0031-3998/93/3401-0111$03.00/0
PEDIATRIC RESEARCH

Vol. 34, No. 1, 1993
Printed in U.S.A.

Identification of Very-Long-Chain Acyl-CoA Dehydrogenase Deficiency in Three Patients Previously Diagnosed with Long-Chain Acyl-CoA Dehydrogenase Deficiency

SEIJI YAMAGUCHI,[1] YASUHIRO INDO,[2] PAUL M. COATES, TAKASHI HASHIMOTO, AND KAY TANAKA

Department of Genetics, Yale University School of Medicine, New Haven, Connecticut 06510 [S.Y., Y.I., K.T.]; Division of Gastroenterology and Nutrition, Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19104 [P.M.C.]; and Department of Biochemistry, Shinshu University School of Medicine, Matsumoto, Nagano 390, Japan [T.H.]

ABSTRACT. **Long-chain acyl-CoA dehydrogenase (LCAD) deficiency is a disorder of fatty acid β-oxidation. Its diagnosis has been made based on the reduced activity of palmitoyl-CoA dehydrogenation, *i.e.*, in fibroblasts. We previously showed that in immunoblot analysis, an LCAD band of normal size and intensity was detected in fibroblasts from all LCAD-deficient patients tested. In the present study, we amplified via polymerase chain reaction and sequenced LCAD cDNA from three of these LCAD-deficient cell lines, and found perfectly normal LCAD sequences in two of them, indicating that at least these patients were not deficient in LCAD. The third patient was homozygous for an A to C substitution at 997, although it is unknown whether or not 997-C is a normal polymorphism. Although the LCAD sequence data were puzzling, a new enzyme, very-long-chain acyl-CoA dehydrogenase (VLCAD), was recently identified. Because VLCAD also has high activity with palmitoyl-CoA as substrate, it was possible that defective VLCAD may cause reduced palmitoyl-CoA dehydrogenating activity. We performed immunoblot analysis of VLCAD in six "LCAD-deficient" patients; VLCAD was negative in three of them, two of whom had a normal LCAD cDNA sequence. These results indicated that a considerable number of the patients who had previously been diagnosed as having LCAD deficiency in fact have VLCAD deficiency. (*Pediatr Res* 34: 111–113, 1993)**

Abbreviations

LCAD, long-chain acyl-CoA dehydrogenase
VLCAD, very-long-chain acyl-CoA dehydrogenase
MCAD, medium-chain acyl-CoA dehydrogenase
PCR, polymerase chain reaction

LCAD deficiency is an inherited disorder of mitochondrial fatty acid β-oxidation. Since this disease was first identified by Hale *et al.* in 1985 (1), at least 13 patients have been reported (2, 3). The main clinical symptoms of this disease include muscle weakness, hepatomegaly, cardiomegaly, and episodes of hypoketotic hypoglycemia. Some of the patients died with an acute episode without noticeable prodromal symptoms, mimicking sudden infant death syndrome (3). LCAD deficiency is one of a group of diseases caused by defects in fatty acid oxidation (4) that includes, among others, MCAD deficiency (5) and long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (6). The molecular abnormalities responsible for MCAD deficiency have been extensively studied (7). By contrast, the molecular basis for LCAD deficiency remains to be elucidated.

LCAD is a mitochondrial matrix enzyme that catalyzes the first reaction in the β-oxidation of long-chain fatty acids (10–18 carbons). MCAD and short-chain acyl-CoA dehydrogenase catalyze the same reaction using fatty acids of four to 10 and four carbons, respectively (8). Thus, the diagnosis of LCAD deficiency has been made based on the reduced dehydrogenase activity toward palmitoyl-CoA in the soluble cell fraction of cells such as fibroblasts or leukocytes (9). Recently, we reported that, among nine LCAD-deficient cell lines, all exhibited a positive protein band that was immunoreactive with anti-LCAD antibody, had a normal molecular mass and normal intensity (10). This result appeared to suggest that all cases of LCAD deficiency were caused by a point mutation in the LCAD gene. However, such a uniformly normal appearance of the "variant LCAD" protein in all patients with such a rare disease as LCAD deficiency appeared anomalous in view of the recent accumulating evidence about molecular heterogeneity for other mitochondrial defects (11, 12). It is now clear that various rare disease-causing mutations within a gene can be highly heterogeneous, producing variant proteins of different appearance. Many of them are undetectable because of instability, and some are truncated, whereas others may appear to be normal. Thus, the result of the immunoblot study raised questions concerning the cause of LCAD deficiency.

