Catalytically inactive lecithin:cholesterol acyltransferase (LCAT) caused by a Gly 30 to Ser mutation in a family with LCAT deficiency

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Abstract Plasma lecithin:cholesterol acyltransferase (LCAT) plays an important role in early steps of reverse cholesterol transport, i.e., cholesterol efflux from peripheral tissues and cholesterol esterification in HDL. However, structural and functional relationships of LCAT have not been fully elucidated. We described a missense mutation of Gly 30-to-Ser in a patient with classical LCAT deficiency. The proband was homozygous for the mutation and had a very low level of HDL cholesterol (2 mg/dl), with a half of normal LCAT mass (2.75 μg/ml), but no detectable or very low LCAT activity in endogenous and exogenous substrate assays. Both his mother and sister were heterozygous for the mutation, and had slightly decreased levels of HDL cholesterol (34 and 36 mg/dl, respectively). Transient expression study using COS cells indicated that mutant cDNA produces similar amounts of media protein as compared to wild type, but no detectable LCAT activity. The missense mutation may result in a near-native conformation without large effects on cellular secretion but a catalytically defective protein. Thus, the N-terminal domain appears crucial for enzymatic activity, in addition to the catalytically active consensus sequence of Gly 179 to Gly183 and a putative sterol binding domain of Glu154 to Lys173.—**Yang, X-P., A. Inazu, A. Honjo, I. Koizumi, K. Kajinami, J. Koizumi, S. M. Marcovina, J. J. Albers, and H. Mabuchi.** Catalytically inactive lecithin:cholesterol acyltransferase (LCAT) caused by a Gly 30 to Ser mutation in a family with LCAT deficiency. *J. Lipid Res.* 1997, **38:** 585–591.

Supplementary key words LCAT deficiency • gene analysis • mutagenesis • expression

Mature LCAT is a glycoprotein of 416 amino acids that catalyzes the transfer of *sn*-2 fatty acids from phosphatidylcholine to the 3-hydroxyl group of cholesterol, producing lysophosphatidylcholine and cholesteryl ester (1). LCAT is bound to lipoproteins, especially to smaller HDL (preβ-HDL), and catalyzes the conversion of lipoprotein unesterified cholesterol to cholesteryl esters (2). In cooperation with cholesteryl ester transfer protein (CETP) (3), LCAT is thought to play an important role in reverse cholesterol transport (4, 5).

Since the first report of a LCAT gene mutation causing LCAT deficiency in 1990 (6), more than 30 varieties of LCAT gene mutation have been reported in the world (7–28). These mutations included 27 missense variants, 4 insertion variants and 2 deletion variants. They distribute over six exons, mainly exon 5 and exon 6, with mutations in exon 2 less frequently reported. In this study, we describe a missense mutation in exon 2 of LCAT gene causing complete LCAT deficiency in a Japanese family.

MATERIALS AND METHODS

Subjects

Fig. 1 shows a pedigree with LCAT deficiency. The proband, a 37-year-old male, was found to have hypertension (160/90 mm Hg), low body mass index (18.4 kg/m^2), corneal opacity, anemia (13.3 g/dl), proteinuria (<1.0 g/day), and a very low level of HDL-C (2 mg/dl). Slit-lamp examination showed minute dots in all layers of corneal parenchyma, being prominent in the periphery. Electromicroscopy showed a small number of target red blood cells. The creatinine clearance

Abbreviations: LCAT, lecithin:cholesterol acyltransferase; HDL, high density lipoprotein; CER, cholesterol esterification rate; CE, cholesteryl ester; apo, apolipoprotein; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; CLD, classical LCAT deficiency; FED, fish eye disease.

