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.


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

H igh density lipoprotein 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.3 Disruption of the LCAT gene in mice also exhibits severe reduction of plasma HDL4,5 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.11,12 Although the expression of CETP is substantially influenced by factors such as dietary cholesterol intake, plasma cholesterol, hormones, and drugs.12,13 LCAT 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 CETP and LCAT levels. In fact, the plasma CETP activity in Tangier disease is low. To investigate the interregulation of LCAT and CETP in relation to plasma lipoprotein metabolism, we established the 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 X C57BL/6) were kindly provided by Dr. E. Rubin at Laurence Berkeley Laboratory. The human CETP transgenic mice (C57BL/6) were gifts from Japan Tobacco Inc (Tokyo). The homozygous LCAT-deficient mice were cross-bred 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=63, 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 specificity either to the neo-resistant region (5’-AAGCAAATTAAAGGC-3’) or to the targeted region (5’-GTGCTCAAGACAGTTTTCG-3’) and share a common reverse primer that hybridizes to exon 3 of the LCAT gene (5’-GTAATCAACACACATTC-3’). Blood (240 µL) was collected from the intraorbital artery of the mice into a microtube containing 5 µL of 0.5 mol/L EDTA, and blood cells were removed by centrifugation to obtain the plasma. The research was conducted according to the Guideline for the Care and Use of Laboratory Animals of Nagoya City University Medical School. The experimental protocol was approved by the Institutional Animal Care and Use Committee of Nagoya City University Medical School.

LCAT Assay

Human HDL was isolated from fresh human plasma of healthy volunteers by sequential ultracentrifugal flotation as density 1.063 to 1.21 g/mL in NaBr by using a Hitachi CPS5 (Hitachi and a RS50 rotator at 4°C. HDL was dialyzed against 10 mmol/L phosphate buffer, pH 7.4, containing 150 mmol/L NaCl (PBS), treated with 5 mol/L N-ethylmaleimide at 37°C for 3 hours to inactivate endogenous LCAT, and dialyzed against PBS. The activity of LCAT was measured as cholesterol esterification on HDL, as previously described. In principle, 31 mL of the HDL (0.28 µg/µL cholesterol) was equilibrated with 145 µL of HCl-cholesterol (14.5 µL ethanol) at 4°C overnight. The reaction was carried out with 20 µL fresh mouse plasma by incubation with a preincubated mixture of 150 µL labeled HDL, 21 µL of 10% BSA, and 42 µL of 32 mmol/L β-mercaptoethanol at 37°C for 15 minutes. After 90 minutes of incubation at 37°C, the reaction was terminated by adding 1 mL chloroform/methanol (2:1). The organic fraction was collected and analyzed by thin-layer chromatography to detect the radioactivity in FC and CE.

CETP Mass and Activity

CETP mass was measured according to the method of Sasai et al. Briefly, by using a CETP assay kit (Daichii Pure Chemicals Co), 50 µL of 200 time-diluted mouse plasma was added to the antibody (monoclonal antibody 3 to 11D)–bound wells and allowed to stand at room temperature for 2 hours. The antigen solution was removed, and the plates were rinsed 3 times with the washing buffer. Then, 50 µL of the detection antibody, monoclonal antibody 14 to 8F,9 was added. After incubation for 1 hour at room temperature, the unbound antibodies were removed and rinsed 3 times with the washing buffer. Fifty microliters of the color reagent solution was added, and the plate was incubated for 15 minutes at room temperature. The reaction was stopped by adding 50 µL of the stop buffer. Absorbances at 492 nm were measured for each well in a SPECTRE MAX340 (Nihon Molecular Devices).

CETP activity was measured by a method described elsewhere. Briefly, 10 µL of the mouse plasma mixed with H-CE–labeled LDL (54.9 µg of total cholesterol) and HDL (49.6 µg of total cholesterol) and 6% (wt/vol) fatty acid–free BSA in PBS (making a total 237.5 µL) and incubated at 37°C for 2 hours. The transfer reaction was terminated by adding 12.5 µL of 5% sodium heparin/1 mol/L MnCl. The mixture was left to stand at 0°C for 35 minutes and then centrifuged for 20 minutes at 10 500 rpm at 4°C to precipitate the LDL fraction, and 0.1 mL of the supernatant was collected for counting transferred radioactive CE to the HDL particles.

