Effect of Probucol in Lecithin-Cholesterol Acyltransferase–Deficient Mice
Inhibition of 2 Independent Cellular Cholesterol–Releasing Pathways In Vivo

Shigehiro Tomimoto, Maki Tsujita, Mitsuyo Okazaki, Shinichi Usui, Toyohiro Tada, Tatsuya Fukutomi, Shigenori Ito, Makoto Itoh, Shinji Yokoyama

Abstract—Cellular cholesterol release takes place by at least 2 distinct mechanisms: the lecithin-cholesterol acyltransferase (LCAT)-driven net efflux by cholesterol diffusion and the generation of high density lipoprotein (HDL) with cellular cholesterol and phospholipid on the cell-apolipoprotein interaction. Therefore, LCAT deficiency impairs the former pathway, and the latter can be inhibited by probucol, which interferes with the apolipoprotein-cell interaction. Hence, probucol was given to the LCAT-deficient mice in the attempt to suppress both of these pathways. The mice were fed low (0.2%) and high (1.2%) cholesterol diets containing 0.5% probucol for 2 weeks. LCAT deficiency and probucol markedly decreased plasma HDL, and the effects were synergistic. Tissue cholesterol content was lower in the adrenal glands and ovaries in the LCAT-deficient mice and in the probucol-treated mice, suggesting that HDL is a main cholesterol provider for these organs. It was also moderately decreased in the spleen of the low cholesterol–fed female mice and in the thyroid gland of the low cholesterol–fed male mice. On the other hand, the esterified cholesterol content in the liver was substantially increased by the probucol treatment with a high cholesterol diet in the LCAT-deficient mice but not in the wild-type mice. Among the groups, there was no significant difference in the tissue cholesterol levels in other organs, such as the liver, spleen, thymus, brain, erythrocytes, thyroid gland, testis, and aorta, resulting from either LCAT deficiency or probucol. Thus, the apolipoprotein-mediated mechanism plays a significant role in the export of cellular cholesterol in the liver, indicating that the liver is a major site of the HDL assembly. Otherwise, tissue cholesterol homeostasis can largely be maintained in mice even when the assembly of new HDL is inhibited by probucol in the absence of LCAT. Nonspecific diffusion of cholesterol perhaps adequately maintains the homeostasis in the experimental condition. (Arterioscler Thromb Vasc Biol. 2001;21:394-400.)

Key Words: high density lipoproteins ■ lecithin-cholesterol acyltransferase ■ cholesterol efflux ■ cholesterol homeostasis ■ probucol

High density lipoprotein (HDL) is given a key role in the hypothesis of a cholesterol transport pathway from the peripheral tissues to the liver. The physiological importance of this transport system lies in the fact that cholesterol is not catabolized in the peripheral tissues and must be transported to its catabolic sites, mainly, the liver (for its conversion to bile acids) and, to a much lesser extent, the steroidogenic cells. The hypothesis is extended to the “antiatherogenic” function of this transport pathway, which is based on experimental data indicating that HDL removes the accumulated cholesterol from the cells in vitro and epidemiological evidence indicating that the plasma HDL level is negatively correlated with the risk of coronary heart disease.

The most critical biological step of this pathway is the release of cholesterol from the cells. At least 2 distinct mechanisms are identified for this reaction: (1) spontaneous cholesterol efflux by diffusion through the aqueous phase in which lecithin-cholesterol acyltransferase (LCAT)-mediated extracellular cholesterol esterification is a driving force for the net efflux and (2) apolipoprotein-cell interaction to generate new HDL particles with cellular cholesterol and phospholipid. Both mechanisms seem to function in maintaining the plasma HDL level, inasmuch as the genetic defect of LCAT results in low HDL in human patients and in gene-manipulated mice, and low HDL is caused by the impairment of the apolipoprotein-mediated generation of HDL in probucol-treated animals and in patients with Tangier disease, in whom the ATP-binding cassette transporter 1 (ABC1) is mutated. The 2 systems apparently back up each other so that neither LCAT deficiency nor...
Tangier disease produces general or serious cholesterol accumulation in the tissues, except for a few specific organs in each disease. Therefore, the following is an important physiological question: what is the proportional contribution of the LCAT-mediated net cholesterol efflux and the apolipoprotein-mediated HDL generation in cellular cholesterol homeostasis in vivo? The answer to this question may also provide us with a view about the major source organs for plasma HDL cholesterol.

