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Immunochemical and Molecular Analysis of Medium-Chain Acyl CoA Dehydrogenase Deficiency

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Summary Medium-chain acyl CoA dehydrogenase (MCAD) (acyl-CoA: (acceptor) 2,3-oxidoreductase, EC 1.3.99.3) deficiency in two patients, MV and AH, was examined by use of an anti-MCAD antibody and the cDNA for the enzyme. No MCAD protein was detected by immunoblot analysis in the fibroblast extract from the first patient MV, while it was present, but not catalytically active in the second patient AH. In order to clarify the molecular mechanism of these deficiencies, a cDNA encoding MCAD was isolated from a human placenta cDNA library. The cDNA contained 1,263 nucleotides of the coding region, 64 nucleotides of the 5′-noncoding region, and 686 nucleotides of the 3′-noncoding region. The level of mRNA for MCAD in the patients was examined by RNA blot analysis with the cDNA as probe, and the results indicate that the patient MV also had the mRNA and that the level of the mRNA in both patients was almost the same as that of the control subject. Thus it seems that the deficiency in the patients is due to a point mutation(s) and that the position of the mutation(s) in the gene of patient MV is different from that of patient AH.

Key Words: medium chain acyl CoA dehydrogenase, MCAD deficiency, cDNA, mRNA level

Medium-chain acyl CoA dehydrogenase (MCAD) deficiency, which is an inborn error of mitochondrial fatty acid metabolism, has been reported in more

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than 85 patients [1, 2] since its first identification in 1982 [3]. The clinical signs of MCAD deficiency are variable. It has been described that in patients sudden infant death syndrome, recurrent Reye's syndrome, and episodic hypoglycemic coma develop after fasting with absence of frank ketosis [2]. The causes of the phenotypic heterogeneity in MCAD deficiency are still unknown. [^{35}S]Methionine labelling/immunoprecipitation analysis suggested that MCAD deficiency in 13 patients was due to a point mutation(s) of the gene that affected the catalytic activity of the enzyme [4]. Recently, partial cDNAs encoding MCAD have been isolated from human liver [5, 6] and placenta [6] cDNA libraries, and a cDNA for the enzyme has also been obtained from a rat liver cDNA library [7, 8].

In the present study, we isolated a full-length cDNA encoding MCAD from a human placenta cDNA library. By using this cDNA and an anti-MCAD antibody, we analyzed the mode of MCAD deficiency in two patients and found that both patients have mRNA for the enzyme but that one of them does not contain enzyme protein, indicating that they have different kinds of MCAD deficiency.

MATERIALS AND METHODS

Materials. Enzymes for DNA manipulation, M13 sequencing kit, and Random Primer DNA Labeling Kit were purchased from Takara Shuzo Co., Ltd., Kyoto; horseradish peroxidase-conjugated goat anti-rabbit IgG, from Bio-Rad Laboratories, Richmond, CA; dCTP and diaminobenzidine (DAB) enhancement kit, from Amersham International, Buckinghamshire, UK; and acyl CoAs, from Sigma Chemical Company, St. Louis, MO. Anti-pig kidney MCAD antiserum was prepared as described previously [9].

Cell culture. Skin fibroblasts were taken from patients referred to as AH and MV, and those taken from a normal individual were kindly donated by Dr. Y. Nozawa of Gifu University. Details of the clinical features of patient MV have been reported elsewhere [10]. Fibroblasts were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. Monolayers were harvested by trypsinization, washed three times with phosphate-buffered salt solution (without magnesium and calcium), and used for the experiments.

Preparation of extracts of fibroblasts. Fibroblasts (2×10^6 cells) were suspended in 100 μl of 10 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA. The suspension was sonicated for 30 s at 10 watts in an ice bath and centrifuged at $10{,}000\times g$ for 10 min at 4°C. The supernatant was used as the extract.