Recently, a novel enzyme involved in fatty acid β-oxidation, VLCAD, was identified (13). Because VLCAD also has high activity with palmitoyl-CoA as substrate, it was possible that defective VLCAD might cause the reduced dehydrogenase activity toward palmitoyl-CoA. In this paper, we report the results from our further study, indicating that at least some patients who were previously diagnosed as "LCAD-deficient" were in fact not deficient in LCAD, but rather deficient in VLCAD.

MATERIALS AND METHODS

Materials. Human VLCAD was purified by the method previously used for the purification of rat VLCAD (13) and was

Received for rapid publication March 18, 1993; accepted April 21, 1993.
Correspondence: Seiji Yamaguchi, M.D., Gifu University School of Medicine, Department of Pediatrics, 40 Tsukasa-machi, Gifu 500, Japan.
Supported by March of Dimes Grants 1-1230 and 92-0785, Johnson and Johnson, and NIH Grants DK38154 and NS17752. S.Y. was supported by a grant from the Ministry of Education, Japan.
[1] Present address: Department of Pediatrics, Gifu University School of Medicine, Gifu, Japan.
[2] Present address: Department of Pediatrics, Kumamoto University School of Medicine, Kumamoto, Japan.

used to raise antibody in rabbits. Protoblot Kit for immunoblotting was purchased from Promega (Madison, WI).

Source of fibroblasts. Cells from six patients with LCAD deficiency have previously been reported; these were J-1, A-1, R-1, CP14, CP2, and CP3, reported by Indo *et al.* (10). Five normal cell cultures were obtained from the Human Genetic Mutant Cell Repository (Camden, NJ). LCAD-deficient cells and normal controls were cultured in Eagle's minimal essential medium containing 10% FCS.

cDNA synthesis and PCR amplification of LCAD. First-strand cDNA was synthesized from total cellular RNA using Moloney murine leukemia virus reverse transcriptase and a cDNA synthesis kit (Bethesda Research Laboratories, Gaithersburg, MD), as described previously (14).

PCR amplification of the coding region was carried out in three overlapping sections. These sections encompassed positions 37–519, 479–900, and 858–1314. These three regions cover the entire mature LCAD sequence, but the first 12 amino acids in the leader peptide are not included. However, the incomplete information on the leader peptide sequence was not detrimental to the purpose of this study, inasmuch as in our previous immunoblot study (10) LCAD was detected in the mature form in LCAD-deficient cells, indicating that mitochondrial uptake and processing was normal. The primers were all 27-mers, each covering the sequence flanking these sections. The reactions were carried out using 10 μL of first-strand cDNA as a template and 1 μM each of an appropriate pair of the primers. To facilitate the subcloning, two or three mismatches were introduced into the primers to create appropriate restriction sites. The reaction mixture contained 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, 50 mM KCl, 0.01% gelatin, 200 μM each of deoxy-ATP, deoxycytidine triphosphate, deoxyguanosine triphosphate, and deoxythymidine triphosphate, and 0.5 μL (2.5 U) of *Taq* DNA polymerase in 100 μL and was covered with 100 μL of mineral oil (Brand-Nu Laboratories, Meriden, CT). After initial incubation at 94°C for 3 min, 30 cycles of PCR reaction were performed using a DNA Thermal Cycler (Perkin-Elmer-Cetus, Norwalk, CT) according to the following program: 1 min of denaturation at 94°C, 2 min of annealing at 50°C, and 3 min of extension at 72°C. The final extension was for 7 min.

DNA sequencing. The PCR-amplified products were extracted with chloroform, precipitated with ethanol, and digested with *Bam*HI (Boehringer-Mannheim, Indianapolis, IN). The product DNA was purified by electrophoresis using 1.5% low-melting agarose gel (SeaPlaque; FMC Bioproduct, Rockland, ME). The fragments of expected size were cut out and purified with a MARmaid kit (Bio 101 Inc., La Jolla, CA). The recombinant plasmid was amplified by transforming *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA). DNA sequencing was performed directly using the recombinant pBluescript with cDNA inserts by the dideoxy-sequencing method (15).