LCAT deficiency causes severe reduction of plasma HDL in mice. Cholesterol content in the steroidogenic cells is markedly reduced in these animals, perhaps because of the low delivery of esterified cholesterol from HDL via scavenger receptor B1 (SRB1). On the other hand, probucol has been shown to interfere with apolipoprotein-cell interaction and inhibit the generation of HDL in vitro. This compound severely reduced plasma HDL in mice by this mechanism without changing other factors in the regulation of plasma HDL levels, such as the message levels of apoA-I, apoE, LCAT, SRB1, phospholipid transfer protein, and ABC1 and the fractional clearance rate of plasma cholesteryl ester and cholesteryl ester transfer protein, which is consistent with the findings in patients with Tangier disease. On the basis of these findings in the animal models, probucol was given to the LCAT-deficient mice in an attempt to inhibit the 2 systems for cellular cholesterol removal simultaneously.

**Methods**

**Animals**

The LCAT-deficient DBA×C57BL/6 mice [Lcat(-/-) mice] were kindly provided by Dr E. Rubin at Laurence Berkeley Laboratory (Berkeley, Calif.). The control wild-type DBA×C57BL/6 mice [Lcat(+/-) mice] were purchased from the local experimental animal supplier. The Lcat(-/-) mice were bred with the Lcat(+/-) mice in the Animal Experimental Laboratory of Nagoya University Medical School, Nagoya, Japan. The newborn mice were weaned and sexually segregated at 3 weeks. The Lcat(-/-), Lcat(+/-), and Lcat(+/-) mice were identified at 5 weeks by the multiplex polymerase chain reaction genotype analysis of the tail genomic DNA. The forward primers either hybridize specificity to the neo-resistant gene (5'-AAGCAAAACCATAAGGCCC-3') or to the targeted region (5'-GCTCTCAAATGCTGTCCCTC-3') and share a common reverse primer that hybridizes to exon 3 of the LCAT gene (5'-GTACTCAACAGATCCGGTCTGTC-3').

**LCAT Assay and Plasma Lipoprotein Analysis**

One hundred microitters of blood was collected from the caudal vein of the mice at 8 weeks. A 30 μL aliquot was mixed with 30 μL of 1.3 mmol/L EDTA. The diluted blood (45 μL) was further diluted with 405 μL of 10 mmol/L sodium phosphate buffer (pH 7.4) containing 0.15 mol/L NaCl and 0.5 mmol/L EDTA (PBS). Blood cells were removed by centrifugation, and 200 μL of the diluted plasma was removed for the analysis of lipoproteins by a high-performance liquid chromatography (HPLC) system with 4 tandem gel permeation columns (TSK gel Lipopropak XL, 7.5 mm×300 mm, Tosoh Co, Tokyo, Japan) and an online enzymatic detection system for total cholesterol. The remaining 70 μL of blood was mixed with 10 μL of 5 mol/L EDTA and centrifuged to obtain the plasma. Agarose gel electrophoresis was performed for the lipoprotein analysis with the 3 μL plasma in a Beckman LIPO system stained with Sudan black. For the LCAT activity assay, substrate lipoprotein was prepared by sonication of 30 mg human HDL apoprotein with 30 mg egg phosphatidylcholine, 18 mg cholesterol, and 50 μCi [14C]cholesterol in 60 mL PBS for 30 minutes under argon. The substrate solution, 100 μL, was preincubated with 25 μL of the 5% BSA solution and 25 μL of 32 mmol/L β-mercaptoethanol at 37°C for 15 minutes and then incubated with 20 μL of the mouse plasma at 37°C for 1 hour. The reaction was terminated by adding 600 μL of chloroform:methanol (2:1), and the organic fraction was analyzed by thin-layer chromatography to detect the radioactivity in cholesterol and cholesteryl ester by an imaging scanner (Fuji Film BAS-2500). For the mouse plasma whose LCAT activity was measured, the HDL cholesterol concentration was determined as the bottom fraction of the ultracentrifugal separation at 1.21 g/mL in a Hitachi GX Micro Ultracentrifuge.