Plasma Protein and Lipoprotein Profile Analysis

Mouse plasma (30 µL) was diluted to 600 µL with PBS containing 0.5 mmol/L EDTA. For analysis of lipoproteins, 100 µL of the diluted plasma was injected into a high-performance liquid chromatography system with 2 tandem gel permeation columns (TSK-gel Lipopropak XL, 7.5 mm x 300 mm, Tosoh) and an online enzymatic detection system for total cholesterol, FC, phospholipids, and triacylglycerides. Association of CETP with lipoprotein was examined by gel permeation chromatography on a column of Bio-gel A-5 m (1.5 x 50 cm). After equilibration with PBS at 4°C, freshly prepared mouse plasma (150 µL) was applied to the column. Chromatography was operated at a flow rate of 1.16 mL/10 minutes, and the eluate was fractionated every 5 minutes. Total cholesterol, protein, and CETP mass were determined for each fraction.

Analysis of CETP Genomic DNA and mRNA

Genomic DNA was extracted from mouse tails. A 1-cm mouse tail was digested overnight at 50°C in a microtube containing 400 µL of a lysis buffer (0.5% SDS, 0.1 mol/L NaCl, 0.05 mol/L Tris, pH 8.0, and 3 mmol/L EDTA) and 8 µL of 20 mg/mL protease K (final concentration 0.4 mg/mL). Seventy-five microliters of 8 mol/L potassium acetate and 500 µL of chloroform were added to each tube for separation of DNA, RNA, and protein. After centrifugation, the top aqueous phase containing DNA was removed and placed into a fresh tube. To precipitate the DNA, 1 mL of ethanol was added to the tube. The DNA was spooled with a sealed capillary tube, air-dried for a few minutes, and resuspended by immersion into 100 µL of 10 mmol/L Tris and 0.2 mol/L EDTA, pH 7.4. The 50 time-diluted DNA sample was used for PCR.

Total RNA was extracted from the liver of the mice by RNA extraction reagent (Isogen, Nippon Gene). After contaminated genomic DNA was digested by DNase I (Takara Shuzo Co), first-strand cDNA was synthesized by a SuperScript preamplification system (GIBCO-BRL) from 1.28 µg of the total RNA. Primer sequences of the sense and antisense for genomic DNA and cDNA were 5’-ATGGTGCGGAATGGTCAAGA-3’ and 5’-CAGCGCTCTCTGAAGG-3’, respectively, for mouse β-actin and 5’-CCCAGCCACAGGCATGGT-3’ and 5’-GGCACCCTCAGGAGGC-3’, respectively, for human CETP, synthesized by Sawady Technology Co, Ltd. PCR was performed in a PCR thermal cycler (Personal, Takara Biomedics) by using Takara Taq polymerase, and the product was analyzed by electrophoresis in a minigel electrophoresis system (Mupid-2, Cosmos) in 2% agarose gel in the 0.445 mol/L Tris and 0.445 mol/L borate buffer containing 0.11 mol/L EDTA. The gel was gently agitated in freshly prepared SYBR Gold nucleic acid gel stain solution for 20 minutes at room temperature. The PCR product bands were detected by a UV transilluminator (UVP LNM-20E) at a wavelength of 302 nm and recorded with a Mamiya Instant Camera equipped with deep yellow photographic filter and Fuji FP-3000B film. For the quantification of CETP genomic DNA and mRNA, PCR was performed by using SYBR
Plasma HDL-Lipid of the Cross-Bred F2 Mice

<table>
<thead>
<tr>
<th>CETP Mass, µg/mL</th>
<th>lcat(+/-)</th>
<th>lcat(+/-)</th>
<th>lcat(-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>no-CETP (0–1)</td>
<td>low-CETP (15–30)</td>
<td>high-CETP (15–30)</td>
</tr>
<tr>
<td>HDL-CE</td>
<td>74.0±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.

