Cholesteryl Ester Transfer Protein Expressed in Lecithin Cholesterol Acyltransferase–Deficient Mice

Cheng-ai Wu, Maki Tsujita, Kuniko Okumura-Noji, Shinichi Usui, Hajime Kakuuchi, Mitsuyo Okazaki, Shinji Yokoyama

Objective—Regulation of plasma cholesteryl ester transfer protein (CETP) concentration was studied in lecithin-cholesterol acyltransferase (LCAT)-knockout mice.

Methods and Results—LCAT-knockout mice were cross-bred with CETP transgenic mice. The offspring (n=63) were classified for LCAT genotype and plasma CETP levels (no CETP, low CETP, and high CETP). High density lipoprotein (HDL) decreased as LCAT decreased in each CETP-level group. In the lcat(+/+) and lcat(+/−) mice, plasma CETP varied from 0 to 30 μg/mL, whereas it was <10 μg/mL in the lcat(−/−) mice. HDL cholesterol and phospholipid decreased and HDL triglyceride and apolipoprotein B increased in CETP in the lcat(+/+) and lcat(+/−) mice, whereas there was no difference in HDL between low and high CETP. An effect of CETP on HDL was not detected in the lcat(−/−) mice because of the absence of mature HDL. Genomic DNA and mRNA of CETP were correlated and were similar in the lcat(−/−) and lcat(+/+) mice. Plasma CETP was correlated with its genomic DNA and mRNA, but the slope of the increase was much lower in the lcat(−/−) mice. Whereas plasma CETP mostly associates with HDL in the lcat(+/+) mouse, it is found free in the lcat(−/−) mouse.

Conclusions—Plasma CETP is posttranscriptionally downregulated in the lcat(−/−) mice, presumably by its extremely low HDL. (Arterioscler Thromb Vasc Biol. 2002;22:1347-1353.)

Key Words: cholesterol ■ high density lipoprotein ■ lecithin-cholesterol acyltransferase ■ cholesteryl ester transfer protein ■ mice

High density lipoprotein (HDL) is given a key role in the hypothesis of a cholesterol transport pathway from the peripheral tissues to the liver. Lecithin-cholesterol acyltransferase (LCAT) plays a major role in this process by esterifying free cholesterol (FC) in circulating lipoproteins to maintain a FC gradient between the peripheral cells and the HDL particle surface and, accordingly, to promote FC efflux from the cells.1 The importance of LCAT in HDL metabolism has been established by identification and characterization of the patients with LCAT deficiency.2 Mutations in the human LCAT gene cause either familial LCAT deficiency or fish eye disease, which results in the decrease of plasma HDL and accumulation of cholesterol in the cell membrane in certain organs.2 Disruption of the LCAT gene in mice also exhibits severe reduction of plasma HDL3,4 and mimics many features of LCAT deficiency in humans.

The cholesteryl ester (CE) generated by LCAT and present in the HDL core can be transported directly to the liver by selective uptake5,6 and/or potentially as a whole particle.7 Alternatively, HDL CE is transferred to apoB-containing lipoproteins by CE transfer protein (CETP) for liver uptake.8 CETP is a plasma glycoprotein that mediates transfer/exchange of CE and triglycerides between HDL and apoB-containing lipoproteins.9,10 The heteroexchange of CE with triglycerides by CETP leads to the net CE transfer between plasma lipoproteins.9 This reaction is also one of the key steps of cholesterol transport from the peripheral tissues to the liver. Thus, CETP is involved as much as LCAT in regulation of the plasma HDL level and remodeling of HDL particles. In fact, patients with CETP deficiency show a marked CE accumulation in HDL.11 Although the expression of CETP is substantially influenced by factors such as dietary cholesterol intake, plasma cholesterol, hormones, and drugs,12,13 CETP varies little on various factors.14,15 The liver has been shown to be a predominant source of CETP and LCAT, inasmuch as plasma LCAT activity can be considered as a parameter for hepatic protein synthesis,16 and there is a positive correlation between plasma CETP levels and hepatic CETP mRNA abundance.17 Other tissues, such as adipose tissue and muscle, are also found in abundance in the CETP message.18

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In human plasma, CETP and LCAT have been found to be associated with HDL particles. Therefore, although both function to regulate the plasma HDL level, the HDL level may also be a determinant of plasma LCAT and CETP levels. In fact, the plasma LCAT activity in Tangier disease is low. In mixed-breed offspring of the CETP transgenic and LCAT-deficient mice. Our findings indicate that the plasma CETP level is posttranscriptionally downregulated by the LCAT deficiency that induces low HDL in plasma.

