Targeted Disruption of the Mouse Lecithin:Cholesterol Acyltransferase (LCAT) Gene

GENERATION OF A NEW ANIMAL MODEL FOR HUMAN LCAT DEFICIENCY*

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We have established a mouse model for human LCAT deficiency by performing targeted disruption of the LCAT gene in mouse embryonic stem cells. Homozygous LCAT-deficient mice were healthy at birth and fertile. Compared with age-matched wild-type littermates, the LCAT activity in heterozygous and homozygous knockout mice was reduced by 30 and 99%, respectively. LCAT deficiency resulted in significant reductions in the plasma concentrations of total cholesterol, HDL cholesterol, and apoA-I in both LCAT −/− mice (25, 7, and 12%; p < 0.001 of normal) and LCAT +/− mice (65 and 59%; p < 0.001 and 81%; not significant, p = 0.17 of normal). In addition, plasma triglycerides were significantly higher (212% of normal; p < 0.01) in male homozygous knockout mice compared with wild-type animals but remained normal in female knockout LCAT mice. Analyses of plasma lipoproteins by fast protein liquid chromatography and two-dimensional gel electrophoresis demonstrated the presence of heterogenous preβ-migrating HDL, as well as triglyceride-enriched very low density lipoprotein. After 3 weeks on a high-fat high-cholesterol diet, LCAT −/− mice had significantly lower plasma concentrations of total cholesterol, reflecting reduced levels of both proatherogenic apoB-containing lipoproteins as well as HDL, compared with controls. Thus, we demonstrate for the first time that the absence of LCAT attenuates the rise of apoB-containing lipoproteins in response to dietary cholesterol. No evidence of corneal opacities or renal insufficiency was detected in 4-month-old homozygous knockout mice. The availability of a homozygous animal model for human LCAT deficiency states will permit further evaluation of the role that LCAT plays in atherosclerosis as well as the feasibility of performing gene transfer in human LCAT deficiency states.

Lecithin:cholesterol acyltransferase (LCAT) is a 63-kDa glycoprotein synthesized primarily by the liver, which plays a major role in the metabolism of HDL (1). In plasma, LCAT is preferentially associated with HDL (2) but may also interact with low density lipoproteins (3), where it catalyzes the transfer of a fatty acid from the sn-2 position of phosphatidylcholine to the 3-hydroxyl group of cholesterol, generating cholesteryl esters and lysolecithin. The newly formed cholesteryl esters (CE) are then transferred to the core of the HDL lipoprotein particle, a process that results in the formation and maturation of spherical HDL (4). LCAT-mediated esterification of free cholesterol in HDL helps maintain a concentration gradient for efflux of cholesterol from peripheral cells to the HDL particle surface for ultimate transport to the liver (4, 5). Thus, together with hepatic lipase and cholesteryl ester transfer protein LCAT appears to be essential for the process of reverse cholesterol transport, one of several proposed mechanisms by which HDL may protect against atherosclerosis (6, 7).

The important role that LCAT plays in HDL metabolism has been established by the identification and characterization of patients with a deficiency of LCAT who present with marked hypoalphalipoproteinemia and moderate hypertriglyceridemia (1, 8). In man, LCAT deficiency leads to the expression of two phenotypically distinct clinical syndromes: classic LCAT deficiency (CLD) and fish eye disease (FED) (1, 8). Although both groups of patients present with corneal opacities and hypoalphalipoproteinemia, CLD, but not FED, is, in addition, associated with the development of anemia and progressive renal disease. The mechanism leading to these two different clinical manifestations resulting from a functional deficiency of LCAT is poorly understood. However, recent studies indicate that the residual plasma LCAT activity, rather than the location of this activity within different plasma lipoproteins, may in fact modulate the phenotypic expression of LCAT deficiency (9, 10).