Green PCR Master mix reagent in an ABI PRISM 7700 sequence detection system (Applied Biosystems Japan).

Results

The genotype was identified for lcat(+/+), lcat(+/-), and lcat(-/-) mice by multiplex PCR (please see Figure 1A, accessible online at http://atvb.ahajournals.org). Plasma CETP 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 1B). The CETP activity was reduced to ≈50% and 4% that of the wild-type mice for the heterozygous and homozygous CETP 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 1B (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 level (no CETP, <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, 8, and 8 mice, respectively; and 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 3.4 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(+/-) mouse (open squares) and lcat(+/-) mouse (closed triangles), the CETP mass exhibits wide distribution (0 to 30 µg/mL), whereas in the lcat(-/-) mouse (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 of CETP in some lcat(+/-) and lcat(-/-) mice. Contrary to the CETP mass in plasma, the genomic DNA level was...
similar between the 2 LCAT genotype groups (Figure 3A and 3B). The mRNA expression level was also same between the \textit{lcat}(1/1) and \textit{lcat}(2/2) mice (Figure 3C). The highest levels of these 2 parameters were very similar for the 2 genotype groups (mice Nos. 703 and 738).

When plasma CETP mass was plotted against the CETP genomic DNA and the liver mRNA in the \textit{lcat}(1/1) and \textit{lcat}(2/2) mice (Figure 4), the CETP mass increased as the genomic DNA and mRNA increased, but the slope of the increase was much lower in the \textit{lcat}(2/2) mice for both parameters. The data suggest that the lower level of plasma CETP in the \textit{lcat}(2/2) mice is not due to the low expression of the CETP gene but to other posttranscriptional factors regulating CETP concentration in mouse plasma.

When no CETP gene was expressed, no CETP mass was detected in the plasma of the \textit{lcat}(1/1) or \textit{lcat}(2/2) mice (Nos. 701 and 760; see Figure IV, accessible online at http://atvb.ahajournals.org). Plasma CETP mass was lower in the \textit{lcat}(2/2) mice despite the same level of the CETP gene, especially in the high CETP gene group (Nos. 703 and 738; see online Figure IV). The lipoprotein profiles in online Figure IV show that the \textit{lcat}(2/2) mice have almost no mature HDL, which most of the CETP binds in plasma. The results suggest that plasma CETP mass level is downregulated by the low LCAT expression, perhaps through the decrease of HDL.

The results of gel permeation chromatography of mouse plasma are shown in Figure 5. Although CETP is largely associated with HDL in the \textit{lcat}(+/+) high-CETP mouse, it was eluted as a free protein in the \textit{lcat}(2/2) high-CETP mouse. Thus, CETP was shown to be mostly in a free form in the plasma of the LCAT-deficient mice.

![Figure 1](image1.png) **Figure 1.** A and B, Decrease of plasma HDL as a function of plasma CETP mass. Open squares represent wild-type mice, \textit{lcat}(+/+); closed triangles represent the heterozygotes, \textit{lcat}(+/−); and open circles indicate the homozygotes, \textit{lcat}(−/−). Solid line curves are arbitrary power function fit to each set of the data. C and D, Relationship between plasma LCAT activity and CETP mass in mouse plasma. LCAT activity was measured by using human HDL as a carrier of substrate, \textsuperscript{3}H-cholesterol, and CETP mass was measured by ELISA, as described in the text. The LCAT genotypes are indicated as open squares for \textit{lcat}(+/+) mice, closed triangles for \textit{lcat}(+/−) mice, and open circles for \textit{lcat}(−/−) mice.