**Probucol Treatment**

Probucol [4,4′-(isopropylidenedithio)bis(2, 6-di-tert-butylphenol)] was a gift from Daiichi Pharmaceutical Co (Tokyo, Japan). Mouse basal growth chow containing 2.0 mg or 12.0 mg cholesterol per gram (low and high cholesterol diets, respectively) was mixed with probucol (0.5% by weight) and was made into pellets (Chubu Kagaku Shizai Co Ltd). The Lcat(+/-) and Lcat(-/-) mice were each divided into 4 groups at 8 weeks and fed either a low or high cholesterol diet with or without probucol for 2 weeks. The uptake of food and probucol was monitored by weighing the remaining chow, and the animals were weighed weekly. The environment was controlled at 25°C with a light cycle of 12-hour daylight and darkness, and the animals had free access to water and food.

**Plasma and Tissue Cholesterol Analysis**

At the end of the 2-week feeding experiment, 30 μL of blood was collected from the mouse eye orbit, and plasma lipoprotein cholesterol was analyzed by HPLC as described above. After exsanguination by cardiac puncture, the whole animal body was perfused with PBS by infusing the buffer into the heart. The animals were euthanized by cervical dislocation, and the major organs were removed and mechanically homogenized. The free and esterified cholesterol content in these tissue samples was determined by an enzymatic method for total and free cholesterol after the extraction of lipid with the organic solvent.

The experimental procedures described above were approved by the institutional animal care and research advisory committee.

**Results**

The Lcat(+/-), Lcat(+/-), and Lcat(-/-) mice were identified by genotyping by means of the multiplex polymerase chain reaction (Figure I, which can be accessed online at http://atvb.ahajournals.org). Plasma LCAT activity was 15.87 ± 6.13, 12.65 ± 4.30, and 0.8 ± 0.92 nmol/mL per hour in the Lcat(+/-), Lcat(+/-), and Lcat(-/-) mice, respectively. The activity of the Lcat(+/-) mice was lower than the level of the wild-type mice but not to statistical significance (online Figure I). Plasma lipoprotein profiles by HPLC of the mice fed the low cholesterol diet are shown in online Figure I. The level of HDL is a function of the plasma LCAT activity and so is LDL. VLDL seems to be increased in the Lcat(-/-) mice.

The body weight, cholesterol, and probucol intake of the mice in each feeding group are listed in the Table. There is no significant change in these parameters after the 2-week probucol treatment among the 16 feeding groups.

The effects of probucol on the lipoprotein profiles are shown in Figures II and III (which can be accessed online at http://atvb.ahajournals.org). Figure II represents agarose gel electrophoresis of the plasma of the mice fed the low cholesterol diet. Probucol caused marked decrease of HDL and LDL in the Lcat(+/-) and Lcat(-/-) mice. Figure III demonstrates the quantitative lipoprotein profile by HPLC in...
Cholesterol and Probucol Intake by Mice

<table>
<thead>
<tr>
<th>Probucol in Chow, % wt</th>
<th>Body Weight at 2 wk,* g</th>
<th>Average Daily Cholesterol Intake,† μg/g Body Wt</th>
<th>Average Daily Probucol Intake,† μg/g Body Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
</tr>
<tr>
<td>Cholesterol 0.2%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lcat(+/+) 0</td>
<td>27.5±1.6</td>
<td>21.4±1.1</td>
<td>319±21</td>
</tr>
<tr>
<td>0.5</td>
<td>28.1±0.7</td>
<td>20.8±0.2</td>
<td>334±11</td>
</tr>
<tr>
<td>Lcat(−/−) 0</td>
<td>29.1±1.0</td>
<td>22.6±1.7</td>
<td>285±9</td>
</tr>
<tr>
<td>0.5</td>
<td>28.0±0.6</td>
<td>21.8±1.6</td>
<td>319±11</td>
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<tr>
<td>Cholesterol 1.2%</td>
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</tr>
<tr>
<td>Lcat(+/+) 0</td>
<td>25.3±1.7</td>
<td>21.1±0.3</td>
<td>1809±104</td>
</tr>
<tr>
<td>0.5</td>
<td>26.5±0.8</td>
<td>21.5±1.1</td>
<td>2058±51</td>
</tr>
<tr>
<td>Lcat(−/−) 0</td>
<td>26.1±0.2</td>
<td>18.6±0.8</td>
<td>1958±13</td>
</tr>
<tr>
<td>0.5</td>
<td>25.7±0.7</td>
<td>17.4±2.2</td>
<td>2014±114</td>
</tr>
</tbody>
</table>