**Methods**

**Animals**

The LCAT-deficient mice (DBA/×C57BL/6) were kindly provided by Dr. E. Rubin at Lawrence Berkeley Laboratory (Berkeley, Calif). The human CETP transgenic mice (C57BL/6) were gifts from Japan Tobacco Inc (Tokyo). The homozygous LCAT-deficient mice were crossed with the highly expressing CETP transgenic mice to produce the F1 mice, and the F2 mice were derived from a total of 15 matings between the F1 mice. The F2 mice were weaned at 3 weeks (n=65, 32 males and 31 females). Genetic types of LCAT, lcat(+/+), lcat(+/-), and lcat(-/-) were identified for the F2 mice at the age of 5 weeks by multiplex polymerase chain reaction (PCR) genotype analysis of the tail genomic DNA. The forward primers hybridize to the neo-resistant region of the tail genomic DNA. The forward primers hybridize to the neo-resistant region of the tail genomic DNA. The forward primers hybridize to the neo-resistant region of the tail genomic DNA. The forward primers hybridize to the neo-resistant region of the tail genomic DNA. The forward primers hybridize to the neo-resistant region of the tail genomic DNA. The forward primers hybridize to the neo-resistant region of the tail genomic DNA.
Plasma HDL-Lipid of the Cross-Bred F2 Mice

<table>
<thead>
<tr>
<th>CETP Mass,</th>
<th>lcat(+/-)</th>
<th>lcat(+/-)</th>
<th>lcat(-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/mL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>no-CETP</td>
<td>low-CETP</td>
<td>high-CETP</td>
<td>no-CETP</td>
</tr>
<tr>
<td>HDL-CE</td>
<td>74.7±14.5</td>
<td>28.2±10.1</td>
<td>31.1±6.0</td>
</tr>
<tr>
<td>HDL-FC</td>
<td>4.2±1.7</td>
<td>1.95±0.47</td>
<td>2.12±0.39</td>
</tr>
<tr>
<td>HDL-PL</td>
<td>66.0±13.6</td>
<td>34.9±8.4</td>
<td>35.7±5.4</td>
</tr>
<tr>
<td>HDL-TG</td>
<td>1.6±3.3</td>
<td>8.7±2.9</td>
<td>11.1±4.1</td>
</tr>
</tbody>
</table>

Values are mean±SE. ND indicates no data.
*P<0.001 and †P<0.05 for the low- and high-CETP (1–15 and 15–30 µg/mL, respectively) mice, significantly different from no-CETP (0–1 µg/mL) mice. No significant difference was observed between the high- and low-CETP mice.

The genotype was identified for lcat(+/-), lcat(+/-), and lcat(-/-) mice by multiplex PCR (please see Figure IA, accessible online at http://atvb.ahajournals.org). Plasma LCAT activities in these mice were 28.38±2.74, 14.60±1.92, and 1.19±0.36 µg cholesterol/mL per hour, respectively (online Figure IB). The LCAT activity was reduced to ≈50% and 4% that of the wild-type mice for the heterozygous and homozygous LCAT deficiencies, respectively. CETP mass and activity in the plasma of the F2 mice were linearly correlated within a range of 0 to 30 µg/mL and 0 to 350 µg cholesterol/mL plasma per hour, respectively, as demonstrated in online Figure II (accessible online at http://atvb.ahajournals.org).

To analyze the effect of LCAT and CETP on plasma lipoprotein, the 63 F2 mice were classified into 9 groups according to the LCAT genotype and plasma CETP mass (no CETP, 0–1 µg/mL; low CETP, 1 to 15 µg/mL; and high CETP, >15 µg/mL). For the lcat(+/-) mice, no-, low-, and high-CETP categories included 5, 8, and 8 mice, respectively; for the lcat(+/-) mice, no-, low-, and high-CETP categories included 5, 22, and 4 mice, respectively; and for the lcat(-/-) mice, no-, low-, and high-CETP categories included 2, 9, and 0 mice, respectively. Their parental F1 mice were all in the low-CETP category, and the number of the low-CETP mice was highest among the F2 mice, as expected by mendelian inheritance. Interestingly, we found no high-CETP mice in the lcat(-/-) group.