Recently, our laboratory has described the generation of transgenic mice and rabbits that overexpress the human LCAT gene (11, 12). Overexpression of human LCAT in these two different animal models leads to alterations in the concentration, composition, and size of HDL. Thus, both mice and rabbits transgenic for human LCAT have marked increases in plasma HDL cholesterol and apolipoprotein (apo) A-I levels (11, 12). Although LCAT expression reduces aortic atherosclerosis by 86% (13) in rabbits, overexpression of the same transgene in mice results in enhanced diet-induced aortic atherosclerosis (14). In man, LCAT deficiency does not appear to be associated with an increased risk of premature coronary artery disease despite markedly reduced plasma concentrations of HDL cholesterol.

To further evaluate the role that LCAT plays in modulating HDL metabolism, reverse cholesterol transport, and atherosclerosis, we have used gene targeting to create mice that are homozygous for LCAT deficiency. We report that homozygous LCAT-deficient mice are healthy and fertile and like their

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‡The abbreviations used are: LCAT, lecithin:cholesterol acyltransferase; apo, apolipoprotein; HDL, high density lipoprotein; CE, cholesteryl ester(s); CER, cholesterol esterification rate; CLD, classic LCAT deficiency; FED, fish eye disease; ES, embryonic stem; PCR, polymerase chain reaction; kb, kilobase; FPLC, fast protein liquid chromatography.

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human counterparts have markedly reduced plasma concentrations of total cholesterol, HDL cholesterol, apoA-I and apoA-II. In addition, we describe for the first time that the absence of LCAT activity significantly attenuates the rise of apoB-containing lipoproteins in response to a high-fat high-cholesterol diet. The availability of a homozgyous animal model for human LCAT deficiency states will permit further evaluation of the role of LCAT in atherosclerosis as well as the feasibility of performing gene transfer in human LCAT deficiency states.

**MATERIALS AND METHODS**

**Construction of Targeting Vector**—A mouse genomic library (129SV Mouse Genomic Library in the Lambda FIXII Vector, Stratagene, La Jolla, CA) was screened for the mouse LCAT gene using a full-length human LCAT cDNA as a probe. A sequence replacement vector spanning exons 1–6 was constructed (Fig. 1), using a 4-kb BglII-XhoI fragment and a 3-kb BamHI-NotI fragment of the LCAT gene. These two fragments were cloned into BclI and XhoI, and BamHI and NotI restriction enzyme sites, respectively, of the pPL61 (a generous gift from Dr. Michael Lenardo, NIDDS, National Institutes of Health, Bethesda, MD). The targeting plasmid was amplified and purified using CsCl gradient ultracentrifugation (Lafstrand Laboratories Ltd., Gaithersburg, MD) and linearized with NotI prior to electroporation.

**ES Cell Culture and Generation of Chimeric Mice**—Mouse ES cells (RW-4) derived from 129/SvJ mice and neomycin-resistant mouse embryo fibroblasts were purchased from Genome System Inc. (St Louis, MO). Culture and electroporation of ES cells were conducted as reported previously (15). For Southern blot analysis, 10 μg of DNA isolated from G418 (350 μg/ml) and 1-2-deoxy-2-fluoro-β-arabinofuranosyl-5-iodouracil (2 μM)-resistant clones was digested with KpnI and probed with a 516-base pair PCR product 2 kb downstream of the termination codon in the 3′-untranslated region of the LCAT gene (Fig. 1). ES cells from four independently targeted clones were microinjected into C57BL/6J blastocysts followed by implantation into pseudopregnant C57BL/6J female mice. Male chimeric mice generated from two out of four targeted clones transmitted ES cell genome to their offspring. Genotyping was performed by Southern blot analysis of KpnI-digested genomic DNA (15–20 μg). All characterizations were performed in the F2 generation descendants, which were hybrids between C57BL/6J and 129/SvJ mice.

**Northern Blot Hybridization Analysis**—Total RNA was isolated from the liver from 2–3-month-old mice using the guanidinium thiocyanate/cesium chloride method (16). Analysis of liver RNA was performed by fractionation on a 1% formaldehyde-agarose gel, transfer to a nylon membrane, and hybridization with the full-length human LCAT cDNA (17) as a probe.