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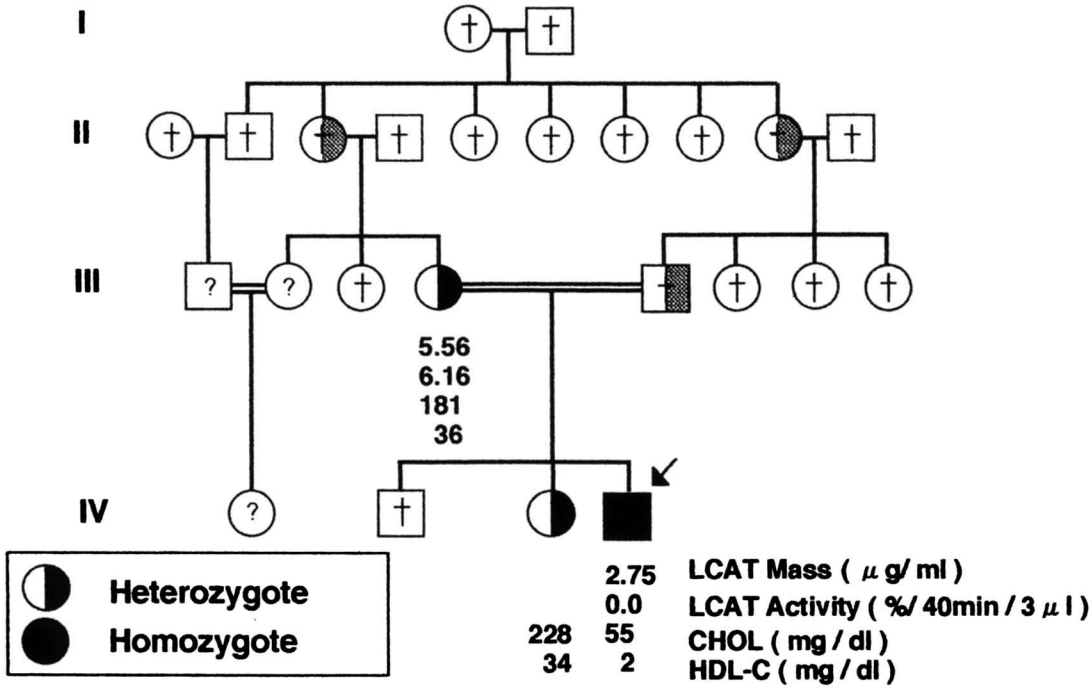

Fig. 1. The pedigree of familial LCAT deficiency. Arrow indicates proband who is identified as the homozygote. His mother and sister are identified as the heterozygotes. His parents have a first cousin marriage.

was within normal range (93.3 ml/min). His parents had a first cousin marriage. His father died from carbon dioxide toxemia. His mother and sister had slightly low levels of HDL-C (34 mg/dl and 36 mg/dl, respectively), but had no evidence of corneal opacities and anemia. His mother had idiopathic thrombocythemia and arteriosclerosis obliterans.

Fasting venous blood samples were collected in the tubes containing Na_2 EDTA. Plasma was separated from blood cells by clinical centrifuge (1200 *g* for 15 min at 4°C), and stored in 4°C for chemical assay. Plasma aliquots for LCAT assay were kept at −80°C. Genomic DNA was extracted from white blood cells according to a modified method of Triton X-100 lysis (29).

Lipoprotein and apolipoprotein analysis

The following lipoprotein fractions were obtained by sequential ultracentrifugation: VLDL (d < 1.006 g/ml), IDL (d 1.006–1.019 g/ml), LDL (d 1.019–1.063 g/ml), and HDL (d 1.063–1.21 g/ml). Plasma cholesterol and triglyceride concentrations were measured by enzymatic methods (30, 31). HDL cholesterol was determined by a heparin-calcium chloride precipitation method (32). Apolipoprotein levels were measured by an immunoturbidimetry method (33).

