Case Report

Molecular Analysis of a Novel LCAT Mutation (Gly179→Arg) Found in a Patient with Complete LCAT Deficiency

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Lecithin-cholesterol acyltransferase (LCAT) is an important enzyme involved in the esterification of cholesterol. Here, we report a novel point mutation in the LCAT gene of a 63-year-old female with characteristics of classic familial LCAT deficiency. The patient’s clinical manifestations included corneal opacity, mild anemia, mild proteinuria and normal renal function. She had no sign of coronary heart disease. Her LCAT activity was extremely low. DNA sequencing revealed a point mutation in exon 5 of the LCAT gene: a G to C substitution converting Gly179 to an Arg, located in one of the catalytic triads of the enzyme. In vitro expression of recombinant LCAT proteins in HEK293 cells showed that the mutant G179R protein was present in the cell lysate, but not the culture medium. LCAT activity was barely detectable in the cell lysate or medium of the cells expressing the G179R mutant. This novel missense mutation seems to cause a complete loss of catalytic activity of LCAT, which is also defective in secretion.


Key words; LCAT, HDL, Cholesterol, Mutation

Introduction

Lecithin-cholesterol acyltransferase (LCAT) is a plasma enzyme that esterifies free cholesterol present in circulating plasma lipoproteins by catalyzing the transfer of fatty acid from the sn-2 position of lecithin to the 3-hydroxy group of cholesterol1). Mutations in the LCAT gene lead to either familial LCAT deficiency (FLD) or fish-eye disease (FED), both rare autosomal recessive disorders2, 3). FLD was first described in 1967 in a Norwegian family4). Patients with this disease often exhibit some degree of corneal opacity, anemia, proteinuria and renal disease3). More than 60 mutations in the LCAT gene have been identified to date (www.hgmd.cf.ac.uk/ac/index.php).

In the present study, we report a Japanese female with corneal opacity, mild anemia, mild proteinuria and normal renal function, who was carrying a novel point mutation of the LCAT gene: a G to C substitution converting Gly179 to an Arg, which is one of the components of the catalytic triad conserved in all animal species examined5). The functional significance of Gly179 was studied by in vitro expression of the mutant LCAT enzyme.

Materials and Methods

Case

The patient was a 63-year-old female diagnosed with hypcholesterolemia when hospitalized for an operation on the left meniscus 8 years ago. She was also found to have corneal opacity, anemia, and proteinuria. She also had hypothyroidism, which had been treated with levothyroxin for 2 years. She was referred to our hospital for a precise evaluation of low
plasma levels of HDL-cholesterol in 2009. She had no history or signs of coronary heart disease (CHD). Her parents were first cousins (Fig. 1); otherwise her family history was not remarkable. Physical examination revealed no clinical abnormalities except bilateral corneal opacity and moderate loss of hearing. She had no signs of coronary heart disease.

The study adhered to the principles of the Declaration of Helsinki and was approved by our institutional ethics committee. We obtained informed consent from the patient.

**Blood and Urine Collection, Plasma Lipid Analyses, and Other Clinical Parameters**

After an overnight fast, blood was collected for isolation of plasma. Urine samples were collected for 24 hours for the determination of protein levels and creatinine clearance.

Total cholesterol and triglyceride concentrations were determined by enzymatic methods using commercial kits (Determiner-L TC II, Kyowa Medex and Eludia-XL TG II; Eikeukagaku Co. Ltd, Tokyo). Free cholesterol concentrations were also determined using a kit (L-type free cholesterol; Wako), as were HDL cholesterol concentrations (Determiner-L HDL-C; Kyowa Medex), plasma levels of apolipoproteins (Apolipoproteins auto N; Sekisui Medical) and cholesterol ester transfer protein concentrations (CETP ELISA-DAIICHI; Sekisui Medical). Fifteen min after iv injection of 30 U/kg heparin, plasma was collected for the measurement of lipoprotein lipase protein concentration (LPL ELISA-DAIICHI; Sekisui Medical).

**LCAT Activity Assay**

LCAT activity in plasma was measured using a proteoliposome substrate in a LCAT assay kit (Anasorb® LCAT; Sekisui). When intracellular extracts and cell culture medium were used as the LCAT source, proteoliposomes were prepared by 30-min incubation of apoA-I (Sigma) with the proteoliposomes provided by the kit at a molar ratio to cholesterol of 0.8:12.5 at 37°C.

**DNA Isolation**

Genetic DNA was isolated from 2 mL peripheral blood using a QIAamp® DNA Blood Midi Kit (Qiagen) and stored at 4°C.

**DNA Amplification by PCR**

Oligonucleotide primers were synthesized by Oligo® Sigma Genosys. The primers used in the PCR of genomic DNA and the PCR program have been reported previously.

