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A molecular defect causing fish eye disease: An amino acid exchange in lecithin–cholesterol acyltransferase (LCAT) leads to the selective loss of α-LCAT activity

(high density lipoprotein deficiency/molecular diagnostics/gene analysis/site-directed mutagenic PCR primers/structural basis of disease)

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ABSTRACT **Epidemiological as well as biochemical evidence of recent years has established that a low plasma level of high density lipoprotein–cholesterol is a predictor for the risk of coronary artery disease. However, there is a heterogeneous group of rare familial disorders, characterized by severe high density lipoprotein deficiency, in which the predicted increased risk is not clearly apparent. One such disorder has been called fish eye disease to reflect the massive corneal opacification seen in these patients. In this report, we describe the biochemical and genetic presentation of two German fish eye disease homozygotes and their family members. Vertical transmission of a decrease in the specific activity of lecithin–cholesterol acyltransferase (EC 2.3.1.43) indicated that this enzyme was a candidate gene for harboring the defect responsible for this disorder. Direct sequencing of DNA segments amplified by the polymerase chain reaction (PCR) that encode the exons of the lecithin–cholesterol acyltransferase gene led to the identification of a homozygous mutation resulting in the substitution of threonine at codon 123 for an isoleucine residue in both individuals. Family analysis in an extended pedigree was used to establish a causal relationship between this mutation and the biochemical phenotype for fish eye disease. The homozygous presence of this mutation in two phenotypically homozygous members of an unrelated Dutch family with fish eye disease further supports this finding.**

Epidemiologic studies of recent years have identified low plasma high density lipoprotein (HDL) cholesterol concentrations as a major predictor for the development of coronary artery disease (1–3). These associations have been substantiated by the detection of genetic defects at the *APOLP1* locus that cause the absence of HDL cholesterol from plasma and were strongly associated with premature coronary artery disease (4, 5). In conflict with this proposed role of HDL in the pathogenesis of coronary artery disease are findings in some rare familial disorders in which, despite a complete absence of plasma HDL cholesterol, no increased prevalence of coronary artery disease has been noted (6, 7). One such heritable anomaly of HDL metabolism is fish eye disease (FED) that was originally identified in Sweden (8) and was subsequently also observed in Canada (9).

The majority of cholesteryl esters present in plasma are the product of the reaction catalyzed by the enzyme lecithin–cholesterol acyltransferase (LCAT; EC 2.3.1.43) in which cholesterol is esterified with the *sn*-2 fatty acid of phosphatidylcholine. The resulting cholesteryl esters are packed into the hydrophobic core of lipoproteins and thereby contribute to the formation of normal-size HDL particles from one or several precursors (10). Vertically transmitted impairments of LCAT activity have been observed in several syndromes that are associated with HDL deficiency. Familial LCAT deficiency is one such disorder that is characterized by the inability of plasma to esterify cholesterol *in vitro*, the virtual absence of LCAT activity and of LCAT mass, and a significant increase in plasma unesterified cholesterol (7). FED differs from this disorder by the retention of the ability to esterify cholesterol in plasma that results in a near normal ratio of unesterified cholesterol/total cholesterol. The hallmark of this disease appears to be the specific inability of LCAT in FED plasma to esterify cholesterol in HDL or in small proteoliposome substrates. Carlson and Holmquist (11–13), therefore, proposed that FED represents a variant of LCAT deficiency in which there is a loss of LCAT activity toward HDL particles. They termed this activity α-LCAT.

METHODS

Description of the Patients. Two brothers, 57 and 68 years old, clinically represented a phenotype with massive bilateral corneal opacities that almost completely covered the irises. Thereby and by their biochemical characteristics the patients resembled the FED phenotype described by Carlson and Philipson (8). The patients reported several consanguinous marriages in their family history. In their village, the kindred are known to have been affected with "sick eyes" for several generations. The younger brother suffers from angiographically assessed two-vessel coronary disease with a 60% stenosis of ramus interventricularis anterior and a 50% stenosis of the arteria coronaria dextra since the age of 50. The elder brother is reported to suffer from angina pectoris since the age of 60 but he refused to undergo detailed cardiological examinations. The family history, however, is not compatible with any increased prevalence of myocardial infarctions. Except for largely reduced serum HDL–cholesterol concentrations routine laboratory analyses in the two brothers gave normal values.

Quantitation of Lipids, Apolipoproteins, LCAT Activities, and LCAT Mass. Concentrations of cholesterol and triglycerides were determined using an autoanalyzer (Hitachi/Boehringer Mannheim). Low density lipoprotein–cholesterol was determined by the method of Friedewald *et al.* (14).