Measurement of LCAT activity and endogenous cholesterol esterification rate (CER)

LCAT activity of plasma and cell cultured media was measured by the method of Chen and Albers (34). The proteoliposome was prepared by ethanol injection. It contained 7.7 mg of egg yolk phosphatidylcholine (Sigma Chemical Co., St. Louis, MO), 116 μg of cholesterol, 0.2 ml of [^{3}H]cholesterol (0.017 mg/ml, 22.5 Ci/mmol) and 200 μg of apoA-I (Cosmo Bio Co., Tokyo, Japan) for 40 assays. Incubation was stopped by adding 2 ml methanol–chloroform 2:1 for each assay, and lipid was extracted and centrifuged at 4°C at 500 *g*. After drying under a stream of nitrogen, the precipitate was dissolved with 60 μl of isopropanol (35). Labeled cholesteryl ester was separated by thin-layer chromatography using silica gel layers (E. Merck, Darmstadt, Germany) with hexane–diethyl ether–acetic acid–methanol 85:20:1:1, and detected with iodine vapor. The radioactivity was determined by liquid scintillation counting.

Endogenous cholesterol esterification rate was determined as previously described (36).

LCAT mass assay

The samples of plasma and cell cultured media were collected, stored at −80°C and sent to University of Washington Northwest Lipid Research Laboratories, Seattle, WA. LCAT mass was measured using radioimmunoassay (37).

Polymerase chain reaction (PCR)

The LCAT gene is located on chromosome 16 q, and consists of 6 exons (38, 39). Seven pairs of primers, based upon published LCAT gene sequence, were used

TABLE 1. Levels of serum lipids, HDL-cholesterol, and LCAT mass and activity

Subject	Total Cholesterol	Triglyceride	Phospholipid	HDL-Cholesterol	LCAT Mass	LCAT Activity	CER
	mg/dl		*mg/dl*		*μg/ml*	*%/40 min/3 μl*	*nmol/ml/h*
Proband	55	111	146	2	2.75	0	14
Mother	181	92	180	34	5.50	6.2	ND
Sister	228	225	228	36	ND	ND	ND
Normal values	132–220	32–150	159–283	34–86	5.80	10.0	38–116

ND, not determined.

for PCR. The PCR products were confirmed by sequence analysis. One μg genomic DNA from a patient and control subject was amplified by PCR. Thirty cycles of the reaction were carried out in a thermal cycler, with the following conditions: denaturation at 95°C for 45 sec, annealing at 52–58°C for 1 min, and extension at 72°C for 2 min.

Sequence analysis

Amplified DNA fragments were separated from 3% NuSieve agarose gel (FMC Bio Products, Rockland, ME) and purified with Microcon™ 30 (Amicon, Beverly, MA). The purified double strand DNA from the first PCR was directly sequenced with the dideoxynucleotide chain termination method (40), using a ΔTth polymerase and a non-radioisotopic DNA sequencing kit of Sequencing high and Imaging high™ (Toyobo, Osaka, Japan).

Restriction fragment length polymorphism (RFLP) analysis

The mutation identified in exon 2 of LCAT gene creates a novel Pvu II restriction site. Ten μl of the amplified product was digested with 5 unit Pvu II for 10 h at 37°C, then electrophoresed on 3% NuSieve agarose gel, and stained with ethidium bromide.

In vitro mutagenesis and subcloning of LCAT cDNA

LCAT cDNA pUC19 was kindly provided by Dr. John W. McLean (Genentech, Inc, San Francisco, CA). The mutation was introduced into LCAT cDNA using the PCR overlap technique (41, 42) with the following pairs of primers: 5′ATCCTCGTGCCCAGCTGCCTG contains a mismatched base; 5′CAGGAAAACAGCTATGAC and 5′GTTTTCCCAGTCACGAC are complementary to polylinker sequence of pUC19; 5′CAGGTCCACTATAGATGAT has a mismatched nucleotide to destroy a Bam HI restriction site. After digestion with Eco Rl and Bam HI, the mutant and wild type LCAT cDNA were subcloned into expression vector pSG5 (Stratagene, La Jolla, CA) with a ligation kit (Amersham Life Science, Buckinghamshire, England) and transformed into *E. coli* AGI strain. The positive clones were screened by restriction enzyme mapping and sequencing. The plasmids were prepared in large quantities using an alkaline lysis method (43) and purified with QIAGEN-tip column (QIAGEN, Catsworth, CA).