The effect of LCAT and CETP on lipoprotein profiles were characterized by high-performance liquid chromatography. Typical lipoprotein profiles are shown in online Figure III (accessible online at http://atvb.ahajournals.org), representing mice in the 8 categories, and the overall quantitative results of the analysis are summarized in the Table. HDL concentration in plasma is influenced by LCAT activity and plasma CETP level. Within the same LCAT genotype, HDL was reduced, and apoB-containing lipoproteins (VLDL and LDL) were increased by increasing the plasma CETP level. In the group with the same CETP level, the HDL level was dependent on the LCAT genotype (or plasma LCAT activity). ApoB-containing lipoproteins seemed to be increased in the lcat(-/-) mice. From the data in online Figure III, reduction of HDL by CETP expression was more prominent in the lcat(+/-) mice than in the lcat(+/-) mice, indicating a synergistic effect of the decrease of LCAT and the presence of CETP on HDL metabolism. The data in the Table suggest that in the lcat(+/-) mice, HDL CE, FC, and phospholipid decreased in the low-CETP mice to 37.8%, 46.2%, and 52.9%, respectively, of the values in the no-CETP group (CETP wild type). HDL triglyceride, in contrast, increased 5.4 times in CETP expression. There was no significant difference between the low- and high-CETP groups in the lcat(+/-) mice. Similar results were obtained in the lcat(-/-) mice, except for the more remarkable reduction of HDL by CETP expression as mentioned above. In contrast, in the lcat(-/-) mice, almost no mature HDL was detected, and the effect of CETP on HDL lipid compositions could not be determined. Figure 1A and 1B demonstrates plasma HDL level as a function of plasma CETP. HDL decreased when CETP was expressed, but no further reduction was observed when CETP increased beyond a few micrograms per milliliter in plasma. The effect of expression of CETP on the HDL level was more prominent in the lcat(+/-) mice than in the lcat(+/-) mice.

Figure 1C and 1D displays the relationship between the CETP mass and the LCAT activity of the F2 mice. In the lcat(+/-) mice (open squares) and lcat(+/-) mice (closed triangles), the CETP mass exhibits wide distribution (from 0 to 30 µg/mL), whereas in the lcat(-/-) mice (open circles), the CETP mass exhibits narrow distribution (<10 µg/mL). Plasma CETP masses in the lcat(+/-), lcat(+/-), and lcat(-/-) mice were 11.44±9.09, 8.59±5.71, and 4.47±3.07 µg/mL, respectively. There was no difference between males and females. A similar result was obtained with the CETP activity (data not shown); ie, no LCAT (−/−) mouse had high CETP activity in its plasma. These data suggest that the LCAT genotype influences the CETP level in plasma.

To determine whether the low CETP mass and low CETP activity in the lcat(-/-) mice are caused by low expression of the transfected human CETP gene, the levels of the genomic DNA and liver mRNA of CETP were determined in the lcat(+/+) and lcat(-/-) mice. As shown in Figure 2, the same positive correlations were observed between the genomic DNA and liver mRNA of CETP in both groups of mice. Figure 3 shows the levels of genomic DNA and mRNA in the quantification of CETP genomic DNA and mRNA. PCR was performed by using SYBR Green PCR Master mix reagent in an ABI PRISM 7700 sequence detection system (Applied Biosystems Japan).
Correlation between the amounts of CETP genomic DNA and the liver mRNA in the lcat(-/-) mice. When no CETP gene was expressed, no CETP mass was detected in the plasma of the lcat(+/-) or lcat(-/-) mice (Nos. 701 and 760; see Figure IV, accessible online at http://atvb.ahajournals.org). Plasma CETP mass was lower in the lcat(-/-) mice despite the same level of the CETP gene, especially in the high CETP gene group (Nos. 703 and 738).

Figure 3. Genomic DNA and cDNA of CETP in lcat(+/-) and lcat(-/-) mice. Genomic DNA and cDNA were determined for CETP and β-actin as described in the text. A, PCR products of the genomic DNA for CETP (24 and 28 cycles) and β-actin (23 and 27 cycles) for individual lcat(+/-) mice (Nos. 701, 716, and 703) and lcat(-/-) mice (Nos. 760, 747, and 738). The bands were detected by a UV transilluminator. B and C, Quantification of the genomic DNA and mRNA of CETP by PCR using a SYBR Green PCR Master mix reagent in an ABI PRISM 7700 sequence detection system, as described in the text. The lcat(+/-) mice are Nos. 701, 703, 707, 712, 716, and 727, and the lcat(-/-) mice are Nos. 705, 714, 720, 721, 733, 738, 739, 747, and 760.