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Abbreviations: LCAT, lecithin–cholesterol acyltransferase; HDL, high density lipoprotein; FED, fish eye disease; apo, apolipoprotein.
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HDL–cholesterol was measured after precipitation with phosphotungstic acid/$MgCl_2$. Apolipoprotein (apo) A-I was quantified by turbidimetry as described (15). The rate of cholesterol esterification in plasma was determined by two methods. (*i*) The cholesterol esterification rate was measured by the method of Dobiasova (16). It reflects the esterification of cholesterol in endogenous substrates (i.e., in lipoproteins of the plasma sample) and was determined by the rate of esterification of radioactive unesterified cholesterol to cholesteryl esters. (*ii*) Cholesterol esterification in an exogenous substrate, in the following referred to as LCAT activity, was determined by the addition of artificial proteoliposomes that contain normal apo A-I as well as phosphatidylcholine and unesterified [^{3}H]cholesterol to the plasma samples (17, 18). LCAT mass was determined immunologically as described (18).

Gene Sequencing and Genotype Determination. Oligonucleotides, 21–27 bases long, were used as primers for PCR amplification. Primers were synthesized using the Gene Assembler (Pharmacia). Primer pairs were matched for calculated melting temperature (68°C) but not for length. Tandem and inverse repeats as well as homologies between primers were avoided. The two most 3′ bases were guanine or cytosine; the 3′ ends were placed approximately 50 bases 5′ of the gene segments that were to be sequenced. Amplification primers were also used as sequencing primers. The amplification reaction was carried out using 100 μl of buffer recommended by the supplier of the *Taq* polymerase (Cetus), 0.5–1 μg of DNA, and all four dNTPs (each at a final concentration of 200 μM), and 0.1 μM primers. Initial denaturation at 100°C for 10 min was followed by the addition of 2–5 units of *Taq* polymerase and 30 cycles of 96°C (2 min), 60°C (1 min), and 70°C (1 min) incubations. Product purification was done by electrophoresis in 4% NuSieve agarose (FMC); DNA of the expected size was excised from the gel and electroeluted in 0.5× TAE [1× TAE = 40 mm Tris acetate/1 mM EDTA (pH 8.0)] for 1.25 h to 1.5 h at 200 V. DNA was desalted and concentrated to 70 μl by ultrafiltration centrifuging 3 times at 5000 rpm in a JA-20.1 rotor (Beckman) for 20 min in Centricon X-100 tubes (Amicon), refilling to 2 ml with water between the centrifugations. Single-strand production was done by the method of Gyllensten and Erlich (20) using the same reaction and purification scheme as above. Primer concentration was 0.1 μM; a second primer was not used. Sequencing was done either from single strands or from double strands after phenol extraction and LiCl/ethanol precipitation using T7 polymerase (Pharmacia) and following the protocol of the polymerase supplier. Labeling times varied between 0.5 and 5 min. After termination the sample volume was reduced to 6 μl in an evaporation centrifuge; half of the sample was applied to the sequencing gel containing 6% polyacrylamide gel [29:1 (wt/vol) acrylamide/*N,N′*-methylenebisacrylamide] in 135 mM Tris, 45 mM boric acid, and 2.5 mM EDTA. In long runs acrylamide concentration was 5% and both of the gel plates were covered with Bind Silan (Pharmacia).

Dra III restriction fragment length polymorphisms were examined using PCR-amplified DNA. The enzyme cutting site was introduced specifically into the wild-type allele by site-directed mutagenic PCR primers (21). The oligonucleotide 5′-CATTGTTGACCAGGTTCTCACCCAGT-3′ was used as the lower strand primer in the PCR (mismatched nucleotides are underlined). The sequence of the reaction product was thus modified to contain a *Dra* III restriction site ($CACN_3$/GTG, where N is any nucleotide) in the absence of the mutation causing FED. Reaction conditions for the PCR were unchanged.

RESULTS AND DISCUSSION

Tables 1 and 2 present the biochemical data for a German family with FED. The 57- and 68-year-old patients (Ic and Ie in Fig. 2) were investigated because of massive bilateral corneal opacities. In contrast to other previously described FED patients, the index patient (Ic) suffered from angiographically assessed coronary heart disease at age 50. There

Table 1. Lipid and apo A-I plasma concentrations in members of a German FED family