Transient expression of LCAT in COS cell

Lipofection method (44) was used to transfect pSG5-LCAT cDNA into COS7 cells. COS7 cells (1×10^6) were seeded into each of 60-mm tissue culture plates in Dulbecco's modified Eagle's medium (DMEM) with 10% heat-inactivated fetal calf serum (FCS), and were incubated at 37°C in 5% CO_2. When the cells were 60% confluent, the following solutions were prepared in polystyrene sterile tubes: solution A, 3 μg of DNA diluted with 200 μl serum-free medium (Opti-MEM medium, Gibco-BRL, Gaithersburg, MD) and solution B, 15 μl Lipofectin Reagent (Gibco-BRL) diluted with 200 μl Opti-MEM medium. Solutions A and B were combined and then incubated at room temperature for 15 min. The medium in the culture plate was replaced with 1.6 ml Opti-MEM medium, then plasmid DNA–lipofectin reagent complex was layered over cells. After 7 h incubation at 37°C in a CO_2 incubator, the DNA containing medium was replaced with DMEM containing 10% FCS. After 48 h incubation, media were collected for LCAT activity and mass assay.

RESULTS

Biochemical analyses of patient's plasma lipoproteins

The levels of plasma lipid and lipoprotein are shown in **Table 1.** The free cholesterol level of the proband was 50 mg/dl (% CE 9%). His mother and sister had normal % CE levels (73 and 74, respectively). The percent CE in VLDL, IDL, LDL, and HDL in the proband was decreased to less than 15%. He had a very low level of HDL-C, and his HDL-CE was zero. His mother and sister had reduced HDL-C. The plasma apolipoprotein levels are summarized in **Table 2.** The proband showed low level of apoA-I and apoA-II (37 mg/dl and 9 mg/dl) as compared with normal subjects, and high level of apoE (10.4 mg/dl) as compared with controls. The

TABLE 2. Serum apolipoprotein concentrations

Subject	ApoA-I	ApoA-II	ApoB	ApoC-II	ApoC-III	ApoE
	mg/dl			*mg/dl*		*mg/dl*
Proband	37	9	25	2.4	2.2	10.4
Mother	76	27	88	2.1	2.3	3.5
Sister	79	26	144	8.0	10.9	5.6
Normal values	95–170	20–45	45–125	0.7–5.0	2.0–14.0	1.8–6.5

apoA-I levels of his mother and sister decreased to 50% of normal values.

Plasma LCAT mass in proband and his mother was 2.75 μg/ml and 5.5 μg/ml as compared to the normal level of LCAT mass of 5.8 μg/ml. The LCAT activity of proband was undetectable, and his mother had 60% of normal LCAT activity by exogenous substrate assay. LCAT activity by endogenous substrate assay was very low in the proband.

Gene analysis

Sequencing six exons with intronic primers, a G to A transition was identified at the position 1729 nucleotide of proband's LCAT gene (**Fig. 2**). This mutation changes the glycine (Gly) at amino acid 30 to serine (Ser) in the mature protein.

The G to A mutation of exon 2 creates a novel Pvu II restriction site. The PCR product of exon 2 was 253 base pairs (bp) in length. After digestion with Pvu II, the homozygote showed 211 bp fragment and the heterozygote showed both the mutant 211 bp and normal 253 bp fragments (**Fig. 3**). However, upon screening for the mutation in 80 controls with Pvu II digestion, none was found.

