

Fish Eye Syndrome: A Molecular Defect in the Lecithin-Cholesterol Acyltransferase (LCAT) Gene Associated with Normal α -LCAT-specific Activity

Implications for Classification and Prognosis

Hanns-Georg Klein, Silvia Santamarina-Fojo, Nicolas Duverger, Michel Clerc,* Marie-France Dumon,* John J. Albers,† Santica Marcovina,† and H. Bryan Brewer, Jr.

Molecular Disease Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892;

*Laboratoire de Biochimie Médicale A, Université de Bordeaux II, Bordeaux, France; and †Department of Medicine, Northwest Lipid Research Laboratories, University of Washington School of Medicine, Seattle 98195

Abstract

We have identified the molecular defect in two siblings presenting with classical clinical and biochemical features of Fish Eye disease (FED), including corneal opacities, HDL cholesterol < 10 mg/dl, normal plasma cholesteryl esters, and elevated triglycerides. In contrast to previously reported patients with FED who are unable to esterify HDL-associated cholesterol, our patients' plasma lecithin-cholesterol acetyltransferase (α -LCAT)-specific activities assayed using an HDL-like proteoliposome substrate were 12.7–25.7 nmol/ μ g (19.5 \pm 1.8 in controls). In addition, significant residual cholesterol esterification was present in VLDL/LDL-depleted plasma, confirming the presence of HDL-associated α -LCAT activity. DNA sequence analysis of the proband's LCAT gene identified deletion of the triplet coding for leu³⁰⁰, which resulted in the loss of a restriction site for MlnI. Digestion of PCR-amplified DNA using MlnI established that both siblings are homozygous for this defect. Expression of LCAT^{300-del.} in human embryonic kidney-293 cells revealed normal mRNA and intracellular LCAT concentrations. However, reduced amounts of LCAT^{300-del.}, which had a normal specific α -LCAT activity, were present in the media.

In summary, we report the first case of FED associated with a mutant enzyme that has a normal α -LCAT-specific activity. The functional significance of this LCAT gene defect has been established in an in vitro expression system, which demonstrates that very small amounts of this functional LCAT mutant enzyme accumulate in the media. Characterization of LCAT^{300-del.} established that selective α -LCAT deficiency is not a prerequisite for the development of FED. On the basis of our combined results, we propose that the residual amounts of total plasma LCAT activity and not its distribution on lipoproteins primarily determines the heterogeneity in phenotypic expression observed in familial LCAT deficiency syndromes. (*J. Clin. Invest.* 1993. 92:479–485.) Key words: Fish Eye disease • hypoalphalipoproteinemia • DNA sequence analysis • LCAT • restriction fragment length polymorphism

Address reprint requests to Hanns-Georg Klein, Building 10, Room 7N117, 9000 Rockville Pike, Bethesda, MD 20892.

Received for publication 11 December 1992 and in revised form 24 February 1993

The Journal of Clinical Investigation, Inc.
Volume 92, July 1993, 479–485

Introduction

Lecithin-cholesterol acyltransferase (LCAT)¹ is a key enzyme necessary for extracellular cholesterol metabolism. In plasma, LCAT esterifies cholesterol after association with alpha- (α -LCAT) and, to a lesser extent, with beta- (β -LCAT) lipoprotein particles. The cholesteryl esters become part of the lipoprotein core or are transferred by cholesteryl ester transfer protein to other lipoproteins. The preferred substrate for LCAT are small HDL particles that contain apo A-I, the most potent activator of the enzyme (1–3). LCAT may facilitate the uptake of cholesterol from peripheral tissues into HDL particles by maintaining a concentration gradient for the efflux of free cholesterol (4) and may play a major role in reverse cholesterol transport.

Two phenotypically distinct syndromes of LCAT deficiency have been reported in the literature: classic LCAT deficiency and Fish Eye disease (FED). Patients with FED, a disorder reported to have a selective defect in HDL- or α -associated LCAT activity, present with pronounced corneal opacification and a marked reduction in HDL cholesterol. Despite HDL cholesterol levels < 10 mg/dl, these patients do not develop coronary artery disease (1, 5–7). In contrast to FED, patients with classic LCAT deficiency have significant glomerulosclerosis and normochromic anemia (8). Classically, these two clinical entities have been differentiated biochemically by analysis of total plasma LCAT activity (plasma cholesterol esterification rate; pCER) and HDL-associated α -LCAT activity (1). Thus, in most patients with FED, the CER is normal, but α -LCAT activity is significantly reduced to < 2% that of controls, indicating that LCAT activity in these individuals is primarily associated with apo B-containing lipoproteins (9). Normally, only 5% of LCAT mass and activity reside on apo B-containing particles (10). In classic LCAT deficiency, the LCAT activities on both HDL and apo B-containing lipoproteins are virtually absent, resulting in a drastically reduced pCER. These findings have led to the concept that a selective deficiency of HDL-associated α -LCAT activity is pathognomonic for FED.

Several molecular defects in the LCAT gene of patients with FED have been recently identified by DNA sequencing (11–13). Analysis of the location of these mutations within the LCAT gene indicates that they involve all regions of the enzyme and result in the synthesis of a functionally defective enzyme with α -LCAT-specific activity < 2% of control (6, 11,

1. Abbreviations used in this paper: CER, cholesterol esterification rate; FED, Fish Eye disease; LCAT, lecithin-cholesterol acyltransferase; pCER, plasma CER.

13). Characterization of the mutations identified in patients with FED and a comparison with defects present in patients with classic LCAT deficiency have provided no further insights into the underlying molecular basis for the different clinical manifestations observed in FED versus classic LCAT deficiency.