![Figure 2](image2.png) **Figure 2.** Correlation between the amounts of CETP genomic DNA and cDNA, representing CETP mRNA. The genomic DNA and cDNA were determined for CETP and β-actin as described in the text. For the \textit{lcat}(+/+) mice (open squares), 2 mice were randomly selected from each no-CETP, low-CETP, and high-CETP group. The parameters were measured for all the \textit{lcat}(−/−) mice (open circles). Solid line and broken line represent least-squares regression with \textit{r} = 0.970 and 0.837 for \textit{lcat}(+/+) and \textit{lcat}(−/−) mice, respectively.
**Discussion**

LCAT is one of the key proteins that modulate plasma HDL, because it mediates esterification of cholesterol mainly on HDL 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 \textit{lcat}\(^{2/2}\) mice, whereas the LCAT activity of \textit{lcat}\(^{1/2}\) mice was reduced to half normal, being consistent with autosomal codominant inheritance. Change of HDL in the \textit{lcat}\(^{2/2}\) mice reproduced symptoms similar to those observed in patients with LCAT deficiency, such as extremely low plasma HDL and very low CE level in lipoproteins.\(^2,4\)

CETP is another major potential determinant of a plasma lipoprotein profile by its action to transfer CE from HDL to apoB-containing lipoproteins in exchange for triglyceride.\(^31\) However, it is still unclear what exactly is the physiological role of this protein in plasma lipoprotein metabolism. To date, many studies have shown that higher CETP levels are related to lower plasma HDL levels in certain conditions\(^32,33\) and that complete loss of CETP activity results in a marked increase of HDL in humans.\(^11\) On the other hand, mice have negligible CETP activity in plasma, and expression of human CETP reduces their plasma HDL.\(^24,34\) Thus, CETP seems to play a major role in the catabolism of HDL CE.\(^35\)

The levels of LCAT in the plasma of experimental animals vary little with factors such as age, sex, hormones, alcohol, diet, and drugs.\(^34,15\) 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.\(^36–41\) Plasma CETP also increases in certain hyperlipoproteinemic conditions.\(^27,42\) 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 \textit{trans}\-activating factor sterol regulatory element binding protein-I contributes to basal CETP expression in animals on a chow diet,\(^43\) whereas dietary cholesterol regulation involves liver X receptor and retinoid X receptor interactions with a DR4 promoter element.\(^44\)

LCAT and CETP are associated with HDL in plasma.\(^19,20\) Human CETP is shown to have higher affinity for human

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**Figure 3.** Genomic DNA and cDNA of CETP in \textit{lcat}\(^{+/+}\) and \textit{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 \textit{lcat}\(^{+/+}\) mice (Nos. 701, 716, and 703) and \textit{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 \textit{lcat}\(^{+/+}\) mice are Nos. 701, 703, 707, 712, 716, and 727, and the \textit{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 \textit{lcat}\(^{+/+}\) mice (open squares) and \textit{lcat}\(^{--}\) mice (open circles). The left panel (A) represents the correlation with the genomic DNA \(r = 0.967\) and 0.697 for \textit{lcat}\(^{+/+}\) and \textit{lcat}\(^{--}\) mice, respectively. The right panel (B) represents correlation with cDNA \(r = 0.896\) and 0.833 for \textit{lcat}\(^{+/+}\) and \textit{lcat}\(^{--}\) mice, respectively. Difference of slope is 4.46 for panel A and 3.57 for panel B.
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(1/1) and lcat(1/2) 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 the lcat(2/2) 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(2/2) mice, CETP mass in the plasma did not increase to the equivalent level in the lcat(1/1) 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.

In the lcat(2/2) mice, the concentrations of cholesterol were significantly lower than those in lcat(1/1) and lcat(1/2) 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(1/1) and lcat(2/2) mice; thus, this possibility has been excluded. Because the lcat(2/2) 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(2/2) mice (Figure 5). The increase of CETP mass is proportional to the genomic DNA and mRNA in the lcat(1/1) and lcat(2/2) 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(2/2) 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 hypoalphalipoproteinemic 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. 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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Cholesteryl Ester Transfer Protein Expressed in Lecithin. Cholesterol Acyltransferase-Deficient Mice
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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 bound 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.
Figure IV, Wu et al.