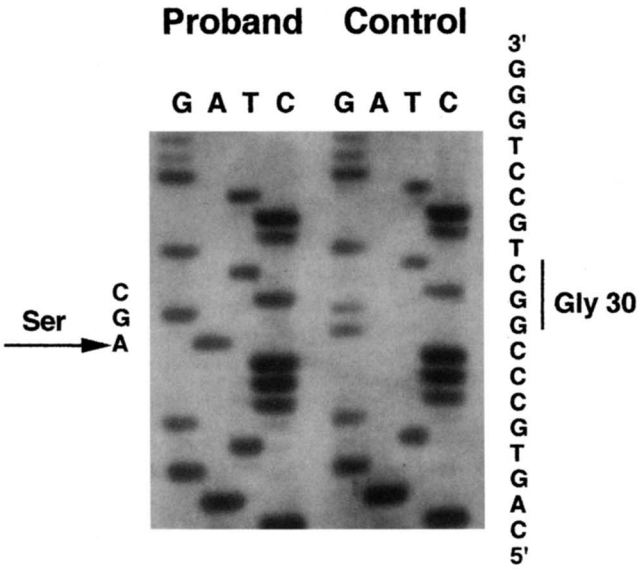

Fig. 2. Illustration of direct sequence of the PCR products of LCAT gene from proband and normal control. A G-to-A transition was found at the 1729th nucleotide in exon 2 of LCAT gene. Arrow indicates a base A. This substitution changes glycine (GGC) to serine (AGC) in 30th amino acid (Gly 30 Ser).

Expression study

The LCAT activity and mass in expression media are shown in **Table 3**. The wild type LCAT activity was 5.3 nmol/ml per h, and the mutant type LCAT activity was undetectable. Nevertheless, wild type and mutant type had approximately the same amount of LCAT protein mass in conditioned media.

DISCUSSION

A complete loss of LCAT activity is classified as classical LCAT deficiency (CLD). Partial LCAT deficiency is classified as fish eye disease (FED), in which anemia and proteinuria are not found. In our case, the proband had a typical syndrome of corneal opacity, proteinuria, anemia, and a very low level of HDL-C. His plasma LCAT activity was undetectable by exogenous substrate assay, and very low by endogenous substrate assay. By gene analysis, the proband was identified as a homozygote of a G to A transition at 1729 nucleotide in exon 2 of LCAT gene, causing the Gly to Ser substitution in amino acid codon 30 (Gly 30-to-Ser). The proband's mother and sister were identified as heterozygotes for the mutation. They had a slightly low level of HDL-C and a half normal level of LCAT activity. RFLP analysis of 80 subjects of the general population excluded the possibility of Gly 30-to-Ser being a polymorphism. When mutant cDNA was expressed in COS cells, LCAT activity was not detected in the culture media. We confirm that the Gly 30-to-Ser missense causes classical LCAT deficiency in this Japanese family.

Gly 30-to-Ser mutation may involve the catalytic active region of a serine protease-type LCAT enzyme. Although the tertiary structure of LCAT is unknown, several functional regions of enzyme have been identified (5, 45, 46). These include the serine esterase consensus

