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In vitro probe acylcarnitine profiling assay using cultured fibroblasts and electrospray ionization tandem mass spectrometry predicts severity of patients with glutaric aciduria type 2☆

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ABSTRACT

Glutaric aciduria type 2 (multiple acyl-CoA dehydrogenase deficiency, MAD) is a multiple defect of mitochondrial acyl-CoA dehydrogenases due to a deficiency of electron transfer flavoprotein (ETF) or ETF dehydrogenase. The clinical spectrum are relatively wide from the neonatal onset, severe form (MAD-S) to the late-onset, milder form (MAD-M). In the present study, we determined whether the in vitro probe acylcarnitine assay using cultured fibroblasts and electrospray ionization tandem mass spectrometry (MS/MS) can evaluate their clinical severity or not. Incubation of cells from MAD-S patients with palmitic acid showed large increase in palmitoylcarnitine (C16), whereas the downstream acylcarnitines; C14, C12, C10 or C8 as well as C2, were extremely low. In contrast, accumulation of C16 was smaller while the amount of downstream metabolites was higher in fibroblasts from MAD-M compared to MAD-S. The ratio of C16/C14, C16/C12, or C16/C10, in the culture medium was significantly higher in MAD-S compared with that in MAD-M. Loading octanoic acid or myristic acid led to a significant elevation in C8 or C12, respectively in MAD-S, while their effects were less pronounced in MAD-M. In conclusion, it is possible to distinguish MAD-S and MAD-M by in vitro probe acylcarnitine profiling assay with various fatty acids as substrates. This strategy may be applicable for other metabolic disorders.

1. Introduction

Fatty acid oxidation disorders (FAODs) potentially cause energy crises that are triggered by stress brought on by starvation or infection, and manifest themselves through nonketotic hypoglycemia, acute encephalopathy, or symptoms similar to those of Reye's syndrome. Among FAODs, medium-chain acyl-CoA dehydrogenase deficiency (MCAD-def) is most common among Caucasian [1], whereas very long-chain acyl-CoA dehydrogenase deficiency (VLCAD-def) and carnitine palmitoyl-CoA transferase 2 deficiency (CPT2-def) are common in Japanese, followed by glutaric aciduria type 2 (multiple acyl-CoA dehydrogenase deficiency: MAD) [2].

In MAD, multiple mitochondrial FAD-dependent dehydrogenases are impaired due to a defect in α- or β-subunits of electron transfer flavoprotein (ETF-α and ETF-β; OMIM 608053 and 130410 respectively) or ETF dehydrogenase (ETF-DH; OMIM 231675) [3,4]. The clinical forms of MAD include the neonatal-onset form (severe form: MAD-S) and the late-onset form (milder form: MAD-M). MAD-S occurs during the neonatal period, and is fatal. MAD-M often becomes symptomatic after infancy, and has episodic symptoms of hypotonia, tachypnea, skeletal muscle symptoms such as myalgia or rhabdomyolysis, and biochemical abnormalities including liver dysfunction, hypoglycemia, or hyperammonemia [5]. These 2 clinical forms can be sharply separated. Biochemical diagnosis is made by blood acylcarnitine analysis using electrospray ionization tandem mass spectrometry (MS/MS) that measures increases in C4 to C18 acylcarnitine, or through urinary organic acid analysis using gas chromatography mass spectrometry (GC/MS) that detects an increase of ethylmalonic acid, glutaric acid, isovalerylglycine, or dicarboxylic acids. However, it is not always feasible to make a definitive diagnosis of MAD by GC/MS or MS/MS, especially during the stable phase of MAD-M [6]. While genetic diagnosis may represent alternative strategy to make accurate diagnosis of MAD, the genotype/phenotype correlation is not clear.

The usefulness of in vitro probe acylcarnitine assay using cultured fibroblasts and MS/MS for the diagnosis of many FAODs has recently been reported [7]. Other reports showed that severity are associated with acylcarnitine prolife in deficiencies of MCAD, VLCAD, CPT2 and long-chain L-3-hydroxyacyl-CoA dehydrogenase (LCHAD) [8–12]. Subsequent reports also demonstrated that it

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is feasible to distinguish deficiency with carnitine–acylcarnitine translocase (CACT) from CPT2 as well as LCHAD from MTP deficiency [13,14]. Herein, we investigated if the severity of MAD can be determined by in vitro probe acylcarnitine assay.