**Measurement of Plasma Lipids, Lipoproteins, Apolipoproteins, LCAT Activity, and Cholesterol Esterification Rate (CER)**—Plasma total cholesterol and triglycerides (Sigma) as well as free cholesterol and phospholipids (Wako, Osaka, Japan) concentrations were measured in 10-μl aliquots of plasma using commercial kits and the Cobas Mira Plus automated chemistry analyzer (Roche Diagnostic Systems, Inc., Branchburg, NJ). CE values were calculated by subtracting free cholesterol from total cholesterol concentrations. Plasma HDL cholesterol was determined after dextran sulfate-Mg2+ (Ciba-Corning, Oberlin, MA).

**TABLE I**

| Plasma lipids, lipoproteins, and apolipoproteins in wild-type, heterozygous, and homozygous LCAT-KO mice |
|--------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Males (n = 10) | Females (n = 10) | Males (n = 10) | Females (n = 10) | Males (n = 10) | Females (n = 10) |
|----------------|-----------------|----------------|-----------------|----------------|-----------------|
| TC | 134 ± 22 | 130 ± 16 | 109 ± 20a | 85 ± 19a | 31 ± 12a | 32 ± 14a |
| TG | 108 ± 23 | 113 ± 23 | 162 ± 38a | 99 ± 19 | 231 ± 101b | 54 ± 83 |
| PL | 244 ± 39 | 195 ± 23 | 226 ± 36 | 156 ± 23a | 99 ± 32b | 70 ± 18b |
| FC | 27 ± 6 | 24 ± 3 | 24 ± 5 | 14 ± 3a | 20 ± 6a | 14 ± 4a |
| CE | 106 ± 18 | 105 ± 14 | 85 ± 17a | 70 ± 17a | 12 ± 6b | 18 ± 10b |
| CE/TG | 79 ± 3 | 82 ± 2 | 78 ± 3 | 83 ± 2 | 34 ± 11b | 52 ± 9b |
| HDL cholesterol | 92 ± 17 | 81 ± 9 | 69 ± 17a | 48 ± 13a | 6 ± 6a | 6 ± 4b |
| Non-HDL cholesterol | 42 ± 13 | 48 ± 12 | 40 ± 10 | 37 ± 11a | 28 ± 8a | 25 ± 12a |
| ApoA-I | 99 ± 19 | 81 ± 29 | 112 ± 31 | 66 ± 24 | 14 ± 5a | 10 ± 3a |
| ApoA-II | 19 ± 11 | 13 ± 5 | 25 ± 18 | 17 ± 14 | 9 ± 7b | 4 ± 7b |
| LCAT activity | 44 ± 3 | 49 ± 4 | 30 ± 2b | 34 ± 4a | 0.3 ± 0.1b | 0.3 ± 0.1b |
| CER | 70 ± 7 | 46 ± 2 | 47 ± 1b | 40 ± 2b | 0.0 ± 0.0b | 0.1 ± 0.1b |

*a p < 0.05.

b p < 0.01 (compared with controls).

c CER for each animal group was determined in quadruplicate on pooled plasma (n = 5).
RESULTS AND DISCUSSION

Fig. 1A illustrates the strategy utilized to generate the LCAT knockout mice. A sequence replacement vector was constructed using two regions of homology which spanned bases −3500 to +500 and +2200 to +5200 relative to the transcriptional start site of the endogenous mouse LCAT gene. Homologous recombination of the targeting vector results in the replacement of exons 2–5 of the mouse LCAT gene (1.7 kb) by the neo gene and the deletion of the residues 28–216 from the amino-terminal of the mouse LCAT gene. The deleted locus encodes for serine 181 which has been proposed to be the catalytically active serine involved in the initial hydrolysis of phosphatidicholine (23). Thus, elimination of exons 2–5 is predicted to inactivate the mouse LCAT gene.