Assay of MCAD activity. Fifty microliters of the extract of fibroblasts was incubated with 1 μl of the anti-MCAD antiserum or normal rabbit serum at 4°C overnight. Fifty microliters of Protein A-Sepharose suspension (100 mg/ml) was then added to the mixture and incubated for 2 h at 4°C. After centrifugation for 1 min at $800\times g$, MCAD activity in the supernatant was assayed by the phenazine methosulfate (1.5 mM)/dichlorophenolindophenol (DCIP) (0.048 mM) method

[11]. Net MCAD activity was expressed as the value suppressed by the addition of the antibody.

Isolation and sequencing of cDNA for human placenta MCAD. cDNAs for MCAD were isolated by immunoscreening of a human placenta cDNA library constructed in λgt 11 (Clontech Laboratories, Inc., Palo Alto, CA) according to the method of Young and Davis [12], as described previously [8]. *Eco*RI fragments of the cDNA were ligated into the *Eco*RI site of pUC 19, and deletion mutants of a subcloned pUC 19 plasmid were prepared by digestion with exonuclease III and mung bean nuclease [13]. The DNA sequence of the deleted inserts was determined by the dideoxy chain termination method [14]. Analysis of sequence data was performed by use of the GENETYX programs (SDC, Tokyo).

RNA blot analysis. RNAs were isolated from fibroblasts of the patients AH and MV and the control subject by the guanidinium thiocyanate extraction method described by Chirgwin *et al.* [15]. The MCAD probe, a *Hap*II-*Hin*dIII fragment of the cDNA, was radiolabeled with a Random Primer DNA Labeling Kit. For RNA dot blot analysis, the RNA samples were dissolved in 50% formamide and 6% formaldehyde, incubated for 1 h at 50°C to denature the RNA, and then chilled in ice. After the samples had been spotted directly onto a GeneScreen *Plus* membrane (DuPont Co., Boston, MA), the membrane was incubated overnight at 60°C with the ^{32}P-labeled MCAD probe in 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), and 1 M NaCl. After washing with 2×SSC (1×SSC, 0.15 M NaCl-0.015 M sodium citrate) containing 1% SDS four times at 25°C, the membrane was exposed to an X-ray film for 2–6 days at −80°C.

Immunoblotting analysis. The fibroblast extracts (10 μl) were subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) followed by immunoblotting [16]. The membrane was stained with 50 mM Tris-HCl buffer (pH 7.5) containing 2 mg/ml of DAB tetrahydrochloride and 0.02% H_2O_2. Then the membrane was washed three times for 5 min each time with 10 mM Tris-HCl (pH 7.5) containing 0.15 M NaCl and then dried. DAB on the membrane was amplified by use of a DAB enhancement kit.

Protein concentration. Protein content was determined according to the method of Smith *et al.* [17] with bovine serum albumin used as the standard.

RESULTS AND DISCUSSION

MCAD activity in fibroblasts from patients

Dehydrogenase activities toward acyl CoA compounds were measured in fibroblasts from MCAD deficient patients. The enzyme activity measured with octanoyl CoA as substrate in extracts of fibroblasts from the MCAD-deficient patients was 10–15 units/mg protein. However, this activity was not abolished by treatment of the extracts with the anti-MCAD antiserum, indicating that the observed activity cannot be ascribed to MCAD. In contrast, the enzyme activity of the fibroblasts of the normal subject was 15–22 units/mg protein, and was suppres-

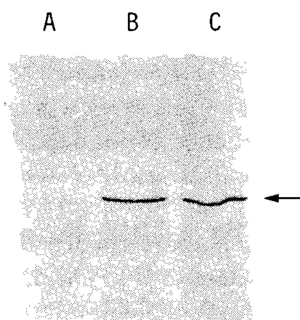

Fig. 1 Immunoblot analysis for extracts of cultured fibroblasts. Extracts (10 μl) were subjected to 12% SDS-PAGE. Proteins were transferred to a nitrocellulose membrane, and the membrane was then immunostained with the anti-MCAD antibody. Lane A, extract of fibroblasts from patient MV; lane B, that from patient AH; lane C, that from the control.

sed by the antiserum to the same level as that of the cells of the patients, indicating the net MCAD activity in the cells of the control subject to be 5–7 units/mg protein. The apparent MCAD activity found in the fibroblasts of the patients should be attributed to another enzyme, viz., long-chain acyl CoA dehydrogenase (LCAD), since LCAD is known to be also active toward octanoyl CoA. In fact, LCAD measured with pentadecanoyl CoA as substrate was in the same range (10–20 units/mg protein) in the fibroblasts of the normal subject as in those of the patients. Short-chain acyl CoA dehydrogenase (SCAD) activity in the fibroblasts of the patients measured with butyryl CoA as substrate was 4–9 units/mg protein, which is 50–60% of the control level. This decrease can be ascribed to the fact that MCAD has a substantial activity toward short-chain acyl CoAs [18, 19].