2. Materials and methods

2.1. Patients with MAD

The 14 patients with MAD were studied. MAD was biochemically diagnosed using GC/MS and/or MS/MS, and further conclusively diagnosed using gene analysis and immunoblotting. Their clinical features were previously described [15]. They included four cases with ETF-α deficiency, two subjects with ETF-β deficiency, and eight patients with ETF-DH deficiency. The clinical phenotype included 3 cases with MAD-S, 10 subjects with MAD-M, and 1 asymptomatic child who was detected before disease onset by the neonatal screening. No obvious correlation between clinical severity and the specific defective enzyme was seen. All 3 cases with MAD-S died during the neonatal period. Seven of the 10 cases of MAD-M developed the disease during infancy with nonketotic hypoglycemia, acute encephalopathy, or Reye-like syndrome. The remaining 3 cases with MAD-M showed muscle symptoms such as myalgia and rhabdomyolysis or occasional general fatigue in later childhood or later.

2.2. Cultured fibroblasts

Fibroblasts from 14 Japanese patients with MAD were used. We also analyzed cultured fibroblasts from 4 healthy controls, one case each of MCAD-def, VLCAD-def, CPT2-def, or mitochondrial trifunctional protein deficiency (MTP-def) and primary carnitine deficiency (PCD) to validate the specificity of our in vitro probe acylcarnitine assay using cultured fibroblasts with MS/MS and to compare with MAD samples. Cells were cultured in modified eagle medium (MEM; Nissui) with 2 mM L-glutamine, 10% BSA (Sigma) and 1% penicillin/streptomycin until achieving confluency at 37 °C and 5% of CO_2.

2.3. In vitro probe acylcarnitine assay of fibroblasts using MS/MS

An in vitro probe assay was performed as described by J.G. Okun et al. with some minor modification [8]. Briefly, the cultured fibroblasts were seeded into a 6-well plate, and washed twice with PBS when they reached confluent. Cells were subsequently cultured in MEM loaded with 0.2 mM palmitic acid. In some experiments, palmitic acid was replaced with either octanoic acid or myristic acid. After incubating for 96 h, the culture medium was collected to analyze acylcarnitines by MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA). Statistic analysis was performed using Mann–Whitney's *U*-test.

3. Results

3.1. Acylcarnitine in cultured fibroblasts with MAD-S and MAD-M shows distinct profile

In the pilot experiments, we confirmed that our in vitro acylcarnitine probe assay demonstrates specific metabolic profile for