Figure 4. Increase of plasma CETP mass as the CETP gene increases in lcat(+/-) mice (open squares) and lcat(-/-) mice (open circles). The left panel (A) represents the correlation with the genomic DNA \( r = 0.967 \) and 0.697 for lcat(+/-) and lcat(-/-) mice, respectively. The right panel (B) represents correlation with cDNA \( r = 0.896 \) and 0.833 for lcat(+/-) and lcat(-/-) mice, respectively. Difference of slope is 4.46 for panel A and 3.57 for panel B.
The role of this protein in plasma lipoprotein metabolism. To date, it is still unclear what exactly is the physiological function of LCAT in plasma, thereby converting a discoidal nascent HDL particle to a mature spherical HDL containing a core of CE. An absence of LCAT activity was confirmed in LCAT-deficient mice, which express the high level of LCAT gene and message. Plasma was fractionated every 5 minutes by using a column of Bio-gel A-5 m (1.5 x 50 cm) with a flow rate of 1.16 mL/10 minutes, as described in Methods. Total cholesterol (circles), protein (solid lines), and CETP mass (closed triangles) were measured in each fraction.

The results of gel permeation chromatography of mouse plasma are shown in Figure 5. Although CETP is largely regulated by the low LCAT expression, perhaps through the decrease of HDL. The levels of LCAT in the plasma of experimental animals vary little with factors such as age, sex, hormones, alcohol, diet, and drugs. On the other hand, CETP biosynthesis seems to undergo upregulation by cholesterol. In human and animal models, high cholesterol diets increase plasma CETP levels, accompanied by an increase in CETP mRNA. Plasma CETP also increases in certain hyperlipoproteinemic conditions. The increase of plasma CETP associated with these conditions may reflect enhanced delivery of lipoprotein-derived cholesterol to responsive tissues, such as liver, where CETP gene expression is upregulated. It has also been shown that the trans-activating factor sterol regulatory element binding protein-I contributes to basal CETP expression in animals on a chow diet, whereas dietary cholesterol regulation involves liver X receptor and retinoid X receptor interactions with a DR4 promoter element.

LCAT and CETP are associated with HDL in plasma. Human CETP is shown to have higher affinity for human apoA-I HDL than for mouse apoA-I HDL in transgenic mice. It was once proposed that functional HDL is a complex involving apoA-I, LCAT, and lipid transfer activity. Indeed, removal of CE from the HDL core by CETP may enhance cholesterol esterification on HDL. However, analysis of the CETP reaction between lipoproteins suggested that HDL-bound CETP may not be functional.

To investigate the cooperation of LCAT and CETP in the regulation of lipoprotein metabolism in plasma, LCAT-deficient mice and CETP transgenic mice were cross-bred in the present study. Interactive regulation between the 2 genes was also examined by analyzing the mixed-breed mice. Expression of CETP resulted in the reduction of HDL in the lcat(+/+) and lcat(−/−) mice. However, overexpression of CETP beyond a few micrograms per milliliter in plasma may not demonstrate further reduction of HDL, seemingly being consistent with the observation in patients with CETP deficiency. In addition, the reduction of HDL by CETP expression was more prominent in the lcat(+/−) mice than in the lcat(+/+) mice. In the lcat(−/−) mice, the HDL level was extremely low; therefore, the effect of CETP expression was undetectable. Interestingly, despite high levels of genomic DNA and mRNA of CETP in many lcat(−/−) mice, CETP mass in the plasma did not increase to the equivalent level in the lcat(+/+) mice with the same genomic DNA and mRNA levels.

By the same assay method as used in the present study, human plasma CETP concentration has been found to be 1.92 ± 0.53 µg/mL in normolipidemic subjects and 2.57 ± 0.77 µg/mL in hyperlipidemic subjects. Thus, a further increase of CETP beyond this concentration does not seem to influence the plasma HDL level. Indeed, this is consistent with the finding in the heterozygous CETP-
deficient patients that reduction of CETP to half normal may not cause a significant increase of HDL.11