Family member	Age/sex	Concentration, mg/dl				
		Triglycerides	Cholesterol	HDL–C	LDL–C	Apo A-I
Homozygote						
Ic	68/m	203	198	0	163	20
Ie	57/m	182	212	0	140	23
Heterozygote						
IIa	41/f	70	136	47	75	115
IId	39/m	98	145	36	89	117
IIf	29/m	53	155	42	101	132
IIg	27/m	107	149	62	73	146
IIh	25/m	120	194	31	140	111
IIIb	11/m	223	136	31	60	100
IIId	12/m	67	137	40	84	111
IIIe	12/m	62	130	46	72	123
IIIg	8/f	91	232	36	178	104
	Mean ± SD	99 ± 47	157 ± 32	41 ± 9*	97 ± 36	119 ± 19†
Normal						
Id	51/f	82	159	45	101	132
IIb	40/m	185	235	56	142	144
IIc	38/f	148	188	55	103	163
IIe	27/f	143	247	69	149	164
IIIa	17/f	50	212	62	138	147
IIIc	5/m	105	155	50	84	134
IIIh	4/m	101	212	67	125	156
	Mean ± SD	117 ± 42	200 ± 33	54 ± 12	123 ± 24	144 ± 18

Heterozygotes were segregated from normal individuals when the LCAT activity was less than 20 nmol per h per ml, which is two standard deviations below the mean activity of a normal control population. HDL–C, HDL–cholesterol; LDL–C, low density lipoprotein–cholesterol; m, male; f, female. Significance of differences between homozygotes and normal individuals in this family was determined by Student's *t* test. *, $P < 0.05$; †, $P < 0.01$.

Table 2. Cholesterol esterification in members of the German FED family

Family member	% FC	Plasma CER, nmol per h per ml	LCAT		
			Activity, nmol per h per ml	Mass, μg/ml	Specific activity, nmol per h per μg
Homozygote					
Ic	44	39	0.5	2.47	0.20
Ie	40	68	1.0	2.31	0.43
Heterozygote					
IIa	32	52	17.5	4.02	4.35
IId	29	56	17.4	5.62	3.10
IIf	27	71	16.0	4.97	3.22
IIg	30	67	16.7	5.66	2.95
IIh	30	52	16.7	4.79	3.49
IIIb	26	110	19.4	5.21	3.72
IIId	32	101	17.0	5.47	3.11
IIIe	28	86	19.2	5.84	3.29
IIIg	31	75	15.7	4.01	3.92
Mean ± SD	29 ± 2	74 ± 21	17.3 ± 1.3*	5.01 ± 0.60	3.42 ± 0.50*
Normal					
Id	—	48	24.6	4.63	5.31
IIb	—	113	27.2	4.66	5.84
IIc	—	114	31.3	5.72	5.47
IIe	—	117	33.6	4.67	7.20
IIIa	—	67	29.9	5.02	5.96
IIIc	—	98	31.3	5.32	5.88
IIIh	—	102	30.3	5.88	5.15
Mean ± SD	29 ± 2	94 ± 27	29.7 ± 2.6	4.83 ± 0.20	5.64 ± 0.32

Heterozygotes were segregated from normal individuals when the LCAT activity was less than 20 nmol per h per ml, which is two standard deviations below the mean activity of a normal control population. FC, free (unesterified) cholesterol; CER, cholesterol esterification rate. Significance of differences between homozygotes and normal individuals in this family was determined by Student's *t* test. *, $P < 0.001$.

was, however, no evidence for an increased prevalence of myocardial infarction in this family. The homozygosity for FED (absence of α-LCAT activity) was confirmed in these two individuals by the inability of plasma to esterify cholesterol in exogenous apo A-I proteoliposomes. The net ability of plasma, however, to esterify radiolabeled cholesterol in endogenous substrates was unaffected (Table 2). The unesterified cholesterol/esterified cholesterol ratio in the plasma sample was slightly elevated (0.44 in homozygotes versus 0.29 in heterozygous and normal family members) but not to the extent observed in familial LCAT deficiency. The characteristic phenotypic expression of LCAT activity in this family with FED led to the hypothesis that the molecular defect lies at the LCAT locus. Since significant amounts of LCAT mass were found in plasma despite the loss of α-LCAT activity, we proposed that such a defect results in a defective partially active gene product. We have, therefore, sequenced the exons, the splice donor and acceptor sequences, and 67 bases of 5′ sequence of the LCAT gene in this family.