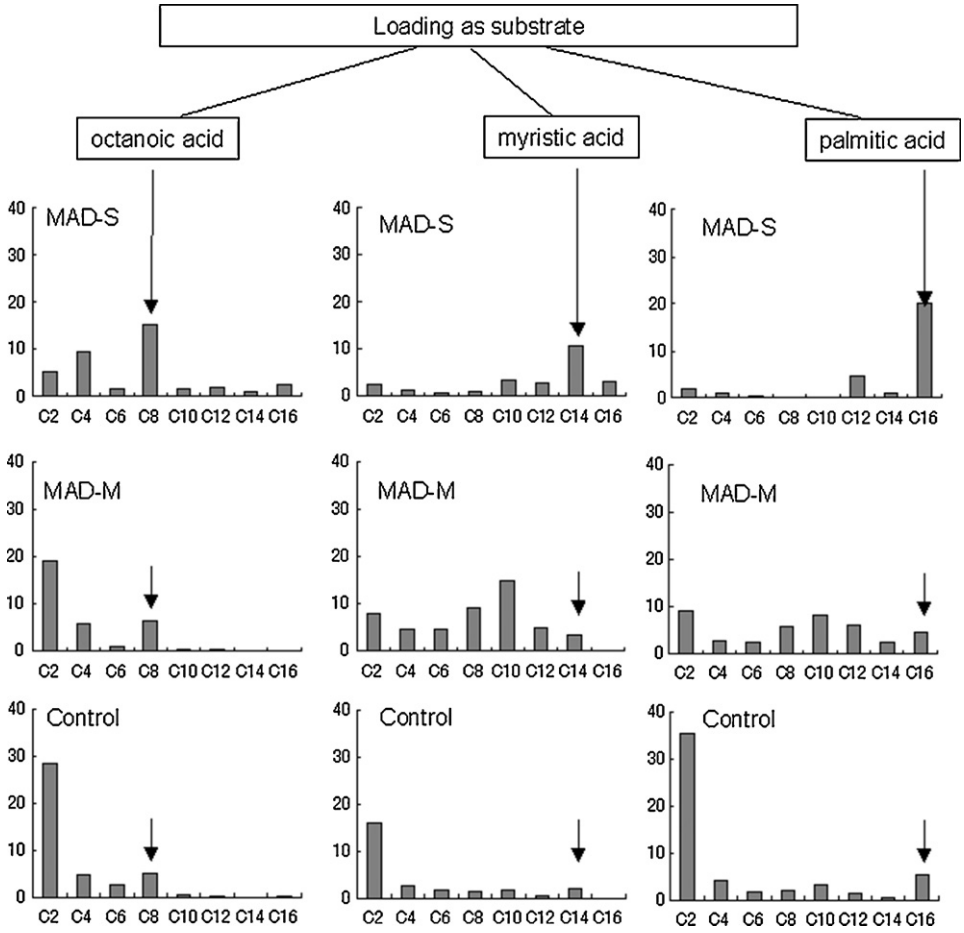

Fig. 1. The acylcarnitine profiles of fibroblasts from MAD-S and MAD-M. Arrows indicate loaded fatty acids; octanoic acid, myristic acid or palmitic acid. The *Y* axis represents values of acylcarnitines expressed as nmol/mg protein/96 h.

Table 1
Acylcarnitine profiles in culture medium of fibroblasts from MAD patients incubated with unlabelled palmitic acid for 96 h.

Clinical type	Acylcarnitine ratio; mean ± SEM				
	Palmitic acid				
	C16/C14	C16/C12	C16/C10	C16/C8	C16/C2
MAD-S (n = 3)	30.1 ± 16.9[*,†] (4.08–61.8)	6.02 ± 1.08[*,†] (3.85–7.12)	59.0 ± 24.6[*,†] (9.85–84.0)	69.1 ± 17.9[*,†] (33.5–89.5)	5.28 ± 1.75[*,†] (1.81–7.48)
MAD-M (n = 10)	4.33 ± 0.83 (1.52–10.0)	0.97 ± 0.17 (0.31–2.11)	1.05 ± 0.58 (0.22–6.20)	1.19 ± 0.70 (0.00–7.38)	0.73 ± 0.43[**] (0.08–4.57)
Asymptomatic (n = 1)	1.63	0.93	0.79	1.06	1.55
Control (n = 4)	7.89 ± 1.10 (5.89–10.8)	1.95 ± 0.64 (0.78–3.73)	0.89 ± 0.33 (0.53–1.89)	1.02 ± 0.16 (0.68–1.46)	0.13 ± 0.03 (0.05–0.23)

Abbreviations: MAD-S and MAD-M: severe and milder forms of MAD, respectively; asymptomatic: a case detected in the neonatal screening.
[*] P = 0.01 compared MAD-S with MAD-M.
[**] P = 0.04 compared MAD-M with control.
[†] P < 0.05 compared MAD-S with control.

Table 2
Acylcarnitine profiles in culture medium of fibroblasts from MAD patients incubated with unlabelled octanoic acid or myristic acid for 96 h.

Clinical type	Acylcarnitine ratio: mean ± SEM					
	Octanoic acid			Myristic acid		
	C8/C6	C8/C4	C8/C2	C14/C12	C14/C10	C14/C2
MAD-S (n = 3)	6.67 ± 3.63 (2.06–13.8)	1.20 ± 0.24 (0.81–1.63)	3.01 ± 1.92[*] (1.02–6.87)	3.66	3.03	4.42
MAD-M (n = 10)	2.73 ± 0.42 (0.88–4.56)	0.93 ± 0.14 (0.31–1.85)	0.73 ± 0.15[**] (0.14–1.38)	0.72	0.24	0.45
Asymptomatic (n = 1)	4.58	1.46	2.15	–	–	–
Control (n = 4)	2.10 ± 0.91 (0.69–4.71)	0.80 ± 0.25 (0.40–1.51)	0.15 ± 0.04 (0.08–0.24)	2.55	1.08	0.14

[*] P < 0.05 compared MAD-S with control.
[**] P < 0.05 compared MAD-M with control.

different FAOD (data not shown). Next, we determined if the acylcarnitine profiles can differentiate the clinical severity of MAD. When palmitic acid was loaded, a substantial accumulation of C16 was observed in the culture medium of cells with MAD-S, whereas the downstream C14, C12, C10, C8 or C2 did not show any increase. In contrast, C14, C12, C10 or C8 were elevated but C16 was lower in MAD-M compared to MAD-S (Fig. 1, right column). Therefore, the ratios of C16/C14, C16/C12, C16/C10, or C16/C8 were significantly higher in MAD-S than in MAD-M (Table 1). These findings suggest that oxidation of palmitic acid is inhibited in MAD-S, resulting in severely impaired production of acetyl-CoA.