The higher MCAD activity in our data as compared with that given in other reports [2, 3, 10] might be due to different assay conditions.

Immunoblot analysis

Figure 1 shows the immunoblot analysis of MCAD protein for extracts of the fibroblasts from the patients and the control. MCAD proteins were detectable in the control (lane C) and patient AH fibroblasts (lane B). The molecular weight of the protein in patient AH was the same as that in the control, suggesting that the deficiency in patient AH is due to a point mutation(s) affecting the enzymatic activity. In contrast, the enzyme protein was not detected in patient MV fibroblasts (lane A).

Isolation and sequencing of cDNA for human placenta MCAD

For DNA and RNA hybridization analyses, we isolated cDNAs encoding MCAD from a human placenta cDNA library. One (HMCAD-5) of them contained 64 nucleotides of the 5′ noncoding region, 1,263 nucleotides of the coding

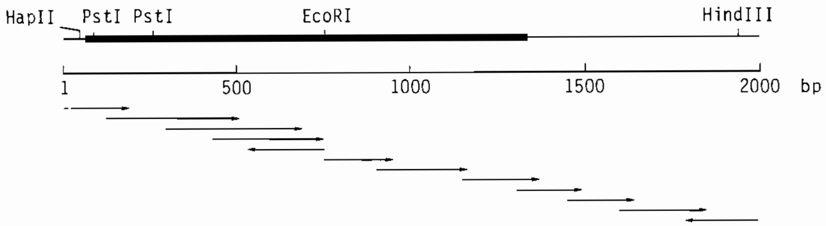

Fig. 2. Partial restriction map and sequence strategy of cDNA insert from human placenta MCAD gene. Closed region denotes the coding region of the MCAD. The arrows represent the direction and length of the sequence runs.

region, and 686 nucleotides of the 3′ noncoding region, as shown in Fig. 2. Figure 3 shows the nucleotide sequence of the cDNA (HMCAD-5). The first ATG codon was located at nucleotides 11–13, but the terminal codon appeared at nucleotides 23–25. This reading frame codes 4 amino acid residues. cDNAs in which the first initiation codon is followed by a stop codon upstream from the second initiation codon have been reported for porcine D-amino acid oxidase [20] and for rat 3-oxoacyl CoA thiolase [21]. The second ATG codon was located at nucleotides 65–67 and the terminal codon, TAA, at nucleotides 1,328–1,330. The 1,263-nucleotide reading frame was completely identical with that of Kelly *et al.* [6], although a minute difference was found in 5′ noncoding region.

RNA blot analysis

In order to estimate the level of mRNA for MCAD in the patients, we carried out RNA dot blot analysis with the ^{32}P-labeled *Hap*II-*Hin*dIII fragment of the cDNA as a hybridization probe. As shown in Fig. 4, the fibroblasts from both patients produced the same level of mRNA as that of the control.

All these results indicate that patient MV is different from patient AH in the mode of the deficiency. Although both patients have mRNA for MCAD at the same level as that of the control, patient MV does not possess immunologically detectable MCAD protein. This finding indicates that in patient MV the enzyme protein is not produced or, if produced, is somehow aberrant. If the protein is not synthesized at all in this patient, the mutation would seem to occur in the 5′ noncoding region of the mRNA or in the coding region containing the initiation codon, resulting in a failure of translation. If the protein is synthesized, it would seem that patient MV synthesizes an abnormal precursor of MCAD which is susceptible to digestion by protease(s) or an unstable enzyme. In a recent preliminary report [22], a patient with MCAD deficiency had an abnormal 5′ noncoding region of the gene, which resulted in a failure of mitochondrial uptake of the precursor. However, in the case of our patient AH, the point mutation(s) might have occurred in the coding region of the gene, resulting in the production of a catalytically inactive enzyme. The detailed structures of the mRNAs from these