For sequence analysis, pairs of oligonucleotides were used to amplify the exons of the LCAT gene by the PCR (22). Direct sequence analysis, which gives simultaneous information for both alleles, showed a homozygous C → T exchange in the second base of codon 123 (Fig. 1). All other analyses yielded the expected wild-type sequence of LCAT (23). This mutation results in the replacement of the threonine at position 123 with an isoleucine residue. Analysis of DNA from the family members for the presence or absence of this mutation yielded a genotype that in all cases corresponded with the inherited biochemical phenotype. Fig. 2 shows the pedigree for this family in which we demonstrate that heterozygosity for this mutation is coinherited with the phenotypic heterozygosity of LCAT activity. As the structural analysis of all exons and large portions of consensus regulatory sequences revealed no further deviation from the wild-type sequence, it can be largely ruled out that this mutation merely represents a marker that is in linkage disequilibrium with the functionally active mutation. The possibility that it represents a common polymorphism that is of no clinical or biochemical significance was further excluded by our inability to identify this mutation in 100 randomly selected DNA samples.

Additional evidence that this mutation is the basic defect in FED was obtained when the homozygous presence of the Thr-123 → Ile substitution was confirmed in two homozygotes of an unrelated Dutch family with FED that was clinically and biochemically indistinguishable from the family presented here (24).

The identification of an amino acid substitution in LCAT as the underlying defect in FED permits us to speculate on the mechanism that results in the unusual phenotypic expression of LCAT activity. The amino acid substituted in FED LCAT is located outside the enzyme's putative catalytic center but in a highly hydrophobic region (25–28). Previous studies of LCAT activity in the plasma of FED homozygotes clearly indicate that the defective LCAT is incapable of esterifying cholesterol in HDL or in exogenous apo A-I proteoliposome substrates (8, 9, 11–13). This probably impairs the maturation of small nascent HDL into larger HDL particles *in vivo*. Conversely, the presence of significant amounts of cholesteryl esters in FED plasma and the ability of at least a portion of LCAT to be active in larger particles suggest that the exchange of a threonine residue for an isoleucine at position 123 only affects the ability of LCAT to bind to or be active in small substrate particles. Since the activity in larger particles does not appear to be impaired, other domains of LCAT may be required for this function that are unaffected by the presence of the FED mutation. This concept of particle-specific domains of the LCAT enzyme gains further support from observations reported by Castro and Fielding (29) and Miida *et al.* (30) who have demonstrated that cellular cholesterol is esterified in small, discoidal, and only apo