In the lcat(−/−) mice, the concentrations of cholesterol were significantly lower than those in lcat(+/+) and lcat(+/−) mice. The low plasma cholesterol could down-regulate CETP mRNA and, subsequently, the protein mass and its activity in plasma. However, the measurement of the CETP genomic DNA and mRNA gave similar results for the lcat(+/+) and lcat(+/−) mice; thus, this possibility has been excluded. Because the lcat(−/−) mice exhibit severe reduction of plasma HDL, CETP secreted into plasma does not associate with HDL, and it can be cleared rapidly from the plasma by an unknown mechanism(s). CETP is indeed found in a free form in lcat(−/−) mice (Figure 5). The increase of CETP mass is proportional to the genomic DNA and mRNA in the lcat(+/+) and lcat(+/−) mice in its total range; therefore, CETP mass is always lower than its expected level from the genomic DNA and mRNA levels in the lcat(−/−) mice. Thus, CETP mass in plasma can be influenced by plasma HDL even with physiological levels of CETP gene expression in humans. It should be confirmed whether this is also the case in humans by measuring CETP concentrations in the plasma of primary hypophalpiliproteinemic subjects, such as those with LCAT deficiency or Tangier disease.

At this moment, we do not have data indicating the mechanism for the clearance of free CETP from plasma. HDL apoproteins, such as apoA-I, may be excreted into the urine in their free form and degraded by the reuptake via the cubilin/megalin-mediated pathway.48 Such a mechanism may have to be investigated for CETP, although it is little less likely because the molecular size of CETP is significantly larger than that of most apolipoproteins.

In summary, we have demonstrated that plasma CETP mass and activity can be influenced not only by expression of the CETP gene but also by posttranscriptional factors. One of these potential factors is deficiency of LCAT, which causes very low plasma HDL levels.

Acknowledgments

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References


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Legends for online Figures

Figure I. The lcat genotype and LCAT activity in the mice. A, Multiplex PCR identification of the mouse genotypes. WT and Neo indicate the wild type and mutant gene, respectively. The heterozygote and homozygote of the mutation are indicated as lcat(+/-) and lcat(-/-). B, LCAT activity of mouse plasma at the age of 5 weeks measured by using human HDL as a substrate carrier. The data represent the mean ± SE for 21 lcat(+/-), 31 lcat(+/-) and 11 lcat(-/-) mice. P< 0.001, between lcat(+/-) and (-/-), and between lcat(+/-) and lcat(+/+).

Figure II. Correlation between plasma CETP mass and CETP activity in the lcat(+/-), (+/-) and (-/-) mice. CETP mass in mouse plasma was determined by using an enzyme-linked immunoabsorption assay system as described in the text. CETP activity was measured as the transfer of the labeled CE from human LDL to HDL as described also in the text. Open squares represent the wild type mice (lcat(+/-)), closed triangles represent the heterozygotes (lcat(+/-)), and open circles indicate the homozygotes (lcat(-/-)) (r = 0.933)

Figure III. Lipoprotein profiles of mice plasma by HPLC, representing the 8 groups of the mixed-bred F2 mice. The 20-times diluted plasma, 100 µL, was injected to a gel permeation HPLC system as described in the text. Lipid was measured with an on-line enzymatic detection system for total cholesterol, FC, phospholipid (PL) and triglyceride (TG). Cholesteryl ester (CE) was calculated by subtracting FC from total cholesterol. The ordinate represents the absorbance at 550 nm (mV) and the abscissa represents retention time (min). Apparent PL and FC peaks after HDL presumably represent those of lysoPL and FC boud to albumin as well as hemoglobin due to hemolysis especially in the lcat(-/-) mice. TG peak at around 35 min represent free glycerol.

Figure IV. Lipoprotein profiles by HPLC of the plasma of the lcat(+/-) and (-/-) mice. The ordinate indicates the absorbance at 550 nm (mV) for colorimetric on-line
assay of cholesteryl ester (CE), free cholesterol (FC), choline-phospholipid (PL) and triglyceride (TG), and then abscissa indicates retention time (min). Mouse ID #701 and #760 represent the lipoprotein profiles of the lcat(+/+) and (-/-) mouse with a negligible level of the CETP genomic DNA. ID #716 and #747 represent the lipoprotein profiles of the lcat(+/-) and (-/-) mouse with the low level CETP genomic DNA. ID #703 and #738 are the lipoprotein profiles of the lcat(+/-) and (-/-) mouse with the high level CETP genomic DNA. Plasma CETP mass of each mice is indicated in the respective panel. Arrows indicate elution position of HDL. gDNA, genomic DNA(CETP/β-actin); Mass, plasma CETP mass in µg/mL.
Figure I, Wu et al.

A

WT →
Neo →

lcat(-/-)  lcat(+/-)  lcat(+-/-)

B

LCAT activity (µg/mL/h)

lcat(+/-)  lcat(+/-)  lcat(-/-)
Figure II, Wu et al.