Incubation with octanoic acid led to higher accumulation of C8-acylcarnitine in MAD-S cells compared to MAD-M (Fig. 1, left column). On the other hand, C2 was lower in MAD-S cells than that of MAD-M, as observed by palmitic acid. The ratio of C8/C2 showed significant increase in MAD-S compared to MAD-M (Table 2). Similarly, C8/C6 was higher in MAD-S (6.67 ± 3.63) than MAD-M (2.73 ± 0.42), though the difference was insignificant. Loading myristic acid to the MAD-S cells led to elevation of C14 acylcarnitine compared to control and MAD-M (Fig. 1, middle column). The ratios of C14/C12 and C14/C10 were also higher compared with those of MAD-M, as seen by palmitic or octanoic acid (Table 2). No difference was observed in the profile between infant and later childhood onset cases of MAD-M. The acylcarnitine profile was not associated with specific enzyme defect or gene; i.e. ETF-α, ETF-β and ETF-DH.

4. Discussion

MAD is a target disease of the neonatal screening using MS/MS. However, abnormalities on blood filter papers may not be detected in the stable condition of MAD-M or the presymptomatic stage, while the biochemical abnormalities are obviously observed in MAD-S and in the acute stage of MAD-M by GC/MS or MS/MS analyses. Actually, accurate biochemical diagnosis of MAD in presymptomatic stage is often difficult. Although genetic mutations of patients with MAD have been reported in various ethnic groups, almost all mutations do not seem to be associated with particular phenotype with a few exceptions [15], making it difficult to predict severity of the patients. The purpose of this study was to determine if the clinical severity in MAD can be evaluated using the in vitro probe acylcarnitine assay. Our data indicates that the in vitro probe acylcarnitine assay can clearly distinguish MAD-S from MAD-M.

An increase in C16 was observed exclusively in cells with MAD-S by loading palmitic acid, as opposed to a reduction in C14, C12, C10, C8 as well as C2. Loading octanoic acid or myristic acid also resulted in specific elevation of C8 or C14, respectively, in MAD-S. In contrast to MAD-S, the increase of C16 by palmitic acid was trivial in cells with MAD-M, whereas elevation of the downstream acylcarnitines C14, C12, C10 or C8 was larger compared to MAD-S. Similar to palmitic acid, such specific increase in C8 or C14 was barely detectable in cells with MAD-M upon incubation with octanotic or myristic acid, respectively. These results suggest that the milder enzyme deficiency in MAD-M allows the exogenous fatty acid substrates to process to some degree, resulting in elevation of downstream metabolites originated from loaded fatty acids. On the contrary, severe enzyme deficiency in MAD-S hampers to metabolize the loaded fatty acids to a shorter product, leading to a dramatic accumulation of the fatty acid corresponding to the substrates added. These hypotheses are consistent with significantly higher ratios between C16 and downstream acylcarnitines; i.e. C16/C14, C16/C12, C16/C12 or C16/C8, in cells with MAD-S compared to

MAD-M (Table 1). These findings strongly suggest that severity of MAD can be evaluated by quantitating the ratio between fatty acids loaded and the downstream metabolites.

Our results demonstrate that elevation of C16 by palmitic acid is one of the markers to characterize MAD-S. However, when compared with other FAODs, elevation of C16 is not specific to MAD-S, since palmitic acid also induced accumulation of C16 acylcarnitine in CPT2 deficiency without augmenting downstream metabolites (data not shown), which make acylcarnitine profile by palmitic acid in MAD-S look alike to CPT2 deficiency. However, a significant increase in C8 or C12 was observed by loading octanoic acid or myristic acid, respectively, in MAD-S, which was not observed in CPT-2 deficiency. This indicates that the enzyme activity for medium-chain fatty acids as well as long chain fatty acids is impaired in MAD-S, allowing MAD-S to be distinguished from CPT-2. However, cells from patients with respiratory chain defects may also show abnormalities similar to FAODs in the in vitro probe acylcarnitine assay [16,17], suggesting that the definitive diagnosis should be made in combination with acylcarnitine profiling and other laboratory tests, including genetic tests and enzymatic analysis.

In conclusion, our study indicates that the in vitro probe acylcarnitine assay using cultured fibroblasts loaded with various fatty acids allows us not just to distinguish MAD from other FAODs, but also clearly identify the severity of MAD. This strategy may be applied to evaluate the severity of the other metabolic diseases.

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