Enzyme assay and immunoblot analysis. LCAD deficiency had been previously demonstrated by measuring acyl-CoA dehydrogenase activity in the soluble fraction of fibroblast extracts using the fluorometric assay based upon reduction of electron transfer flavoprotein (1, 3). It was revealed in this assay that these patients had reduced dehydrogenase activity toward palmitoyl-CoA, with a range from 5 to 35% of control levels, whereas they had normal levels of activities toward octanoyl-CoA and butyryl-CoA. In the present study, cell pellets that had been stored at −80°C were thawed and suspended in 0.2–0.4 mL of 100 mM sodium phosphate, pH 7.2, containing 0.1 mM EDTA/200 mM NaCl/0.2% Triton X-100 (extraction buffer). The suspension was sonicated and centrifuged at 10 000 × *g* at 4°C for 30 min. To confirm that the activity to dehydrogenate palmitoyl-CoA was deficient in the "LCAD-deficient" cells, we repeated the enzyme assay; the same supernatant was used for immunoblotting. Acyl-CoA dehydrogenase activity was determined using the ferricenium ion reduction method as described by Lehman *et al.* (16), with some modifications: 200 mM NaCl and 0.2% Triton X-100 were added to the extraction buffer as mentioned above, and 0.5 mM sodium tetrathionate was used instead of 0.5 mM N-ethylmaleimide in the reaction mixture. Approximately 250–500 μg of total protein was used for each assay. In immunoblot analysis, 50 μg of protein was subjected to SDS/PAGE using 10% gel. After electrophoresis, the gel was electroblotted onto an Immobilon-P sheet (Millipore, Bedford, MA) and the blot was treated according to the manufacturer's instructions (Protoblot kit).

RESULTS AND DISCUSSION

PCR amplification and sequencing of LCAD cDNA from cultured fibroblasts from three patients who were previously diagnosed with LCAD deficiency. As previously reported by Indo *et al.* (17), the human LCAD sequence is peculiar in having two polymorphic sites. In the initial cloning experiment, we obtained two human LCAD cDNAs from two different cDNA libraries that differed at positions 908 and 997. One cDNA contained 908-G (serine-303) and 997-A (333-lysine), while the second cDNA clone contained C at both positions, resulting in 303-threonine and 333-glutamine. Subsequently, we sequenced five independent normal LCAD cDNA, each of which was amplified from five independent normal cultured human fibroblast lines, and found that all contained 303-serine and 333-lysine. Thus, 303-serine and 333-lysine are by far predominant. Because serine to threonine substitution is of a conservative nature, it is likely that 303-serine/threonine represents a normal polymorphism (17, 18). However, the nature of 333-glutamine is questionable, as discussed below.

In the present study, we amplified by PCR LCAD cDNA from three "LCAD-deficient" patients, J-1, A-1 and R-1, and determined their sequences. To our surprise and in spite of repeated experiments, we found perfectly normal LCAD sequence in A-1 and R-1 with 303-serine and 333-lysine. Taken together with the previous results indicating that they had immunoreactive LCAD (10), LCAD deficiency is unlikely to be the cause of the disease in these two patients. The third patient, J-1, was homozygous for both 303-serine and 333-glutamine. It is currently unknown whether or not 333-glutamine renders LCAD inactive; it is stable judging from the previous immunoblot study (10). In considering the pathogenicity of this variant, it is interesting to note that 333-lysine is highly conserved. Normal rat LCAD cDNA has 997-A (333-lysine). Furthermore, 333-lysine is conserved in short-chain acyl CoA dehydrogenase and isovaleryl-CoA dehydrogenase, in both human and rat (17, 18). Substitution of such a conserved basic residue with a neutral residue may be deleterious. It is possible that J-1 is indeed LCAD-deficient because of 333-glutamine substitution, and that the fibroblast cDNA library that we used might have been produced using tissue from a LCAD-deficient person. To determine the nature of 333-glutamine, an expression study of this variant cDNA is necessary.

Immunoblot analysis of VLCAD. Shortly after the LCAD sequence study was completed, Izai *et al.* (13) reported the identification and purification of a previously unknown enzyme, VLCAD, from rat liver mitochondria. Unlike all other acyl-CoA dehydrogenases that are found in the mitochondrial matrix, VLCAD is membrane-bound and oxidizes mainly acyl-CoA with 14–22 carbons. Its molecular size, 71 kD (70 kD for human VLCAD), is considerably larger than that of the other acyl-CoA dehydrogenases, which range from 40 to 45 kD (8). Because the specific activity of purified VLCAD for palmitoyl-CoA is nearly 10 times higher than that of purified LCAD, reduced activity toward palmityl-CoA may be due to deficiency of this enzyme. Thus, it was possible that the disease previously considered to be caused by LCAD deficiency may in fact be caused by a deficiency of VLCAD. To test such a possibility, we performed immunoblot analysis of VLCAD in the above six LCAD-deficient cells. By enzyme assay performed before immunoblot analysis, it was

confirmed that the activity with palmitoyl-CoA in all these cell lines was indeed markedly reduced (17 to 47% of control levels), whereas the activity with octanoyl-CoA was similar to that of normal controls (data not shown).