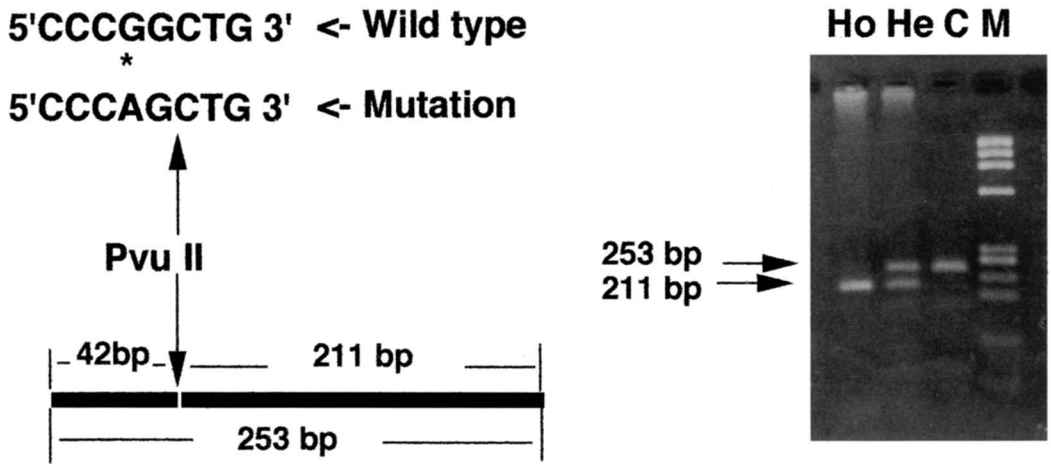

Fig. 3. Illustration of the restriction enzyme analysis of exon 2 PCR products. Left graph: the bar indicates the size of exon 2 PCR product; bp, base pairs;*, the position of G-to-A transition; arrow, indicates Pvu II restriction site. Right graph: 3% NuSieve agarose gel electrophoresis of fragments digested with Pvu II. The DNA bands were stained with ethidium bromide. Ho, homozygote; He, heterozygote; C, control; M, Hae III-digested $\phi \times 174$ marker.

sequence of Gly 179 to Gly 183, including the active site Ser 181; free thiol groups, Cys 31 and 184; two disulfide bonds between Cys 50 and 74 and between Cys 313 and 356; a putative lipoprotein sterol binding domain, which is an α-helical segment extending from Glu 154 to Lys 173; as well as the potential N-linked glycosylation sites at residues 20, 84, 272, and 384. Yang et al. (5) reported that two or more of the hydrophobic regions of LCAT are located around the free-SH groups at Cys 31 and Cys 184; they are adjacent to the active site Ser181 and act as acyl receptor. However, recent studies suggest that an artificial Cys 31-to-Gly variant was unexpectedly found to have near-normal catalytic activity when transiently expressed and that the free SH group of Cys 31 is not crucial for catalytic activity (47, 48). Nevertheless, the Gly 30-to-Ser resulted in complete loss of the enzyme activity shown in vivo and in vitro in our report. As previously reported, two natural mutations in this region were Leu 32-to-Pro (20), a compound heterozygosity Gly 33-to-Arg and 30 bp insertion (24). Interestingly, these natural mutations obviously impaired the enzyme activity. Clearly, the changes in this region of LCAT are critical for the enzymatic activity. Very recently, the same Gly 30-to-Ser mutation was identified in a Indian family (49). The phenotype of the homozygote appears similar to our case. The patient had anemia and serious renal failure and corneal opacity leading to visual failure. His plasma concentration of LCAT was approximately normal but the enzyme catalytic activity was completely defective, indicating that the protein is inactive in vivo.

TABLE 3. LCAT activity and mass of transfected COS cell media

Transfectant	n	LCAT Activity	LCAT Mass
		nmol/ml/h	*μg/ml*
Mock	3	0	<0.010
Mutant type	3	0	0.013
Wild type	5	5.3	0.014

n, sample numbers.

Missense mutations could result in two different phenotypes. One is a transport defective phenotype from rough endoplasmic reticulum to Golgi, which produces no secretory protein caused by incorrect folding. The other is a well-secreted but catalytically defective protein with a near-native conformation. The majority of homozygous missense mutations of LCAT have well-secreted protein mass. Preserved cellular secretion appears in N- or C-terminal mutants. Probably, the amino acid substitutions of N- or C-terminus have near-native conformations. The CLD mutations locate evenly in all regions of LCAT, but the FED mutations only distribute in N- or C-terminal peptides as no mutations have yet to be found in the central portion of LCAT from amino acid 150 to amino acid 290. As the central portion of the enzyme is the catalytically active region of LCAT, the mutations of this portion will induce complete loss of LCAT activity. The Gly 30-to-Ser mutation results in complete LCAT deficiency although it is located in N-terminus and is well-secreted. This further suggests that this point mutation involves unknown specific functions abolishing both α- and β-LCAT activity.

Comparing the homology of LCAT cDNA amino acid sequence among human, baboon, rabbit, and mouse, the sequence encoded by exon 2 of LCAT gene shows high conservation (50–52). The homologies of mouse

versus human, rat versus human, and baboon versus human are as high as 94.4%, 98.2%, and 100%, respectively. Furthermore, Gly 30 and Cys 31 are conserved among these species. This further supports the concept that this N-terminal region of LCAT is a significant functional region.

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