In this manuscript we describe a unique mutation in the LCAT gene of a patient presenting with classic clinical and biochemical features of FED. The functional significance of this mutation was established by site-directed mutagenesis and *in vitro* expression of the mutant LCAT. Our studies indicate that α -LCAT deficiency is not necessary for the expression of FED and provide new insights into the underlying molecular basis for the clinical heterogeneity observed in patients with primary LCAT defects.

Methods

Proband. The 55-yr-old proband was noted to have corneal clouding before the age of 20 and deteriorating vision after the age of 40. Extensive evaluation revealed no evidence of glomerulosclerosis or anemia, typical clinical features of classic LCAT deficiency. The family record revealed a history of consanguinity, with his parents being first cousins. An extensive clinical and biochemical description of the French proband of Mediterranean origin has been recently published (14).

Blood samples. Fasting venous blood was drawn into EDTA tubes. The plasma was separated by low-speed centrifugation (1,500 g, 15 min) and kept at 4°C. Plasma for LCAT assays was frozen on dry ice and kept at -70°C until assayed. The cell pellet was kept at -20°C until DNA extraction was performed.

Lipoprotein and apolipoprotein analysis. Plasma concentrations of cholesterol, triglycerides, and HDL cholesterol were quantitated by an autoanalyzer (model VP Supersystem; Abbott Laboratories, Irving, TX) using the commercially available test kits (Abbott Laboratories). HDL was precipitated from plasma by dextran sulfate using a commercial reagent system (Ciba-Corning Diagnostics, Oberlin, OH). The total free plasma cholesterol esters were assayed by an enzymic colorimetric method (Wako Pure Chemical Industries, Osaka, Japan). Plasma levels of apo A-I and apo A-II were determined by a commercially available turbidometric assay (Boehringer Mannheim GmbH, Mannheim, Germany).

CER and α -LCAT activity. The CER was quantitated by determining the rate of esterification of [¹⁴C]cholesterol using autologous plasma as a substrate as described (15, 16). Briefly, 200 μ l of autologous plasma was labeled by overnight incubation at 4°C with [¹⁴C]cholesterol. The labeled plasma was incubated for 60 min at 37°C, and samples were taken before and after the incubation and were extracted with ethanol. Protein precipitates were removed by centrifugation (5,000 g, 10 min) and the supernatant was evaporated. Free and esterified cholesterol were separated by thin-layer chromatography (silica gel) and radioactivity of the spots was quantitated in a liquid-scintillation counter (model LS 6000 Ta; Beckman Instruments, Inc., Fullerton, CA). The molar esterification rates were calculated by multiplying fractional esterification rates by the concentrations of free plasma cholesterol. The CER in the culture media of transfection experiments was quantitated using 200 μ l of [¹⁴C]cholesterol-labeled heat-inactivated control plasma as a substrate. Samples for quantitation of the radioactivity were taken before and after 6 h of incubation at 37°C.

The HDL-associated α -LCAT activity in plasma and transfection media was determined using an artificial HDL-like proteoliposome substrate as previously described (17). Stable proteoliposomes were synthesized by 30 min of incubation of apo A-I, [¹⁴C]cholesterol, and egg phosphatidylcholine at a molar ratio of 0.8:12.5:250 at 37°C and the α -LCAT activity determined from the rate of formation of [¹⁴C]cholesteryl ester. The molar rate of α -LCAT esterification was calculated by multiplying the percentage of cholesterol esters formed

per hour by the concentration of total cholesterol in the proteoliposomes. The cholesterol esterification rate and α -LCAT activity were determined in both native plasma and plasma depleted of apo B-containing lipoprotein by phosphotungstic acid as previously reported (13).

LCAT mass. LCAT mass was quantitated by radioimmunoassay using a polyclonal antibody and [¹²⁵I]human LCAT as previously described in detail (18).

DNA isolation. Genomic DNA was isolated from white blood cells using an automated nucleic acid extractor (model 340A; Applied Biosystems, Inc., Foster City, CA) and kept at 4°C.

Oligonucleotides and DNA amplification by PCR. Oligonucleotide primers containing restriction sites for EcoRI and HindIII for subcloning were synthesized on a DNA synthesizer (model 380B; Applied Biosystems, Inc.) as previously reported (13). The coding regions of the proband genomic DNA were amplified by PCR as described (19–21) using an automated DNA thermal cycler and Taq DNA polymerase (Perkin-Elmer Cetus Instruments, Norwalk, CT) in a reaction mixture of 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 1.5 mM MgCl₂, and 200 μ M each dATP, dCTP, dGTP, and dTTP. 30 cycles were performed, each consisting of 45 s denaturation at 95°C, 1 min annealing at 55°C, and 1 min polymerization at 72°C.

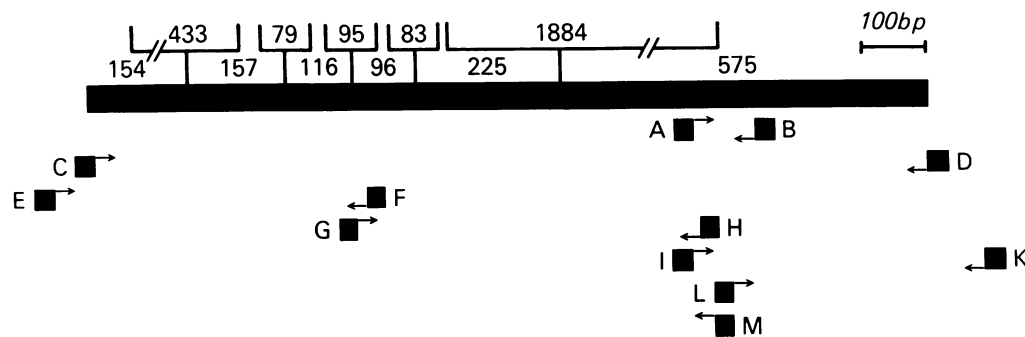
Subcloning of PCR products and DNA sequence analysis. After digestion with EcoRI and HindIII (Bethesda Research Laboratories, Gaithersburg, MD) and electrophoretic separation on a 1.5% low melting point agarose gel (Bethesda Research Laboratories), PCR products were phenol extracted and ligated into the M13 vectors mp18 and mp19 (Bethesda Research Laboratories) and subcloned as described (22). Single-stranded DNA of the coding regions, 150 bp of the promoter region, and the intron/exon splice sites were sequenced using the dideoxynucleotide chain termination method (23) and T7 DNA polymerase (Sequenase; United States Biochemical Corp., Cleveland, OH).