As shown in Figure 1, in three cell lines, J-1, CP14, and CP3 (lanes 1, 4, and 6, respectively), the 70 kD VLCAD band was detectable with intensity comparable to that of normal controls (lane C). In contrast, no VLCAD band was detectable in R-1 and CP2 (lanes 3 and 5, respectively), and only an extremely faint VLCAD was detected in A-1 (lane 2). We also performed immunoblot analysis of the same cell extracts for isovaleryl-CoA dehydrogenase and MCAD as positive mitochondrial protein controls; both proteins were detected in all six LCAD-deficient cell lines at an intensity comparable to that of normal controls (data not shown). The results suggested that at least three of the six LCAD-deficient patients were negative for VLCAD.

Thus, the results from this study unequivocally indicate that patients A-1, R-1, and CP2 have VLCAD deficiency and suggest that a considerable number of the patients who had previously been diagnosed with LCAD deficiency in fact have VLCAD deficiency. At present, it is unknown whether the patients who exhibited normal-looking bands for both proteins are deficient in LCAD or VLCAD activity. Comparison of symptoms of the three VLCAD-negative patients with those of the other three was not revealing. It is important to note that J-1, which had 333-glutamine, exhibited a positive VLCAD band by immunoblot analysis. Therefore, there is still the possibility that 333-glutamine may cause LCAD deficiency.

During the preparation of this report, two groups of investigators reported the identification of patients with VLCAD deficiency based on the low palmitoyl-CoA dehydrogenating activity in the membrane fraction of cultured fibroblast homogenates (19, 20), which was resistant to inhibition by anti-VLCAD antibody (20). However, the result of the present paper is important in indicating that other patients who have been identified as LCAD-deficient need to be reinvestigated, and that VLCAD deficiency should be also considered in the diagnosis of patients who exhibit symptoms such as muscle weakness, hepatomegaly, cardiomegaly, and episodes of hypoketotic hypoglycemia, which had been considered typical of LCAD deficiency.

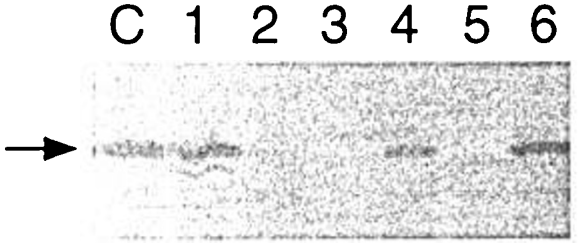

Fig. 1. Immunoblot of VLCAD in fibroblasts from patients who had been previously diagnosed as having LCAD deficiency. *Lane C*, normal control; *lanes 1–6*, patients J-1, A-1, R-1, CP14, CP2, and CP3, respectively. In patients J-1, A-1, and R-1, LCAD cDNA sequence was determined: in J-1 (*lane 1*), 997A to C substitution was detected, whereas in A-1 (*lane 2*) and R-1 (*lane 3*), no seqeunce abnormality was found. An *arrow* indicates the position of mature human VLCAD.

