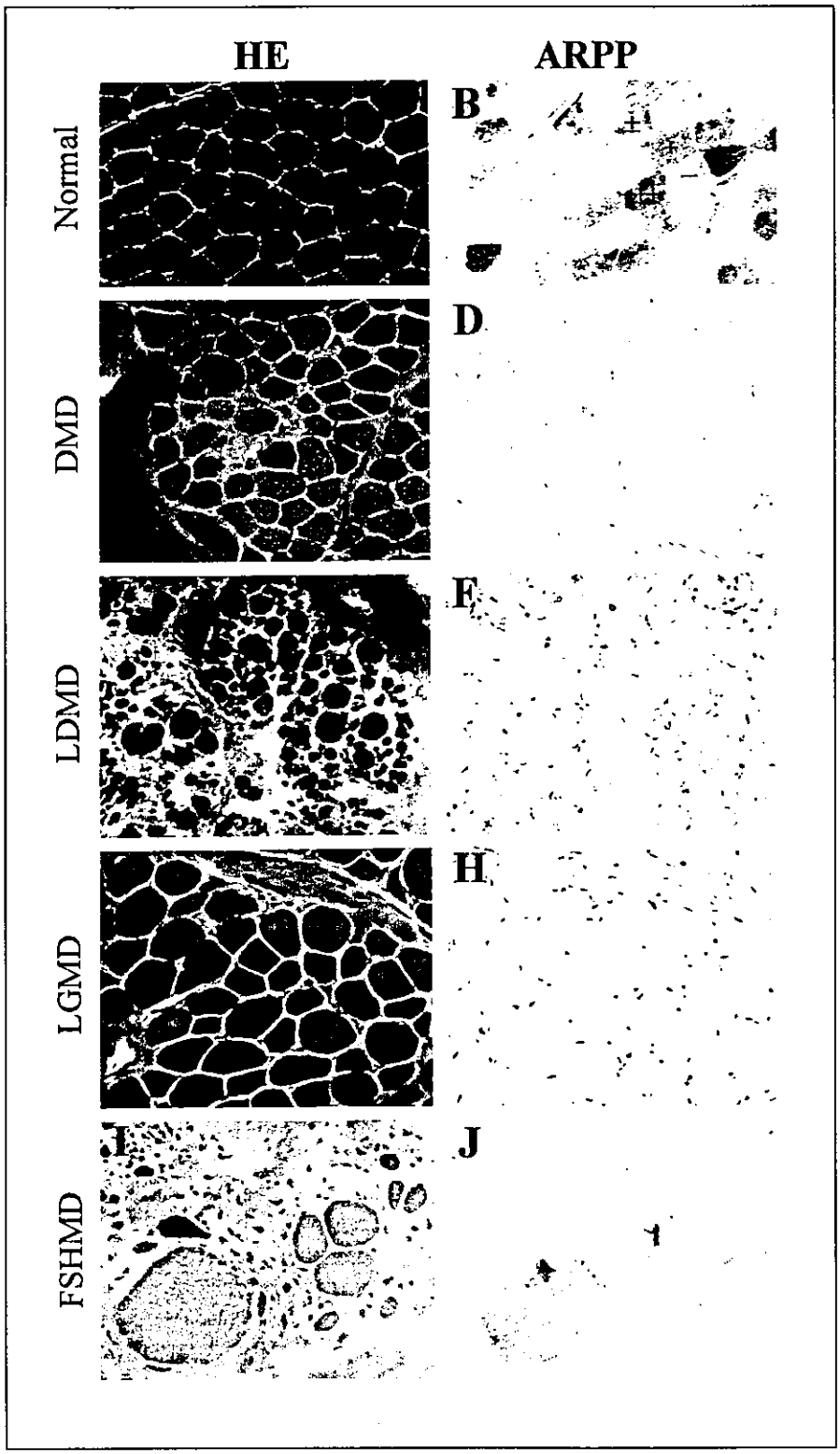

Fig. 1. Down-regulation of ARPP expression in patients with MDs. Frozen skeletal muscle tissue sections from MD patients [DMD (**C** and **D**), laminin-α_2-deficient MD (LDMD; **E** and **F**), LGMD (**G** and **H**) and FSHMD (**I** and **J**)] and a sample of normal control muscle were subjected to immunohistochemical staining with the α-ARPP(FL) Ab (**B**, **D**, **F**, **H** and **J**) and hematoxylin-eosin (HE) staining (**A**, **C**, **E**, **G** and **I**). Original magnification × 100. ARPP-positive fibers were arranged in a checkerboard-like pattern in normal muscle (**B**), whereas no such pattern was observed in muscle from MD patients and their weakly positive fibers were distributed uniformly (**D**, **F**, **H** and **J**). The intensities of ARPP immunoreactivity were classified as strongly positive (++), weakly positive (+), borderline (±) or negative (–).