Southern blot hybridization analysis of KpnI-digested ES cell genomic DNA utilizing the probe illustrated in Fig. 1A demonstrated that 14 out of 79 ES clones (18%) were appropriately targeted, as shown by the presence of a 7.4-kb fragment after digestion with KpnI (data not shown). Four targeted clones were injected into blastocysts, with two resulting in germline transmission. Mice were then genotyped by Southern blot hybridization analysis of KpnI-digested tail genomic DNA (Fig. 1B). A total of 15 matings between LCAT−/− mice yielded 113 offspring, comprising 22 +/+ , 64 +/- , and 27 -/- mice identified by Southern blot hybridization analysis. Fig. 1B illustrates a representative genomic Southern blot that demonstrates the presence of the diagnostic bands for the wild-type (5.8 kb) as well as the disrupted LCAT gene (7.4 kb) after digestion with KpnI.

Evaluation of both LCAT +/- and +/- mice from the F2 progeny revealed healthy, fertile animals. Northern blot hybridization analysis failed to detect mouse LCAT mRNA in homozygous knockout mice (Fig. 1C). Accordingly, plasma LCAT activity was reduced by approximately 30% (male: 30 ± 2 nmol/h/ml; female: 34 ± 4 nmol/h/ml) in heterozygotes and 99% (male: 0.3 ± 0.1; female: 0.3 ± 0.1 nmol/h/mol) in homozygotes, compared with controls (male: 44 ± 3; female: 49 ± 4 nmol/h/mol) (Table I). Plasma CER, a more physiologic measurement of in vivo LCAT activity, was similarly decreased in LCAT-KO heterozygous (male: 47 ± 1 nmol/h/ml; female: 40 ± 2 nmol/h/ml) and homozygous (male: 0.0; female: 0.1 ± 0.1 nmol/h/mol) mice compared with controls (male: 70 ± 7; female: 46 ± 2). Thus, homozygous LCAT-deficient mice had a complete deficiency of both α- and β-LCAT activities in plasma, indicating that biochemically these LCAT deficient mice were most similar to patients with CLD. Further evaluation of both LCAT −/− and LCAT +/- mice at age 2–3 months by slit lamp
exam of the cornea and analysis of plasma albumin, blood urea nitrogen and creatinine levels revealed no evidence of corneal opacities or renal insufficiency which are characteristic of patients with CLD (data not shown). In man, however, the age of onset of these clinical manifestations of CLD is variable (1, 24). Thus, it is possible that with increasing age homozygous LCAT-deficient mice may develop these abnormal physical findings.

The physiological consequences of LCAT deficiency on the plasma lipids, lipoproteins, and apolipoproteins in the F2 litter derived from the mating of F1 LCAT +/+ mice are summarized in Table I. The plasma concentrations of total cholesterol, HDL cholesterol, and apoA-I of fasted LCAT −/− mice were reduced to approximately 24, 6, and 13% of the normal concentrations, which ranged from 58 to 322 mg/dl. The cholesterol ester/total cholesterol ratio in LCAT +/+ and LCAT −/− mice, respectively, did not statistically differ from those of wild-type mice (p = 0.21 and p = 0.13, respectively), although female LCAT −/− mice demonstrated marked variability in plasma triglyceride concentrations, which ranged from 58 to 322 mg/dl. The cholesterol ester/total cholesterol ratio in LCAT −/− and LCAT −/− mice was 34–52, 78–83, and 79–81%, respectively, reflecting the absence of LCAT-mediated cholesterol esterification in the plasma of homozygous mice. The residual cholesterol esters still present in LCAT −/− mice plasma may be originated from intracellular pool formed by acylcoenzyme A:cholesterol acyltransferase as shown by the analysis of the fatty acid pattern of plasma cholesterol esters in human LCAT deficiency (1). Thus, the changes in the plasma lipids and lipoproteins in both +/+ and −/− LCAT-deficient mice are similar to those described for patients with FED or CLD (1, 8).