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          GGGCGGGAGTATGTCAAGGCCGTGACCCGTGTATTATTGTCCGAGTGGCCGGAACGGAGCCAAC          64

ATGGCAGCGGGGTTCGGGCGATGCTGCAGGGTCCTGAGAAGTATTTCTCGTTTTCATTGGAGATCACAGCATACA        139
MetAlaAlaGlyPheGlyArgCysCysArgValLeuArgSerIleSerArgPheHisTrpArgSerGlnHisThr

AAAGCCAATCGACAACGTGAACCAGGATTAGGATTTAGTTTTGAGTTCACCGAACAGCAGAAAGAATTTCAAGCT        214
LysAlaAsnArgGlnArgGluProGlyLeuGlyPheSerPheGluPheThrGluGlnGlnLysGluPheGlnAla

ACTGCTCGTAAATTTGCCAGAGAGGAAATCATCCCAGTGGCTGCAGAATATGATAAAACTGGTGAATATCCAGTC        289
ThrAlaArgLysPheAlaArgGluGluIleIleProValAlaAlaGluTyrAspLysThrGlyGluTyrProVal

CCCCTAATTAGAAGAGCCTGGGAACTTGGTTTAATGAACACACACATTCCAGAGAACTGTGGAGGTCTTGGACTT        304
ProLeuIleArgArgAlaTrpGluLeuGlyLeuMetAsnThrHisIleProGluAsnCysGlyGlyLeuGlyLeu

GGAACTTTTGATGCTTGTTTAATTAGTGAAGAATTGGCTTATGGATGTACAGGGGTTCAGACTGCTATTGAAGGA        439
GlyThrPheAspAlaCysLeuIleSerGluGluLeuAlaTyrGlyCysThrGlyValGlnThrAlaIleGluGly

AATTCTTTGGGGCAAATGCCTATTATTATTGCTGGAAATGATCAACAAAAGAAGAAGTATTTGGGGAGAATGACT        514
AsnSerLeuGlyGlnMetProIleIleIleAlaGlyAsnAspGlnGlnLysLysLysTyrLeuGlyArgMetThr

GAGGAGCCATTGATGTGTGCTTATTGTGTAACAGAACCTGGAGCAGGCTCTGATGTAGCTGGTATAAAGACCAAA        589
GluGluProLeuMetCysAlaTyrCysValThrGluProGlyAlaGlySerAspValAlaGlyIleLysThrLys

GCAGAAAAGAAAGGAGATGAGTATATTATTAATGGTCAGAAGATGTGGATAACCAACGGAGGAAAAGCTAATTGG        604
AlaGluLysLysGlyAspGluTyrIleIleAsnGlyGlnLysMetTrpIleThrAsnGlyGlyLysAlaAsnTrp

TATTTTTTATTGGCACGTTCTGATCCAGATCCTAAAGCTCCTGCTAATAAAGCCTTTACTGGATTCATTGTGGAA        739
TyrPheLeuLeuAlaArgSerAspProAspProLysAlaProAlaAsnLysAlaPheThrGlyPheIleValGlu

GCAGATACCCCAGGAATTCAGATTGGGAGAAAGGAATTAAACATGGGCCAGCGATGTTCAGATACTAGAGGAATT        814
AlaAspThrProGlyIleGlnIleGlyArgLysGluLeuAsnMetGlyGlnArgCysSerAspThrArgGlyIle

GTCTTCGAAGATGTGAAAGTGCCTAAAGAAAATGTTTTAATTGGTGACGGAGCTGGTTTCAAAGTTGCAATGGGA        889
ValPheGluAspValLysValProLysGluAsnValLeuIleGlyAspGlyAlaGlyPheLysValAlaMetGly