Six mice in each group were fed a low (0.2%) or high (1.2%) cholesterol chow diet containing no or 0.5% probucol for 2 wk.

*Mean±SD of 6 mice during 2-wk treatment.
†Mean±SD of 6 mice at 2-wk treatment.

terms of lipoprotein cholesterol. The same effect was observed in the high cholesterol- and low cholesterol-fed animals.

Because the HDL level is already very low in the Lcat(−/−) mice, the effect of probucol on the HDL level was further analyzed as a function of the plasma LCAT activity. As shown in Figure 1, the HDL concentration correlated with the LCAT activity in the control and probucol-fed groups regardless of the LCAT genotypes. The slope of the linear regression was lower with the probucol-fed group, indicating that probucol decreased the plasma HDL level independently of the LCAT activity in plasma.

Tissue cholesterol content was analyzed in the male and female mice with respect to the LCAT mutation, the probucol treatment, and high and low cholesterol diet. In the female mice (Figure 2), the cholesteryl ester content markedly decreased in the ovaries and adrenal glands, presumably because of the reduction of plasma HDL resulting from LCAT deficiency and/or from probucol treatment in the high cholesterol- and low cholesterol-fed groups. In the high cholesterol–fed group, unesterified cholesterol in the adrenal glands of the Lcat(−/−) mice was further decreased by probucol. There was also a decrease of unesterified and esterified cholesterol in the spleen brought about by lowering HDL in the low cholesterol–fed group. A nonsignificant decrease of unesterified cholesterol brought about by lowering HDL was observed in the thyroid glands of the low cholesterol–fed group. In the high cholesterol–fed group, a significant increase of esterified cholesterol was observed in the liver, which was brought about by probucol treatment of the Lcat(−/−) mice. Examination of the aorta demonstrated no significant deposition of lipid by lipid staining with oil red O (data not shown) regardless of the Lcat expression and the probucol treatment. Cholesterol contents of the aortic wall measured for the mice fed 0.2% cholesterol were 14.2±3.3, 13.1±4.3 (n=3), 18.8±3.2 (n=2), and 17.9±4.1 (n=2) mg/g tissue protein for Lcat(+/+) probucol(−), Lcat(+/-) probucol(+), Lcat(−/-) probucol(−), and Lcat(−/-) probucol(+), respectively, showing no differences among the groups either.

The reduction of cholesterol content in the adrenal glands brought about by LCAT deficiency was more prominent in the male mice (Figure 3) than in the female mice. In addition to this reduction, there was another effect of probucol in the male. Esterified cholesterol was significantly decreased in the testis by the lowering of HDL in the high cholesterol–fed mice. The decrease in unesterified cholesterol brought about by lowering HDL was significant in the thyroid gland of the low cholesterol–fed group. The increase of esterified cholesterol was again observed in the liver of the high cholesterol–fed group after probucol treatment of the Lcat(−/−) mice.

Figure 1. Effect of probucol on plasma HDL level. The plasma HDL level measured by the ultracentrifugation method was plotted against LCAT activity determined as described in the text for the probucol-treated (solid symbols) and untreated (open symbols) animals. Each group includes the various genotypes of LCAT: Lcat(−/−), Lcat(+/-), and Lcat(+/-).
Discussion

Two distinct mechanisms are known for cellular cholesterol release. In 1 of these pathways, LCAT is believed to be a driving force for net cholesterol removal from cells. It creates and maintains the gradient of cholesterol content between the lipoprotein surface and cell membrane; therefore, the net flow of cholesterol is generated through its bidirectional diffusion through the aqueous phase. LCAT functions by decreasing the cholesterol influx from lipoproteins to the cells and by increasing the efflux only when cholesterol diffusion from the cell membrane is high enough not to limit the rate of the flow. LCAT reaction may not be the absolute requirement for the net release of cholesterol by diffusion, but it is necessary to synthesize mature and stable HDL with a core of esterified cholesterol. Therefore, the lack of LCAT activity results in reduction of the plasma HDL level.