Analysis of PCR-amplified genomic DNA by digestion with MnlI. Oligonucleotide primers A and B (Fig. 1) were used to amplify a 153-bp DNA fragment by PCR containing the mutant gene region in exon 6 of the proband, six family members, and a control. PCR products were gel purified and subjected to digestion with the restriction enzyme MnlI as recommended by the manufacturer (New England Biolabs, Beverly, MA). Digestion fragments were separated on a 2% agarose (FMC Bio Products, Rockland, ME) gel supplemented with 1% low melting point agarose and DNA was stained with ethidium bromide.

LCAT cDNA expression vector. Restriction sites for XbaI (5'-T/CTAG-3') and HpaI (5'-GTT/AAC-3') were introduced into the human LCAT cDNA by PCR amplification from a pUC19 clone, carrying the entire coding region of the human LCAT gene (kindly provided by Dr. John W. McLean, Department of Cell Biology, Genentech Inc., San Francisco, CA), using oligonucleotides C and D (Fig. 1). The 1.35-kb PCR product was digested by XbaI and HpaI as recommended by the supplier (Bethesda Research Laboratories). After purification on a 1% low melting point agarose gel (Bethesda Research Laboratories), the DNA was ligated into the XbaI and HpaI sites of a pUC18 vector (24) containing the cytomegalovirus immediate early promoter and the SV40 polyadenylation site. The vector was subcloned using competent DH-5 α cells (Bethesda Research Laboratories). Clones carrying the LCAT cDNA were grown overnight at 37°C in LB-Broth (Biofluids, Rockville, MD) and DNA was isolated by one-tube miniprep (25). Double-stranded sequencing of the LCAT cDNA insert was performed using the oligonucleotide primers E–K (Fig. 1) and the dideoxynucleotide chain termination method (23) as described (25). The vector was then large-scale amplified and purified by the cesium chloride double-banding method (26).

Site-directed mutagenesis of the LCAT cDNA. The mutant LCAT cDNA was generated by the PCR overlap technique (27) from the pUC19 cDNA clone using primers E and M, and L and K (Fig. 1) containing the CTC deletion at codon 300. Double-stranded sequencing of the mutant and the wild-type LCAT cDNA from miniprep preparations of the expression vectors confirmed the normal LCAT cDNA

LCAT cDNA



Oligonucleotide Primers

- A:** 5' - CTG CAC TTT GAG GAA GGC TGG TAC - 3'
B: 5' - CGT GTA GGG GAA TTC GTG GTC GTA - 3'
C: 5' - GGC TCT AGA GCT GGA ATG GGG CCG - 3'
D: 5' - AGG GCT TAC GTT AAC AAA GGA AGG - 3'
E*: 5' - TCG CCT GGA GAC GCC ATC CAC GCT GTT - 3'
F: 5' - GAC CAG GTT CTG CAC CAG TGT GTG - 3'
G: 5' - TAC CTG GAC AGC AGC AAG CTG GCA - 3'
H: 5' - CAG GAG GTC ACG TGA CTG GAG CCA - 3'
I: 5' - TTT GCA GAC CTG CAC TTT GAG GAA - 3'
K:** 5' - ATT TGG ATG CTA TTG CTT TAT TTG TAA CC - 3'
L: 5' - CAG TCA CGT GAC CTG GCA GGA CTC - 3'
M: 5' - GAG TCC TGC CAG GTC ACG TGA CTG - 3'

Figure 1. Structure of the human LCAT cDNA and location and sequence of oligonucleotide primers used for restriction analysis, site-directed mutagenesis, and LCAT cDNA sequencing. The black bars represent the six exons present in the LCAT gene. Their sizes, in bp, are indicated. The brackets and numbers indicate the location and size of the introns. The recognition sites for the restriction enzymes XbaI and HpaI in primers C and D are underlined. Primers E(*) and K(**) are located within the 3' sequence of the cytomegalovirus early promoter and the 5' sequence of the SV40 polyadenylation signal.

sequence of the control plasmid (normal LCAT) and the deletion of leu³⁰⁰ in the mutant vector (LCAT^{300-del.}).

In vitro transient expression of the LCAT cDNA in human embryonic kidney-293 cells. 40 µg of the control, the wild-type LCAT (normal LCAT), and the mutant LCAT expression vectors (LCAT^{300-del.}) was used for transient transfections of subconfluent monolayers of kidney-293 cells by the calcium phosphate coprecipitation method (28). All transfections were carried out in triplicate. The cell culture media (DME; Gibco Laboratories, Gaithersburg, MD) were changed after 16 h and were harvested after another 48 h. The intracellular proteins were extracted from collected cells by centrifugation, resuspension of the cell pellet in 0.22 M Tris-HCl pH 8.5 (supplemented with 0.2% sodiumdesoxycholate, 0.008% Nonidet P-40, 0.005% Heparin, 1% BSA, and 0.25 M sucrose) and were sonicated as described (29). Aliquots of the media and the intracellular extracts were kept at -70°C until LCAT assays were performed.