**DNA Sequence Analysis**

PCR-amplified DNA was purified with a QIaquick® Gel Extraction Kit (Qiagen) and re-amplified by PCR using an unequal ratio (10/1) of the same primers with the BigDye Terminator V1.1 cycle sequencing kit (Applied Biosystems). Single-stranded DNA (20 μL) from the second PCR was purified and precipitated in 99% ethanol, resolved in 25 μL Hi-Di Formamide and sequenced by a 3100 Genetic Analyzer (Applied Biosystems).

**ApoE Genotyping**

Restriction isotyping of common APOE isoforms was carried out by gene amplification and cleavage with HhaI as previously described.

**Site-Directed Mutagenesis and Construction of LCAT cDNA Expression Vectors**

Total RNA was isolated from HepG2 cells using TRIzol® Reagent (Invitrogen). The RNA was reverse transcribed with a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), and amplified by PCR using the primers 5’ATGGGGCCCGGCGGCTCC3’ and 5’ AGGAGGCAGGGGCTGCTGG3’. Fig. 1. Pedigree of a Japanese family with FLD. Squares and circles indicate males and females, respectively. Slashes indicate deceased persons. Roman numerals to the left of the pedigree indicate the generation; numerals under each symbol indicate individual family members.

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to obtain whole cDNA of LCAT without a stop codon. This PCR product was then subcloned into pCR2.1-TOPO (Invitrogen) by TA cloning, and the single-stranded DNA of the coding regions was confirmed by sequencing. The T vector and expression vector pcDNA3.1/myc-His A (Invitrogen) were prepared by double digestion with HindIII and XhoI, purified by gel extraction using a QIAquick Gel Extraction Kit (Qiagen) and ligated by T4 DNA ligase using a DNA Ligation Kit, Ver.2.1 (TaKaRa). After confirmation by sequencing, an expression plasmid for LCAT cDNA with a C-terminal myc epitope and the polyhistidine tag was successfully constructed. This plasmid was modified using a QuickChange® Site-Directed Mutagenesis kit (Stratagene) with the primers 5’ CCTGTCTTCTTCATTCGCACACGCCTCGGCTG3’ and 5’ CAGCCGAGGCTGTGGCCGGAATGAGGAAGACAGG3’ by substituting one base, GGC (Gly of the wild type) for CGC (Arg of the mutant), at codon 179 in exon 5. Also, the mutant plasmid was confirmed by sequencing.

**Transient Expression of the LCAT cDNA in vitro**

HEK293 cells (4 × 10⁵) were suspended in 5 mL Dulbecco’s modified Eagle’s medium (DMEM; Sigma) supplemented with 10% fetal calf serum (FCS) and incubated in a 60-mm tissue culture dish at 37°C under an atmosphere of 5% CO₂ and 95% air until 80% confluent. The plasmids (5 μg) were introduced into HEK293 cells in triplicate using the SuperFect® Transfection Reagent (Qiagen). The cell culture medium was changed to FCS-free DMEM after 24 hours and cells were harvested after another 72 hours. Intracellular proteins were extracted from the cells by centrifugation and resuspension of the cell pellet in 0.2 mL RIPA buffer (1% NP-40, 150 mM NaCl, 50 mM Tris-HCl pH7.4, 0.1% SDS, 0.1% sodium deoxycholate) with a cocktail of proteinase inhibitors (1 mM PMSF, 2 μM leupeptin and 0.08 μM aprotinin). The medium was concentrated by Amicon Ultra-30 (Millipore) by about 10-fold. The protein concentration was determined using a BCA kit (Pierce). Aliquots of the medium and the intracellular extracts were kept at −80°C until Western blotting and LCAT assays were performed.

**Western Blot Analysis**

5 × SDS sample buffer (Sigma) was added to 20 μg cell lysate or 20 μL concentrated medium, and boiled for 5 min. After centrifugation, the supernatant was subjected to 10% SDS-PAGE, which was run at 10 mV for 3 hours. Western blot analysis was performed with an anti-myc antibody (Invitrogen). Horseradish peroxidase-conjugated secondary antibody and the ECL Western blotting detection reagents system (Amersham) were used for detection. The signal was visualized with X-ray film (Roche Diagnostics).