The other pathway is the assembly of HDL particles mediated by apolipoprotein-cell interaction brought about by removing cellular cholesterol and phospholipid. This pathway requires an apolipoprotein interaction site on the cell surface to generate HDL with cellular phospholipid, signal transduction to initiate the mobilization of intracellular cholesterol, and a specific intracellular cholesterol transport system for the HDL assembly. Probucol, which reduces plasma HDL, has been shown to interfere with the cell-apolipoprotein interaction to inhibit the generation of HDL in vitro and in vitro and to induce tissue cholesterol accumulation in a certain strain of mouse. Tangier disease was shown to be caused by the impairment of the apolipoprotein-cell interaction to generate HDL, and the causative mutations were identified in the genes of ABC1 for Tangier disease and other types of familial HDL deficiency. Thus, the apolipoprotein-cell interaction was shown to be a major source of plasma HDL.

Patients with LCAT deficiency exhibit cholesterol accumulation only in certain organs, such as erythrocytes and the cornea. Chronic renal problems may develop only later in their lives. Thus, cholesterol efflux is impaired in this disease only in the organs that might lack the other system, the apolipoprotein-cell interaction. As a matter of fact, erythrocytes lack this reaction, and no HDL is generated with apolipoprotein. On the other hand, patients with Tangier disease may suffer from cholesterol accumulation in certain organs, such as the tonsils and spleen. Thus, the 2 systems seem to back up each other to maintain cellular cholesterol homeostasis. There seems to be a variation in the relative dependency of cellular cholesterol homeostasis on the 2 distinct cholesterol release pathways.

Figure 2. Tissue cholesterol content in female Lcat(+/+) and Lcat(−/−) mice. A, Results for low cholesterol (0.2%)–fed mice. Mice were fed a diet containing 0 or 0.5% probucol for 2 weeks. Unesterified and esterified cholesterol levels (FC and CE, respectively) in the tissues were determined as described in Methods. RBC indicates red blood cells. B, Results for high cholesterol (1.2%)-fed mice. Each group contained 6 mice, and the data represent mean±SD of these animals. 

*P<0.05 and **P<0.01 vs control; #P<0.05 and ##P<0.01 vs indicated values.
pathways among the various organs and types of cell. Therefore, it is an important and relevant to ask how these 2 systems contribute to the cholesterol homeostasis in different organs in vivo.

To determine this, we used 2 animal models: (1) LCAT deficiency, in which at least the LCAT-driven net cholesterol efflux through the diffusion is impaired, and (2) probucol treatment, which inhibits apolipoprotein-cell interaction and the subsequent HDL generation. In addition, probucol was given to LCAT-deficient animals in the attempt to inhibit both the potential cell cholesterol removal mechanisms. Probucol induced the reduction of HDL, which was independent of the LCAT activity in plasma (Figure 1).

Conclusions were as follows: (1) There was a marked reduction of cholesterol in certain organs brought about by LCAT deficiency and by probucol treatment. The effects were synergistic in the adrenal glands. (2) Cholesterol was increased by simultaneous inhibition of the 2 pathways in only the livers of the high cholesterol–fed mice.

In the mice, steroidogenic cells were shown to depend heavily on HDL for their sources of cholesterol (Figures 2 and 3). This is consistent with previous reports that SRB1 is responsible for the uptake of HDL cholesteryl ester as a major source of cholesterol in these organs in mice.6,19 The reduction of cholesteryl ester was greater in the adrenal glands than in other steroidogenic organs, such as the ovaries or testes, and the synergistic effect of the LCAT defect and probucol treatment was demonstrated only in the adrenal glands of mice fed a high cholesterol diet. This shows that the adrenal glands are more sensitive to the plasma HDL levels with respect to the external cholesterol supply.