Isolation of mRNA and Northern blot analysis. Extraction of mRNA from cells transfected with the control, the wild-type LCAT (normal LCAT), and the mutant LCAT expression vector (LCAT^{300-del.}) was performed using the guanidinium thiocyanate method (30). Northern blot hybridization was performed by separation of RNA on a 6% formaldehyde-agarose gel followed by transfer to Nytran (Schleicher & Schuell Inc., Keene, NH) and hybridization with a full-length human LCAT cDNA probe. Northern blots were scanned and analyzed on a Betascope 603 (Betagen, Waltham, MA).

Results

Lipoprotein and apolipoprotein data. Table I summarizes the lipoprotein and apolipoprotein values of the proband and six of

his relatives as well as those of previously reported FED patients and healthy controls. The plasma lipid profile of the proband and his homozygous sister exhibited the characteristic anomalies present in classic FED with normal plasma levels of cholesteryl esters, elevated triglycerides, and HDL cholesterol < 10 mg/dl. The HDL cholesterol of the heterozygous family members was in the low-normal range. Total plasma triglycerides were markedly increased not only in the two homozygotes, but also in three of the four analyzed heterozygotes. Plasma levels of apo A-I and particularly apo A-II were significantly reduced in the homozygotes.

pCER, α-LCAT activity, and LCAT mass. Unlike other FED patients who exhibit normal or only slightly reduced pCER, our proband's pCER was reduced to 25 nmol/ml per h (59±11 nmol/ml per h in controls, Table II). Plasma cholesteryl esterification was still detectable (7 vs. 51±4 nmol/ml per h in controls, Table II) after precipitation of apo B-containing lipoproteins with phosphotungstic acid, indicating significant residual HDL-associated LCAT activity. Using an artificial HDL-like proteoliposome substrate, the proband's α-LCAT activity was 14% that of normal controls, a value significantly higher than the residual α-LCAT activity in other FED patients. The calculated specific α-LCAT activities of the proband and his affected sibling using proteoliposomes or VLDL/LDL-depleted plasma as a substrate ranged from 65 to 112% that of control (Table II).

Sequence analysis of genomic DNA. Single-stranded DNA sequence analysis of the coding, promoter, and splicing regions

Table I. Lipoprotein and Apolipoprotein Data of a French Kindred with Partial LCAT Deficiency (Fish Eye disease)

	Proband a	Offspring b	Offspring c	Offspring d	Sister e	Mother f	Uncle g	α -LCAT def. (n = 6)*	Controls (n = 7)
Chol. (mg/dl)	129	197	145	196	207	266	196	219±17	163±24
CE (%)	46	68	71	73	60	74	71	59±2	69±2
TG (mg/dl)	399	310	118	62	165	266	79	276±132	65±18
HDL-C (mg/dl)	9	40	35	47	10	34	43	0-7	65±17
apo A-I (mg/dl)	40	132	104	130	50	106	122	33±11	145±24
apo A-II (mg/dl)	10	36	28	40	10	29	32	7±4	41±5

Values for the homozygote family members are highlighted in bold. Values for other FED patients (α -LCAT def.) represent the mean of six individuals. Chol., cholesterol; CE, cholesterol esters; TG, triglycerides; HDL-C, HDL-cholesterol. * References 6, 11, and 13.

of the LCAT gene from the patient and a control identified the deletion of an entire codon (CTC) in exon 6, resulting in the loss of leu³⁰⁰ (Fig. 2). This mutation was the only defect identified in six of six independent clones. Computer analysis of the proband's mutant LCAT DNA sequence (PC/GENE; IntelliGenetics, Mountain View, CA) revealed the loss of a restriction site for the enzyme MlnI (5'-CCTC/-3') at residue 300.

Analysis of PCR-amplified genomic DNA by digestion with MlnI. Digestion of a PCR-amplified 153-bp LCAT gene fragment containing the mutant codon in a control, the proband, and six of his relatives with MlnI established that the proband and one of his sisters are homozygous for the leu³⁰⁰ deletion (Fig. 3 A). His three children and his mother are heterozygous for this defect, whereas his paternal uncle is not affected. A pedigree of the kindred is shown in Fig. 3 B, illustrating consanguinity in the third generation.

CER, α -LCAT activity, and LCAT mass of in vitro expressed LCAT. To establish the functional significance of the leu³⁰⁰ deletion, the mutant enzyme was expressed in human embryonic kidney-293 cells. Analysis of culture media and intracellular extracts 48 h after transfection with the normal LCAT plasmid revealed that 95% of total α -LCAT activity and LCAT mass was present in the culture media, indicating that most of the normal protein was secreted (Table III). In contrast, the total LCAT activity and mass present in the culture media of cells expressing LCAT^{300-del.} was very low, whereas intracellular activity and mass were comparable to those transfected with the normal LCAT plasmid. Like the plasma LCAT^{300-del.}, the specific α -LCAT activity of the expressed mutant enzyme was similar to normal (43 vs. 46 nmol/ μ g). Northern blot hybridization of normal and patient RNA re-

vealed normal levels of a normal size 1.6-kb LCAT mRNA (data not shown).

Comparison of mammalian LCAT cDNA sequences. The human LCAT gene structure (31) is highly homologous to the mouse (32) and the rat (33) (~ 85% identity). The mutant codon 300 is conserved in all three species.

Primary and secondary structure prediction. Primary and secondary structure prediction models of the normal and the mutant LCAT enzyme were developed using the computer program PC/GENE (IntelliGenetics). The deletion of codon 300 does not alter the average hydrophilicity of the enzyme (34). A minimal reduction in chain flexibility at residue 300 is predicted (35). A stretch of extended conformation from residues 299 to 303 is changed to turn and coil in the mutant protein (36). In an additional model (37), the mutant protein is predicted to have an extended stretch of coil conformation from codons 297 to 308 and a disrupted stretch of helix extending from residues 289 to 301 in the normal protein.