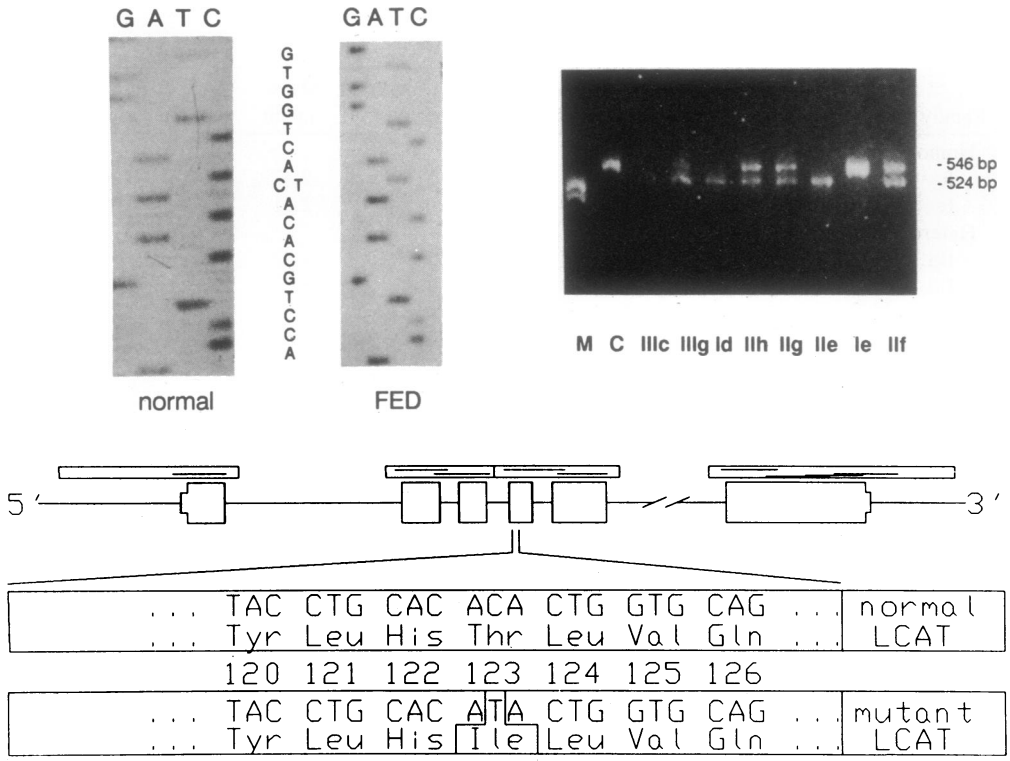

FIG. 1. Single-nucleotide exchange in the fourth exon of the LCAT gene is the underlying defect in FED. (*Lower*) Schematic drawing of the LCAT gene (23); exons are boxed. Boxes above the gene scheme indicate the lengths of single-stranded DNA PCR fragments that were used as templates in sequence analyses. Lines inside these boxes show the lengths of sequence readings; the use of upper- or lower-strand sequencing primers is indicated by the location of these bars in the upper or lower half of the boxes, respectively. The enlarged view of the fourth exon of the LCAT gene (bottom part of the drawing) shows the site and the nature of the nucleotide exchange in codon 123. (*Upper Left*) The partial sequences were obtained from probands IIb (normal) and Ic (homozygous FED). (*Upper Right*) Example of genotype determination in family members with the use of a *Dra* III restriction site. These sites were artificially introduced into the nucleotide sequence. For this purpose TGC in codons 125 and 126 was changed into GTG using the method of Kumar and Dunn (21). The presence of the Thr → Ile mutation abolished this restriction site and led to the detection of a 546-base-pair (bp) fragment instead of the normal 524-bp fragment. Lane M contains molecular size markers of 506 bp and 516 bp. Lane C is an uncut control. IIIc, IIIg, Id, IIh, IIg, IIe, Ie, and IIf refer to the pedigree in Fig. 2. The genotype of all other family members, of two Dutch homozygous FED patients, and of 100 controls from the general population was determined by the same procedure (data not shown).

A-I-containing particles with pre-β mobility upon agarose electrophoresis (pre-β-HDL), whereas low density lipoprotein-derived cholesterol is esterified in larger spherical α-migrating HDL particles.

The description of the underlying defect in FED is a step toward the understanding of the pathophysiology of this clinical presentation. However, the origin of corneal opacification in FED remains at present speculative: both impairment of reverse cholesterol transport and deposition of abnormal lipoproteins may cause this anomaly. In this context it is interesting to note that an HDL-deficient phenotype with corneal opacities as the only clinical symptom, thereby

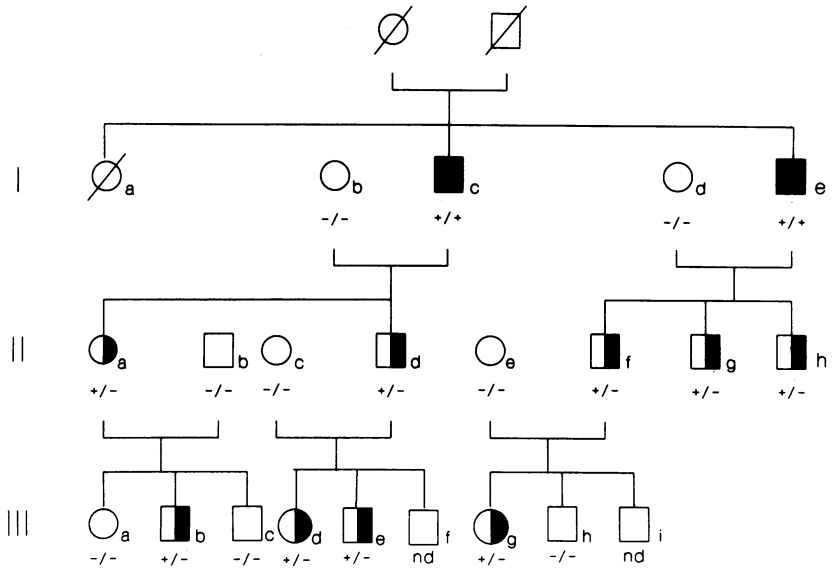

FIG. 2. Pedigree of the German FED family. The biochemical phenotype of FED (reduced LCAT activity and near normal cholesterol esterification rate) in the family was assigned on the basis of LCAT activity as described in the text. Standard symbols are used for the different phenotypes. The genotype for each individual is indicated by the presence (+) or absence (−) of the FED mutation and is presented beneath the biochemical phenotype symbols. nd, Not determined.

resembling FED, can also be the consequence of a frameshift mutation in apo A-I (31). In this condition, however, the associated LCAT deficiency was secondary to the complete loss of HDL and affected both LCAT activity and cholesterol esterification rate. The other important question whether or not α-LCAT deficiency is associated with premature CAD cannot be answered at present because of possible selection bias, since the index patient of the family described in this study was discovered upon screening for HDL deficiency in a cardiology department. Nevertheless, the identification of the molecular defect in FED undoubtedly will facilitate research aimed to answer these questions.

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