLTP activity in huCETPtg mice was comparable with that in wild-type mice. Therefore CETP does not seem to affect the activity of the endogenous mouse PLTP in plasma. In comparison with the lipoprotein profile found in huPLPTg mice [12], huCETPtg/huPLPTg mice have somewhat smaller HDLs, which is in agreement with previous studies in transgenic mice expressing human CETP [35]. The activity profiles of PLTP in huCETPtg mice and huCETPtg/huPLPTg mice were measured after gel filtration of plasma (Figure 3D). The profiles show that PLTP activity elutes at the position corresponding to relatively large-sized HDL particles in both groups of mice. Thus CETP and PLTP co-elute with particles of different sizes, a situation also found in humans [28].

The main purpose of the present study was to determine the relative contribution of PLTP and CETP to preβ-HDL generation. We evaluated the formation of preβ-HDL in plasma using two different methods and demonstrated that preβ-HDL formation is increased in plasma from transgenic mice expressing human PLTP, whereas expression of human CETP did not affect this process. These findings cannot be attributed to differences in preference of the transfer proteins towards human versus mouse HDL, since incubations of plasma from huCETPtg mice with freshly isolated human HDL did not show formation of preβ-HDL (J. T. Lie, R. de Crom, M. Jauhiainen, T. van Gent, R. van Hapener, L. M. Scheek, C. Ehnholm and A. van Tol, unpublished work).

LCAT activity was similar in plasma of the various transgenic mice, which implies that a difference in maturation of preβ-HDL into α-HDL is unlikely. Therefore differences in LCAT activity cannot explain the observed differences in preβ-HDL formation.

The involvement of hepatic lipase in the formation of preβ-HDL has been suggested [36]. In humans, virtually all hepatic lipase is bound to liver endothelial cells, whereas in mice hepatic lipase circulates in plasma [31]. It was therefore important to know whether plasma from huCETPtg/huPLPTg mice displayed higher hepatic lipase activity than plasma from huCETPtg mice. Hepatic lipase activity was found to be lower in plasma from huCETPtg/huPLPTg compared with plasma from huCETPtg mice, so this activity cannot explain the observed differences in preβ-HDL generation.

Other groups have reported a role for CETP in preβ-HDL formation [13,17,18]. Kunikata et al. [13] showed that preβ-HDL formation is stimulated by CETP in vitro. Francone et al. [18], using double-transgenic animals that overexpressed CETP (driven by the metallothionein promoter) as well as human apolipoprotein A-I, observed increased levels of preβ-HDL in these mice. In the mouse models used here, CETP expression was driven by its native promoter and CETP levels and activities were comparable with those in human plasma. An increase in the generation of relative and absolute plasma levels of preβ-HDL formation was only observed in huPLPTg mice. Despite lower HDL concentrations in huCETPtg/huPLPTg mice compared with huCETPtg mice, plasma of huCETPtg/huPLPTg mice had a greater ability to produce preβ-HDL. This finding of a combination of low HDL cholesterol levels and increased preβ-HDL formation was also observed in huPLPTg mice [12], as well as in mice using adenoviral overexpression of PLTP [22,24].

Although CETP and PLTP share similar structural properties and belong to the same gene family, it has been found that they have non-overlapping functions in vivo [11]. The roles of the HDL modulators CETP and PLTP in atherosclerosis have not yet been elucidated. CETP participates in reverse cholesterol transport, a function that may mean it has anti-atherogenic potential [10]. On the other hand, CETP mediates the exchange of cholesteryl esters in HDL for triglycerides in VLDL. This process leads to a decrease in HDL cholesterol and to an increase in potentially pro-atherogenic VLDL and LDL cholesterol [37]. Although substantial overexpression of human CETP was reported to cause increased susceptibility to diet-induced atherosclerosis [32], CETP has been proved to be anti-atherogenic in other (compound) transgenic mouse models [38,39]. In rats, high-level expression of human CETP is highly atherogenic [33], whereas in rabbits inhibition of CETP proved to be anti-atherogenic [40]. The impact of CETP on atherosclerosis in humans remains inconclusive [37]. The present study supports the notion that PLTP, rather than CETP, is responsible for the generation of preβ-HDL. The impact of PLTP on diet-induced atherosclerosis will be the subject of future investigations.

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