Discussion

The phenotypic heterogeneity of patients presenting with hypoalphalipoproteinemia has been puzzling to the clinician. Among these patients, two major clinical syndromes, classic LCAT deficiency and FED, characterized by low plasma HDL and a primary defect in the LCAT gene have been reported (5, 10). Both syndromes are characterized by corneal opacities, hypoalphalipoproteinemia, and the lack of coronary artery disease; however, subjects with classic LCAT deficiency are additionally affected by various degrees of glomerulosclerosis and anemia (1).

Table II. pCER, α -LCAT Activity, LCAT Mass, and Specific α -LCAT Activity in a French Kindred with Partial LCAT Deficiency (Fish Eye disease)

	Proband a	Offspring b	Offspring c	Offspring d	Sister e	Mother f	Uncle g	α -LCAT def. (n = 5)*	Controls (n = 7)
pCER (nmol/ml per h)	25 [7]	78 [27]	52 [14]	54 [25]	16 [5]	71 [36]	60 [36]	60 ± 14 [<1]	59±11 [51±4]
α -LCAT activity (nmol/ml per h)	14	66	67	90	18	85	89	0.9±0.3	99±5
LCAT mass (μ g/ml)	1.1	3.3	3.2	3.8	0.7	3.0	4.0	2.9±0.8	5.2±0.7
Specific α -activity (nmol/ μ g)	12.7	20.0	20.9	23.7	25.7	28.3	22.3	0.3±0.2	19.5±1.8

Values for the homozygote family members are highlighted in bold. The values for other patients with FED (α -LCAT def.) represent the mean of five individuals. Numbers in brackets represent measurements in VLDL/LDL-precipitated plasma. * References 6, 11, and 13.

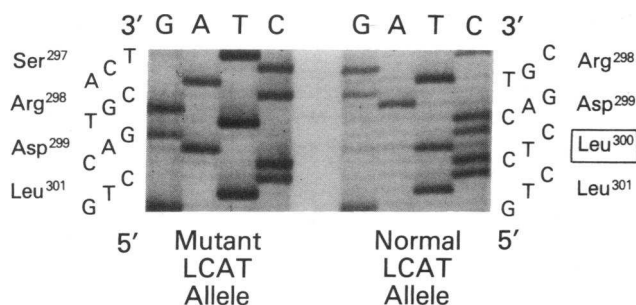


Figure 2. Autoradiogram of DNA sequencing gels of the proband's LCAT allele (left) and control (right). The deleted residue leu³⁰⁰ is boxed.

The first insight into the molecular basis of the phenotypic heterogeneity of LCAT disorders was made by Carlson and co-workers (5–7, 38, 39), who reported that FED was due to selective α -LCAT deficiency. Classic LCAT deficiency, on the other hand, was associated with a complete absence of LCAT enzymic activity (8). Thus, the major biochemical difference between the two clinical syndromes was postulated to be the presence of residual LCAT activity on apo B-containing lipoproteins in patients with FED. It remained, however, to be established whether a selective deficiency of LCAT activity in HDL was indeed a requirement for the expression of FED. In this manuscript, we characterize a unique defect in the LCAT gene of a patient presenting with FED, which provides new insights into the underlying molecular basis for the clinical heterogeneity observed in patients with LCAT deficiency.

Our proband presented with the typical clinical and biochemical features of FED as initially reported in the three index cases of α -LCAT deficiency from Sweden (5, 6) and in other patients with this disorder (11, 13). The proband developed corneal opacities at age 20 and visual impairment by age 40 but had no evidence of glomerulosclerosis and anemia by age 55. Biochemically, the proband's plasma cholesteryl ester and HDL levels were similar to levels observed in other patients with FED. In addition, significant CER was detected in plasma. However, unlike other patients with FED, determination of LCAT activity after precipitation of apo B-containing lipoproteins with phosphotungstate revealed significant residual α -LCAT activity present in the proband and his affected sister. Determination of the specific HDL-associated α -LCAT activities using proteoliposomes or LDL/VLDL-depleted plasma as substrates demonstrated that although total α -LCAT activity in plasma was reduced in both homozygotes to ~14 and 18% that of normal, the specific α -LCAT activity of the mutant enzyme was similar to that of normal plasma LCAT. pCER in the proband and his sister was reduced to 15 and 27% that of normal individuals, respectively. Thus, in this kindred, the clinical phenotype of FED is associated with an overall reduction in both α - and β -LCAT enzymic activity with normal α -LCAT-specific activity rather than the selective deficiency of α -LCAT activity observed in the other FED patients described previously (5–7, 11, 13).

DNA sequence analysis of the proband's LCAT gene identified a mutation in codon 300 that resulted in deletion of leu³⁰⁰. Analysis of the altered DNA restriction pattern using MlnI demonstrated that the proband and his sister were homozygous for the defect. The functional significance of LCAT^{300-del.} was established by expression of the normal and