More important, the liver demonstrated the increase of cholesteryl ester by probucol in the high cholesterol–fed LCAT-deficient mice. This indicates that inhibition of HDL neogenesis caused the cholesteryl ester accumulation in the cholesterol-loaded liver in the absence of an LCAT reaction. Thus, HDL assembly with apolipoproteins is 1 of the major cholesterol release pathways in the mouse liver. This result also suggests that the liver is a major source of plasma HDL supply in mice.

Surprisingly, there was no other drastic change in the tissue cholesterol by inhibiting the 2 cholesterol release mechanisms, even after the HDL levels became virtually zero by giving probucol to the Lcat(−/−) mice. This fact indicates that cellular cholesterol homeostasis can well be maintained even without LCAT and the apolipoprotein-mediated HDL generation on a short-term basis. Even in the erythrocytes, where significant cholesterol accumulation is usually observed in humans with LCAT deficiency, cholesterol accumulation was not visible even in the high cholesterol–fed

Figure 3. Tissue cholesterol content in male Lcat(+/+) and Lcat(−/−) mice. A, Results for low cholesterol (0.2%)-fed mice. Mice were fed a diet containing no or 0.5% probucol for 2 weeks. Unesterified and esterified cholesterol levels in the tissues were determined as described in Methods. B, Results for high cholesterol (1.2%)-fed mice. Each group contained 6 mice, and the data represent the mean±SD of these animals. *P<0.05 and **P<0.01 vs control; #P<0.05 and ##P<0.01 vs indicated values.
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*Leclat (−/−) mice.* This is probably due to the efficient non-specific diffusion from the cells of cholesterol molecules, which can substantially be picked up by albumin, globulin, and other potential “acceptors.” However, the long-term influence of such an environment for the cells may not be completely healthy and may raise the risk for many disorders, including atheromatous vascular lesions.

Probucol has been a controversial drug for a long time. It has been known for a specific effect of reducing skin xanthoma in patients with familial hypercholesterolemia despite the decrease of HDL.28 Also for the prevention of atherogenesis in hypercholesterolemic experimental animals.29,30 These effects are thought to be due to its strong antioxidative activity and hydrophobic nature, which prevent LDL-lipid oxidation. It has also been implicated that this compound has an anti-inflammatory effect, the prevention of cell proliferation.31 However, under certain conditions, opposite observations have been reported; in mice, probucol has been shown to induce the deposition of cholesterol in tissues.27,32 The controversy may be related to the balance between the antioxidative and anti-inflammatory actions of the drug and its strong HDL-reducing effect.33 Therefore, the effect of probucol on tissue cholesterol accumulation may have to be evaluated carefully. In the context of the dual effect of probucol, the lack of general cholesterol accumulation in the tissues may have resulted in part from such antioxidative or anti-inflammatory effects. Also, if cholesterol is substantially provided by the HDL-SRB1 pathway not only to the specific steroidogenic organs but also to other tissues in mice, low HDL itself may also contribute to the prevention of cholesterol accumulation in the tissues.

Tissue cholesterol deposit varies by mouse strains, and the C57BL/6 strain is known for the induction of cholesterol deposit by probucol.27 The LCAT-deficient mice used in the present study were DBA × C57BL/6, and the behavior of this mixed strain was apparently different from that of the C57BL/6 strain with respect to the response to probucol. Thus, the use of a strain more susceptible to tissue cholesterol accumulation may yield a more distinct outcome.

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**References**


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Figure I, Tomimoto et al.

A

WT   Neo

+ / +   + / -   - / -

B

Lcat (+/+)  Lcat (+/-)  Lcat (-/-)

Retention time (min.)

Cholesterol (absorbance at 555 nm, mV)

VLDL  LDL  HDL

C

Lcat (+/+)  Lcat (+/-)  Lcat (-/-)

VLDL  LDL  HDL

Retention time (min.)

Lcat activity (nmol/ml/hr)

+ / +   + / -   - / -
Figure II, Tomimoto et al.
Figure III, Tomimoto et al.

0.2% cholesterol chow

Retention time (min.)

0.2% cholesterol chow

1.2% cholesterol chow

Control  Probucol  Control  Probucol

Lcat (+/+)

Lcat (-/-)