Like the homozygous LCAT knockout mice, not all patients with functional LCAT deficiency present with hypertriglyceridemia (1, 25), indicating that this clinical manifestation may be modulated by variation at other genetic loci.

Characterization of the mouse plasma lipoproteins by FPLC (Fig. 2) demonstrated a moderate to severe reduction in the apoA-I-containing HDL particles present in plasma for LCAT +/+ and −/− mice, respectively. In addition, the very low density lipoprotein fractions from both LCAT +/+ and −/− mice appeared to be triglyceride-enriched, compared with that of control animals. LCAT −/− mice accumulated a smaller, cholesterol-poor apoA-I-containing particle (see inset, elution volumes 32–33) whose major lipids consisted of phospholipids.

![Figure 4](image_url)  
**FIG. 4.** Elution profile of wild-type, LCAT +/+ , and LCAT −/− mice plasma on FPLC after 3 weeks on a high-fat high-cholesterol diet. Fifty μl of pooled plasma from wild-type (n = 3), LCAT +/+ (n = 4), and −/− (n = 3) mice were analyzed by FPLC. The concentrations of cholesterol (top), triglycerides (middle), and phospholipids (bottom) in each fraction are indicated in the y axis.

Analysis of plasma lipoproteins by two-dimensional gel electrophoresis followed by immunoblotting with mouse apoA-I antibody (Fig. 3) demonstrated a significant increase in apoA-I-containing preβ-migrating lipoproteins present in the plasma of LCAT −/− mice. These preβ-HDL lipoproteins were very heterogeneous in size, forming several subspecies visible after immunoblotting reminiscent of the small disc-like HDL li-
poproteins described in patients presenting with CLD (26). In addition, the size and levels of the α-migrating lipoproteins in LCAT −/− mice were significantly reduced compared with those in LCAT +/+ mice.

To investigate the impact of the absence of LCAT on the lipoprotein response to cholesterol feeding, three groups of female mice, aged 2–3 months, were fed a high-fat high-cholesterol diet which contained 15% fat (ratio of polyunsaturated to saturated fatty acids = 0.69), 1.25% cholesterol, and 0.5% cholic acid as described previously (27). Table II summarizes the plasma lipid, lipoprotein, and apolipoprotein concentrations in the three groups of mice. After being fed a high-fat high-cholesterol diet for 3 weeks, all three groups of animals demonstrated significant increases in their base-line plasma total cholesterol, free cholesterol, cholesteryl ester, and non-HDL cholesterol concentrations. ApoB levels also significantly increased in control and homozygote groups. However, compared with control animals, LCAT −/− mice had significantly lower plasma levels of total cholesterol, free cholesterol, cholesteryl ester, non-HDL cholesterol, and apoB. Further analysis of the plasma lipoproteins by FPLC (Fig. 4) confirmed that the apoB-containing lipoproteins increased in a dose-dependent manner with increasing LCAT activity. Thus, we demonstrate that LCAT modulates the mouse lipid response to a high-fat high-cholesterol diet.

In the present paper we describe the generation of an animal model of human LCAT deficiency. Like patients presenting with CLD and FED, homozygous LCAT knockout mice have severe hypoalphalipoproteinemia, variable hypertriglyceridemia, and accumulation of heterogeneous preβ HDL. In addition, we demonstrate that the increase in apoB-containing lipoproteins in response to dietary cholesterol is attenuated in LCAT −/− mice, thus indicating that LCAT modulates the lipoprotein response to dietary cholesterol. The availability of a viable, homozygous animal model for human LCAT deficiency states will permit further investigation of the mechanisms by which a defect in LCAT can result in the expression of two distinct clinical syndromes, CLD and FED. In addition, evaluation of the processes of reverse cholesterol transport and atherosclerosis in homozygous LCAT knockout mice will provide further insights into the role that LCAT plays in HDL metabolism and the development of premature coronary artery disease.

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