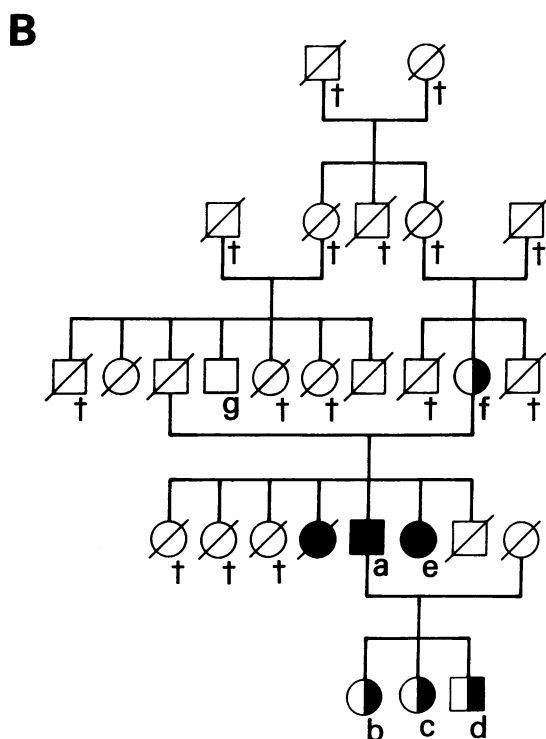
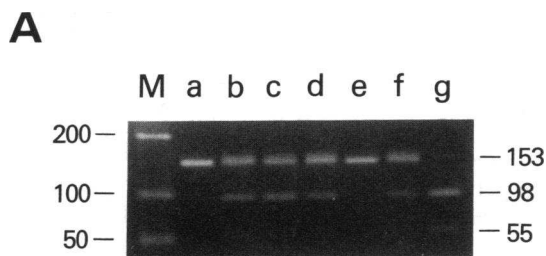


Figure 3. Screening of family members for the leu³⁰⁰ deletion by restriction analysis with the enzyme MlnI (A). A 153-bp portion of the LCAT gene from the proband, family members, and a control, spanning the region of the mutation, was amplified from genomic DNA by PCR. The amplified fragments were digested with MlnI, separated on a 3% agarose gel, and visualized by staining with ethidium bromide. Because of the lost restriction site for MlnI, the proband's (a) and his homozygous sister's (e) DNA was not cleaved. The proband's offspring (b–d) and his mother (f) are heterozygous for this defect as demonstrated by the incomplete MlnI restriction, whereas the uncle (g) had the same restriction pattern as the control (not shown) demonstrated by complete cleavage of the 153-bp fragment into a 98- and a 55-bp fragment. M, molecular size standard; ○, female; □, male family members (filled symbols, homozygotes; half-filled symbols, heterozygotes; diagonal bars, family members not available for analysis); †, deceased.

the mutant enzyme in human embryonic kidney-293 cells. The specific α -LCAT activity of the expressed mutant LCAT enzyme was similar to that of normal LCAT, which confirmed the normal α -LCAT-specific activity determined with the plasma enzyme; however, in contrast to the control, where >90% of the LCAT mass and activity was secreted into the culture media, only a small fraction of mass and activity of LCAT^{300-del.} was detected extracellularly, suggesting either a defect in LCAT secretion or enhanced degradation. Although

Table III. CER, α -LCAT Activity, LCAT Mass, and Specific α -LCAT Activity in the Culture Media and Intracellular Extracts of In Vitro Expressed Normal and Mutant LCAT

	Cell culture media		Intracellular extracts	
	Normal LCAT	LCAT ^{300-del.}	Normal LCAT	LCAT ^{300-del.}
Total CER (nmol/h)	40±1	3.1±0.6	—	—
Total α -LCAT activity (nmol/h)	394±26	20±10	28±1	15±2
Total LCAT mass (μ g)	9.0±2.2	0.4±0.2	0.5±0.1	0.3±0.1
Specific α -activity (nmol/ μ g)	46±14	43±13	54±14	43±6

Total amount of cholesteryl esters formed per hour in the cell culture media (4.5 ml) and in intracellular extracts (0.4 ml) using plasma (CER) or proteoliposomes (α -activity) as substrates, as well as LCAT mass and calculated specific α -LCAT activity are indicated. Values represent means±SD of triplicate measurements. The background values for cultured cells only and cells transfected with the vector without insert, which was 2% of control for CER and α -LCAT activity and 0 for LCAT mass, have been subtracted.

the intracellular concentration of the mutant enzyme was similar to that of normal, the LCAT^{300-del.} levels detected in the cell culture media were significantly reduced, a finding consistent with the 80% reduction of LCAT mass present in the proband's plasma. The mechanism by which the deletion of leu³⁰⁰ alters the plasma levels of LCAT^{300-del.} is unclear. Transcription of the mutant gene does not appear to be affected, since mRNA levels are normal; however, the deletion of leu³⁰⁰ may disrupt posttranslational processing, leading to either abnormal translocation or defective secretion of the protein. Surprisingly, the loss of leu³⁰⁰ does not affect the functional properties of LCAT^{300-del.}, indicating that this residue is not essential for enzymic activity and ruling out major structural changes in the mutant LCAT enzyme. The defect does not affect any of the four potential *N*-glycosylation sites of the LCAT polypeptide (40).

The data presented in this study suggest a novel functional explanation for the expression of FED that does not involve a selective loss of HDL-associated α -LCAT activity. Thus, deletion of leu³⁰⁰ results in the synthesis of an enzyme that has normal specific activity but has decreased plasma levels. Despite the reduced quantity of LCAT present in the patient's plasma, the distribution of the mutant enzyme on α - and β -lipoproteins, as demonstrated by the analysis of native and LDL/VLDL-depleted plasma, is similar to that of normal plasma LCAT, establishing that in this patient cholesterol esterification by the mutant LCAT is not restricted to apo B-containing lipoproteins. In fact, significant levels of LCAT activity were detected in the patient's HDL fraction.