REFERENCES

1. Hale DE, Batshaw ML, Coates PM, Frerman FE, Goodman SI, Singh I, Stanley CA 1985 Long-chain acyl coenzyme A dehydrogenase deficiency: an inherited cause of nonketotic hypoglycemia. Pediatr Res 19:666–671
2. Amendt BA, Moon A, Teel L, Rhead WJ 1988 Long-chain acyl-coenzyme A dehydrogenase deficiency: biochemical studies in fibroblasts from three patients. Pediatr Res 23:603–605
3. Hale DE, Stanley CA, Coates PM 1990 The long-chain acyl-CoA dehydrogenase deficiency. In: Tanaka K, Coates PM (eds) Fatty Acid Oxidation: Clinical, Biochemical and Molecular Aspects. Alan R Liss Inc., New York, pp 303–311
4. Coates PM, Tanaka K 1992 Molecular basis of mitochondrial fatty acid oxidation defects. J Lipid Res 33:1099–1110
5. Matsubara Y, Narisawa K, Tada K 1992 Medium-chain acyl-CoA dehydrogenase deficiency: molecular aspects. Eur J Pediatr 151:154–159
6. Hale DE, Thorpe C, Braat K, Wright JH, Roe CR, Coates PM, Hashimoto T, Glasgow AM 1990 The L-3-hydroxyacyl-CoA dehydrogenase deficiency. In: Tanaka K, Coates PM (eds) Fatty Acid Oxidation: Clinical, Biochemical and Molecular Aspects. Alan R Liss Inc., New York, pp 503–510
7. Tanaka K, Yokota I, Coates PM, Strauss AW, Kelly DP, Zhang Z, Gregersen N, Andresen BS, Matsubara Y, Curtis D, Chen Y-T 1992 Mutations in the medium chain acyl-CoA dehydrogenase (MCAD) gene. Human Mutation 1:271–279
8. Ikeda Y, Okamura-Ikeda K, Tanaka K 1985 Purification and characterization of short-chain, medium-chain, and long-chain acyl-CoA dehydrogenases from rat liver mitochondria. Isolation of the holo- and apoenzymes and conversion of the apoenzyme to the holoenzyme. J Biol Chem 260:1311–1325
9. Hale DE, Stanley CA, Coates PM 1990 Genetic defects of acyl-CoA dehydrogenases: studies using an electron transfer flavoprotein reduction assay. In: Tanaka K, Coates PM (eds) Fatty Acid Oxidation: Clinical, Biochemical, and Molecular Aspects. Alan R Liss Inc., New York, pp 333–348
10. Indo Y, Coates PM, Hale DE, Tanaka K 1991 Immunochemical characterization of variant long chain acyl-CoA dehydrogenase in cultured fibroblasts from nine patients with long chain acyl-CoA dehydrogenase deficiency. Pediatr Res 30:211–215
11. Vockley J, Parimoo B, Tanaka K 1991 Molecular characterization of four different classes of mutations in the isovaleryl-CoA dehydrogenase gene responsible for isovaleric acidemia. Am J Hum Genet 49:147–157
12. Fukao T, Yamaguchi S, Kano M, Orii T, Osumi T, Hashimoto T 1992 Molecular basis of 3-ketothiolase deficiency. In: Coates PM, Tanaka K (eds) New Developments in Fatty Acid Oxidation. Wiley-Liss, New York, pp 573–581
13. Izai K, Uchida Y, Orii T, Yamamoto S, Hashimoto T 1992 Novel fatty acid β-oxidation enzymes in rat liver mitochondria. I. Purification and properties of very-long-chain acyl-coenzyme A dehydrogenase. J Biol Chem 267:1027–1033
14. Yokota I, Indo Y, Coates PM, Tanaka K 1990 Molecular basis of medium chain acyl-CoA dehydrogenase deficiency. An A to G transition at position 985 that causes a lysine-304 to glutamate substitution in the mature protein is the single prevalent mutation. J Clin Invest 86:1000–1003
15. Sanger F, Nicklen S, Coulson AR 1976 DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74:5463–5467
16. Lehman TC, Hale DE, Bhala A, Thorpe C 1990 An acyl-coenzyme A dehydrogenase assay utilizing the ferricenium ion. Anal Biochem 186:280–284
17. Indo Y, Yang-Feng T, Glassberg R, Tanaka K 1991 Molecular cloning and nucleotide sequence of cDNAs encoding human long-chain acyl-CoA dehydrogenase and assignment of the location of its gene (*ACADL*) to chromosome 2. Genomics 11:609–620
18. Matsubara Y, Indo Y, Naito E, Ozasa H, Glassberg R, Vockley J, Ikeda Y, Kraus J, Tanaka K 1989 Molecular cloning and nucleotide sequence of cDNAs encoding the precursors of rat long chain acyl-coenzyme A, short chain acyl-coenzyme A, and isovaleryl-coenzyme A dehydrogenases. Sequence homology of four enzymes of the acyl-CoA dehydrogenase family. J Biol Chem 264:16321–16331
19. Bertrand C, Largilliere C, Zabot MT, Mathieu M, Vianey-Saban C 1993 Very long chain acyl-CoA dehydrogenase deficiency: identification of a new inborn error of mitochondrial fatty acid oxidation in fibroblasts. Biochim Biophys Acta 1180:327–329
20. Aoyama T, Uchida Y, Kelley RI, Marble M, Hofman K, Tonsgard JH, Rhead WJ, Hashimoto T 1993 A novel disease with deficiency of mitochondrial very-long-chain acyl-CoA dehydrogenase. Biochem Biophys Res Commun 191:1369–1372