**Results**

**Clinical Data**

Table 1 summarizes the clinical and laboratory data of the patient and her family members. The
The patient had mild anemia with poikilocytosis, such as target cells in blood smears and mild proteinuria (0.23 g/24 h). No abnormalities were detected in liver, renal and thyroid function tests or in plasma electrolytes. Fasting blood glucose was 100 mg/dL. Plasma haptoglobin level (52 mg/dL) was low, indicating the presence of hemolysis. Total cholesterol and HDL cholesterol levels were markedly reduced. The ratio of cholesterol ester to total cholesterol (0.105) was also markedly decreased. Plasma levels of apo A-I, A-II, B, C-II and C-III were low (33, 3.5, 40, 1.4 and 2.6 mg/dL, respectively). Apo E level was 5.9 mg/dL. Lipoprotein (a) was undetectable. Lipoprotein X was positive. RLP-C (33.6 mg/dL) was increased. LCAT activity (<50 nmol/mL/hr) was almost undetectable. Post-heparin lipoprotein lipase mass (85 ng/mL) was decreased. Plasma CETP concentration (2.5 ng/mL) was normal. Creatinine clearance was 113 mL/min. Titers of anti-thyroglobulin and anti-thyroid peroxidase antibodies were 3.6 and <0.3 U/L, respectively. Plasma Ig G level was mildly elevated (1,816 mg/dL). Anti-nuclear antibody was negative. The patient was a carrier of APOE ε3/ε3 genotype.

Plasma HDL-C levels of the family members (II-1, II-4 and III-1) were slightly reduced (Table 1).

**DNA Sequence Analysis**

Direct sequencing showed that this patient was homozygous for a novel mutation in exon 5 of the LCAT gene (Fig. 2). A nucleotide transition in exon 5 (c.607 G>C) was found which results in an amino acid change at codon 179 from glycine (GGC) to arginine (CGC).

**Western Blot Analysis**

Immunoblotting of the lysate of transfected cells expressing the wild-type or mutant LCAT showed a single band with a molecular weight of about 52 kDa. Although the medium from the cells expressing wild-type LCAT showed a single band of about 67 kDa, corresponding to the fully glycosylated form of LCAT, the mutant LCAT (LCAT G179R) was not detected in the medium (Fig. 3A).
**Discussion**

Since 1967, over 60 variations in the LCAT gene have been described, which are classified into two rare autosomal recessive disorders: FLD and FED, characterized by a low HDL-C concentration, corneal opacity, hemolytic anemia and renal involvement.

In this case, the deficiency of plasma LCAT activity, which was associated with corneal opacity, mild hemolytic anemia and a very low HDL-C level, is supportive of the diagnosis of classical LCAT deficiency. Direct sequencing of the LCAT gene revealed a novel point mutation in exon 5 (c.607 G > C), which is predicted to result in an amino acid change at codon 179 from glycine (GGC) to arginine (CGC). This mutation is located in the catalytic triad of the enzyme, formed by Ser<sup>181</sup>, Asp<sup>345</sup> and His<sup>377</sup>, and also part of the conserved strand of β-pleated sheet 5, which is important for the proper folding of the enzyme<sup>5</sup>. Mutations of the other two key amino acids of this motif (S<sup>181</sup> and G<sup>183</sup>) have been reported, all of which can disrupt the LCAT activity.<sup>3, 17</sup>

Since other FLD mutants are also located at positions that are strictly conserved<sup>5</sup>, we believe that the homozygous mutation G179R in LCAT accounts for the clinical manifestations in this case of FLD.

As predicted from the structure, LCAT activity was barely detectable in the cell lysate or in the medium of cultured cells transiently expressing the mutant LCAT (G179R) (Fig. 3). Furthermore, the mutant protein itself was not detected in the culture medium, although it was detectable in the cells, suggesting that LCAT G179R is defective in secretion. Taramelli et al. reported a similar non-secreting LCAT mutant, R147W, in a patient who lacked LCAT activ-
ity in plasma\textsuperscript{18}. The R147W mutation substitutes a bulky tryptophan for arginine, thereby presumably inhibiting the formation of a salt bridge\textsuperscript{5}.

Three of the family members exhibited a mild decrease in plasma HDL-cholesterol levels (Table 1). Although we have not measured LCAT activity or genotyped for the mutation in these family members, they may be obligate heterozygotes for the mutation, being responsible for the relatively low plasma HDL-cholesterol levels\textsuperscript{19}. Renal disease is the major cause of morbidity and mortality in FLD patients\textsuperscript{20}, but the clinical manifestations of FLD patients vary even among family members carrying same mutations\textsuperscript{17, 20}. The GFR of the patient was nearly normal, despite the presence of mild proteinuria. The progression of nephropathy may have been delayed in this patient because she was a vegetarian.

Lipoprotein (a) in the patient was almost undetectable. Although it is possible that the low lipoprotein (a) level was caused by apo (a) genotype, absence of LCAT activity may have caused a failure to produce LDL particles of the right morphology and chemical composition to allow lipoprotein (a) formation\textsuperscript{21}.

Baass A et al. have recently reported that the APOE genotype significantly influences the phenotypic expression of familial LCAT deficiency\textsuperscript{19}. Consistent with their observations, the patient, who had ε3/ε3 genotype, had a relatively low plasma level of triglyceride.

In summary, we report a novel point mutation, G179R, in the LCAT gene of a homozygous FLD patient. This mutation is located in the catalytic triad of the enzyme and may affect the secretion and activity of LCAT protein.

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