On the basis of these results we propose that the phenotypic heterogeneity of primary LCAT deficiency syndromes is primarily dependent on the residual levels of activity of the mutant LCAT enzyme rather than on its location on the lipoprotein particles. Classic LCAT deficiency and FED may represent two ends of a clinical spectrum in which the amount of residual LCAT activity determines the clinical manifestations. Thus, previous studies have indicated that a profound deficiency of total plasma cholesterol esterification is required for the expression of classic LCAT deficiency (41), whereas patients with the FED phenotype had normal rates of plasma cholesterol esterification (6, 11, 13). In the two homozygotes reported here, 27–42% of normal plasma cholesterol esterification and 14–18% of normal α -LCAT activity are sufficient to protect from renal disease and anemia but not to prevent hypophthalipoproteinemia and corneal opacification. Thus, the

residual LCAT activity that protects against renal disease and hemolytic anemia may reside either on the apo B-containing lipoproteins, as originally described by Carlson and Holmquist (7, 9), or on both α - and β -containing lipoproteins, as reported here, or potentially only on the α -containing lipoproteins. Thus, it is not the location on the lipoprotein particles but the presence of residual enzymic activity that protects against renal disease and hemolytic anemia.

In summary, we describe a unique defect in the LCAT gene of a patient presenting with FED. In vitro transfection of LCAT^{300-del.} results in reduced expression of a mutant enzyme with normal specific α -LCAT activity, thus establishing the functional significance of this defect. Characterization of LCAT^{300-del.} establishes that a combined partial deficiency of both α - and β -LCAT activity can lead to FED. Thus, selective deficiency of α -LCAT activity is not essential for the development of this syndrome. On the basis of the present study we propose that classic LCAT deficiency and FED represent different clinical manifestations of the same genetic disorder in which the heterogeneity of phenotypic expression is dictated primarily by the residual levels of total LCAT activity in the patient's plasma.

Acknowledgments

This study was supported in part by National Institutes of Health Grant HL 3-00H6 to Dr. J. Albers. H.-G. Klein is the recipient of a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft. N. Duverger is the recipient of a postdoctoral fellowship from Rhone-Poulenc-Rorer, Vitry sur Seine, France.

References

1. Norum, K. R., E. Gjone, and J. A. Glomset. 1989. Familial lecithin:cholesterol acyltransferase deficiency, including fish eye disease. *In* The Metabolic Basis of Inherited Disease. M. C. R. Scriver, A. L. Beaudet, W. S. Sly, and D. Valle, editors. McGraw-Hill, Inc., New York. 1181–1194.
2. Fielding, C. J. 1990. Lecithin:cholesterol acyltransferase. *In* Advances in Cholesterol Research. M. Esfahani and J. B. Swaney, editors. The Telford Press, Caldwell. 271–314.
3. Jonas, A. 1991. Lecithin-cholesterol acyltransferase in the metabolism of high-density lipoproteins. *Biochim. Biophys. Acta.* 1984:205–220.
4. Fielding, C. J. 1984. Increased free cholesterol in plasma low and very low density lipoproteins. *J. Lipid Res.* 25:1624–1628.
5. Carlson, L. A., and B. Philipson. 1979. Fish-eye disease: a new familial condition with massive corneal opacities and dyslipoproteinemia. *Lancet* 2:921–923.
6. Carlson, L. A. 1982. Fish eye disease: a new familial condition with massive corneal opacities and dyslipoproteinemia. *Eur. J. Clin. Invest.* 12:41–53.
7. Carlson, L. A., and L. Holmquist. 1985. Evidence for deficiency of high