GCTTTTGATAAAACCAGACCTGTAGTAGCTGCTGGTGCTGTTGGATTAGCACAAAGAGCTTTGGATGAAGCTACC        964
AlaPheAspLysThrArgProValValAlaAlaGlyAlaValGlyLeuAlaGlnArgAlaLeuAspGluAlaThr

AAGTATGCCCTGGAAAGGAAAACTTTCGGAAAGCTACTTGTAGAGCACCAAGCAATATCATTTATGCTGGCTGAA       1039
LysTyrAlaLeuGluArgLysThrPheGlyLysLeuLeuValGluHisGlnAlaIleSerPheMetLeuAlaGlu

ATGGCAATGAAAGTTGAACTAGCTAGAATGAGTTACCAGAGAGCAGCTTGGGAGGTTGATTCTGGTCGTCGAAAT       1114
MetAlaMetLysValGluLeuAlaArgMetSerTyrGlnArgAlaAlaTrpGluValAspSerGlyArgArgAsn

ACCTATTATGCTTCTATTGCAAAGGCATTTGCTGGAGATATTGCAAATCAGTTAGCTACTGATGCTGTGCAGATA       1189
ThrTyrTyrAlaSerIleAlaLysAlaPheAlaGlyAspIleAlaAsnGlnLeuAlaThrAspAlaValGlnIle

CTTGGAGGCAATGGATTTAATACAGAATATCCTGTAGAAAAACTAATGAGGGATGCCAAAATCTATCAGATTTAT       1264
LeuGlyGlyAsnGlyPheAsnThrGluTyrProValGluLysLeuMetArgAspAlaLysIleTyrGlnIleTyr

GAAGGTACTTCACAAATTCAAAGACTTATTGTAGCCCGTGAACACATTGACAAGTACAAAAATTAAAAAAATTAC       1339
GluGlyThrSerGlnIleGlnArgLeuIleValAlaArgGluHisIleAspLysTyrLysAsn***

TGTAGAAATATTGAATAACTAGAACACAAGCCACTGTTTCAGCTCCAGAAAAAAGAAAGGGCTTTAACGTTTTTT       1414

CCAGTGAAAACAAATCCTCTTATATTAAATCTAAGCAACTGCTTATTATAGTAGTTTATACTTTTGCTTAACTCT       1489

GTTATGTCTCTTAAGCAGGTTTGGTTTTTATTAAAATGATGTGTTTTCTTTAGTACCACTTTACTTGAATTACAT       1564

TAACCTAGAAAACTACATAGGTTATTTTGATCTCTTAAGATTAATGTAGCAGAAATTTCTTGGAATTTTATTTTT       1639

GTAATGACAGAAAAGTGGGCTTAGAAAGTATTCAAGATGTTACAAAATTTACATTTAGAAAATATTGTAGTATTT       1714

GAATACTGTCAACTTGACAGTAACTTTGTAGACTTAATGGTATTATTAAAGTTCTTTTTATTGCAGTTTGGAAAG       1789

CATTTGTGAAACTTTCTGTTTGGCACAGAAACAGTCAAAATTTTGACATTGATATTCTCCTATTTTACAGCTACA       1864

AGAACTTTCTTGAAAATCTTATTTAATTCTGAGCCCATATTTCACTTACCTTATTTAAAATAAATCAATAAAGCT       1939

TGCCTTAAATTATTTTTATATGACTGTTGGTCTCTAGGTAGCCTTTGGTCTATTGTACACAACCTCATTTCCCC        2013
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Fig. 3. Nucleotide sequence of cDNA and deduced amino acid sequence of human placenta MCAD. ***Terminal codon.

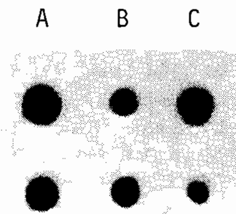

Fig. 4. Dot blot analysis of mRNA for MCAD from fibroblasts. The blot was hybridized with labeled MCAD cDNA, followed by washing and autoradiography. The blot was exposed to the film for 1 day. Lane A, mRNA from the control fibroblasts; lane B, that from patient AH; lane C, that from patient MV. Amount of mRNA: upper dots, 3.5 μg; and lower dots, 1.75 μg.

patients are currently under investigation in our laboratory.

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