- density lipoprotein lecithin:cholesterol acyltransferase activity (alpha-LCAT) in fish eye disease. *Acta Med. Scand.* 218:189–196.
8. Norum, K. R., and E. Gjone. 1967. Familial plasma lecithin:cholesterol acyltransferase deficiency. Biochemical study of a new inborn error of metabolism. *Scand. J. Clin. Lab. Invest.* 20:231–243.
9. Carlson, L. A., and L. Holmquist. 1985. Evidence for the presence in human plasma of lecithin:cholesterol acyltransferase activity (beta-LCAT) specifically esterifying free cholesterol of combined pre-beta- and beta-lipoproteins. Studies of fish eye disease patients and control subjects. *Acta Med. Scand.* 218:197–205.
10. Chen, C. H., and J. J. Albers. 1982. Distribution of lecithin-cholesterol acyltransferase (LCAT) in human plasma lipoprotein fractions. Evidence for the association of active LCAT with low density lipoproteins. *Biochem. Biophys. Res. Commun.* 107:1091–1096.
11. Funke, H., A. von Eckardstein, P. H. Pritchard, J. J. Albers, J. J. P. Kastelein, C. Droste, and G. Assmann. 1991. A molecular defect causing fish eye disease: an amino acid exchange in lecithin-cholesterol acyltransferase (LCAT) leads to the selective loss of alpha-LCAT activity. *Proc. Natl. Acad. Sci. USA.* 88:4855–4859.
12. Skretting, G., and H. Prydz. 1992. An amino acid exchange in exon I of the human lecithin:cholesterol acyltransferase (LCAT) gene is associated with fish eye disease. *Biochem. Biophys. Res. Commun.* 182:583–587.
13. Klein, H.-G., P. Lohse, P. H. Pritchard, D. Bojanovski, H. Schmidt, and H. B. Brewer, Jr. 1992. Two different allelic mutations in the lecithin-cholesterol acyltransferase gene associated with the fish eye syndrome. Lecithin-cholesterol acyltransferase (Thr₂₃ → Ile) and lecithin-cholesterol acyltransferase (Thr₃₄₇ → Met). *J. Clin. Invest.* 89:499–506.
14. Clerc, M., M. F. Dumon, D. Sess, M. Freneix-Clerc, M. Mackness, and C. Conri. 1991. A 'fish-eye disease' familial condition with massive corneal opacities and hypoalphalipoproteinemia: clinical, biochemical and genetic features. *Eur. J. Clin. Invest.* 21:616–624.
15. Stokke, K. T., and K. R. Norum. 1971. Determination of lecithin:cholesterol acyltransferase in human blood plasma. *Scand. J. Clin. Lab. Invest.* 27:21–27.
16. Dobiasova, M., and M. Schutzova. 1986. Cold labelled substrate and estimation of cholesterol esterification rate in lecithin cholesterol acyltransferase radioassay. *Physiol. Bohemoslov.* 35:319–327.
17. Chen, C. H., and J. J. Albers. 1982. Characterization of proteoliposomes containing apoprotein A-I: a new substrate for the measurement of lecithin:cholesterol acyltransferase activity. *J. Lipid Res.* 23:680–691.
18. Albers, J. J., J. C. Adolphson, and C. H. Chen. 1981. Radioimmunoassay of human lecithin-cholesterol acyltransferase. *J. Clin. Invest.* 67:141–146.
19. Saiki, R. K., S. Scharf, F. Faloona, K. B. Mullis, G. T. Horn, H. A. Erlich, and N. Arnheim. 1985. Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science (Wash. DC).* 230:1350–1354.
20. Scharf, S. J., G. T. Horn, and H. A. Erlich. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science (Wash. DC).* 233:1076–1078.
21. Mullis, K. B., and F. A. Faloona. 1987. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. *Methods Enzymol.* 155:335–350.
22. Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* 101:20–78.
23. Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA.* 74:5463–5467.
24. Beg, O. U., M. S. Meng, S. I. Skarlatos, L. Previato, J. D. Brunzell, H. B. Brewer, Jr., and S. Santamarina-Fojo. 1990. Lipoprotein lipase Bethesda: a single amino acid substitution (Ala176 → Thr) leads to abnormal heparin binding and loss of enzymic activity. *Proc. Natl. Acad. Sci. USA.* 87:3474–3478.
25. Del Sal, G., G. Mantioletti, and C. Schneider. 1988. A one-tube plasmid DNA mini-preparation suitable for sequencing. *Nucleic Acids Res.* 16:9878.
26. Radloff, R., W. Bauer, and J. Vinograd. 1967. A dye-buoyant-density method for the detection and isolation of closed circular duplex DNA: the closed circular DNA in HeLa cells. *Proc. Natl. Acad. Sci. USA.* 57:1514–1521.
27. Ho, S. N., H. D. Hunt, R. M. Horton, J. K. Pullen, and L. R. Pease. 1989. Site-direct mutagenesis by overlap extension using the polymerase chain reaction. *Gene (Amst.).* 77:51–59.
28. Rosenthal, N. 1987. Identification of regulatory elements of cloned genes with functional assays. *Methods Enzymol.* 152:704–720.
29. Chait, A., P. H. Iverius, and J. D. Brunzell. 1982. Lipoprotein lipase secretion by human monocyte-derived macrophages. *J. Clin. Invest.* 69:490–493.
30. Chirgwin, J. M., A. E. Przybyla, R. J. McDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources rich in ribonuclease. *Biochemistry.* 18:5294–5299.
31. McLean, J. M., K. Wion, D. Drayna, C. Fielding, and R. Lawn. 1986. Human lecithin-cholesterol acyltransferase gene: complete sequence and sites of expression. *Nucleic Acids Res.* 14:9397–9406.
32. Warden, C. H., C. A. Langner, J. I. Gordon, B. A. Taylor, J. McLean, and A. J. Lusis. 1989. Tissue-specific expression, developmental regulation, and chromosomal mapping of the lecithin:cholesterol acyltransferase gene. Evidence for expression in brain and testes as well as liver. *J. Biol. Chem.* 264:21573–21581.
33. Meroni, G., N. Margaretti, P. Magnaghi, and R. Taramelli. 1990. Nucleotide sequence of the cDNA for lecithin-cholesterol acyl transferase (LCAT) from the rat. *Nucleic Acids Res.* 18:5308.
34. Hopp, T. P., and K. R. Woods. 1981. Prediction of protein antigenic determinants from amino acid sequences. *Proc. Natl. Acad. Sci. USA.* 78:3824–3828.
35. Karplus, P. A., and G. E. Schultz. 1985. Prediction of chain flexibility in proteins. *Naturwissenschaften.* 72:212–213.
36. Garnier, J., D. J. Osguthorpe, and B. Robson. 1978. Analysis of the accuracy and implications of simple methods for predicting the secondary structure of globular proteins. *J. Mol. Biol.* 120:97–120.
37. Gascuel, O., and J. L. Golmard. 1988. A simple method for predicting the secondary structure of globular proteins: implication and accuracy. *Comput. Appl. Biosci.* 4:357–365.
38. Carlson, L. A., and L. Holmquist. 1985. Paradoxical esterification of plasma cholesterol in fish eye disease. *Acta Med. Scand.* 217:491–499.
39. Holmquist, L., and L. A. Carlson. 1987. Alpha-lecithin:cholesterol acyltransferase deficiency. Lack of both phospholipase A2 and acyltransferase activities characteristic of high density lipoprotein lecithin:cholesterol acyltransferase in fish eye disease. *Acta Med. Scand.* 222:23–26.
40. Collet, X., and C. J. Fielding. 1991. Effects of inhibitors of N-linked oligosaccharide processing on the secretion, stability, and activity of lecithin:cholesterol acyltransferase. *Biochemistry.* 30:3228–3234.
41. Klein, H.-G., P. Lohse, N. Duverger, J. J. Albers, D. J. Rader, L. A. Zech, S. Santamarina-Fojo, and H. B. Brewer, Jr. 1993. Two different allelic mutations in the lecithin:cholesterol acyltransferase (LCAT) gene resulting in classic LCAT deficiency: LCAT (tyr⁸³ → stop) and LCAT (tyr¹⁵⁶ → asn). *J. Lipid Res.* 34:49–58.