Table 1. Expression of ARPP protein in congenital myopathies

Case No.	Histology	Age	Sex	Positive rates	Intensities of immunoreactivity
1	nemaline (S)	4 months	M	++	+
2	nemaline (B)	9 years	M	++	++
3	nemaline (B)	7 months	M	+	+
4	nemaline (S)	11 months	M	+++	++
5	myotubular (S)	5 months	M	+++	++
6	myotubular (S)	1 years	M	++	++
7	myotubular (S)	8 months	M	++	++
8	central core (B)	6 years	M	+++	±
9	central core (B)	34 years	M	+	+
10	central core (B)	8 months	M	+++	±
11	CFTD (B)	2 years	M	+++	+
12	CFTD (B)	28 years	M	++	+
13	CFTD (B)	10 months	F	++	+

S = Severe infantile form; B = benign congenital form. Positive rates were classified based on the proportions of the positive fibers: +++ = >90%; ++ = 90–50%; + = 50–5%; – = 5–0%. Intensities of immunoreactivity were classified into three groups: +++ = strong; ++ = weak; ± = borderline.

Semi-Quantitative Analysis of ARPP-Positive Myofibers

Intensities of immunoreactivities of ARPP-positive myofibers were classified into 4 groups. As shown in figure 1B, strongly positive (++), moderately positive (+), weakly positive (±) and negative (–) myofibers were diagnosed according to their intensities.

Results

Down-Regulation of ARPP Expression in MD Patients

To evaluate ARPP expression in skeletal muscle of patients with MDs, we subjected skeletal muscle biopsy samples from 15 MD patients to immunohistochemical analysis. Consistent with the results of our previous study [10], the ARPP-positive and negative myofibers in control normal muscle were arranged randomly in a checkerboard-like pattern (fig. 1B and 3A1–3). The ARPP expression levels of the ARPP-positive myofibers were not all the same. As shown in figure 1B, myofibers that were strongly positive, weakly positive and negative for ARPP were admixed in normal muscle, whereas in muscle from MD patients, the ARPP expression levels appeared to be lower than those in normal muscle (fig. 1D, F, H and J). Although the expression levels were low in most fibers, we noticed that hypertrophic myofibers, which were occasionally scattered among dystrophic myofibers, tended to express ARPP at comparable levels to normal control muscle fibers (fig. 1J).

ARPP Is Highly Induced in Myofibers of Congenital Myopathy Patients

To evaluate ARPP expression in myofibers of patients with congenital myopathies, we subjected muscle tissues from 13 patients with congenital myopathies, which included 4 with nemaline myopathy, 3 with myotubular myopathy, 3 with central core disease and 3 with CFTD (table 1), to immunohistochemical analysis. All 13 samples were ARPP positive (table 1).

Nemaline Myopathy. Although all 4 muscle samples were found to express ARPP, the proportions of ARPP-positive myofibers differed among the patients (cases 1–4 in table 1). Small atrophic myofibers tended to express ARPP (fig. 2A and B), and positive and negative myofibers were randomly admixed (fig. 2B). Interestingly, analysis of serial tissue sections by Gomori trichrome staining and immunohistochemical staining of ARPP showed that ARPP-positive myofibers tended to contain nemaline rods (fig. 2A and B). Conversely, ARPP-negative myofibers (arrowheads in fig. 2B) rarely contained these rods. This trend was also observed in the other 3 patients. Double-labeled immunohistochemistry with the α-ARPP(FL) and α-slow-MHC Abs revealed that, in patients with nemaline myopathy, the ARPP-positive myofibers coincided with the slow-MHC-positive myofibers (fig. 3B1–3). This suggests that the increased numbers of ARPP-positive myofibers may be associated with increased numbers of type 1 fibers.

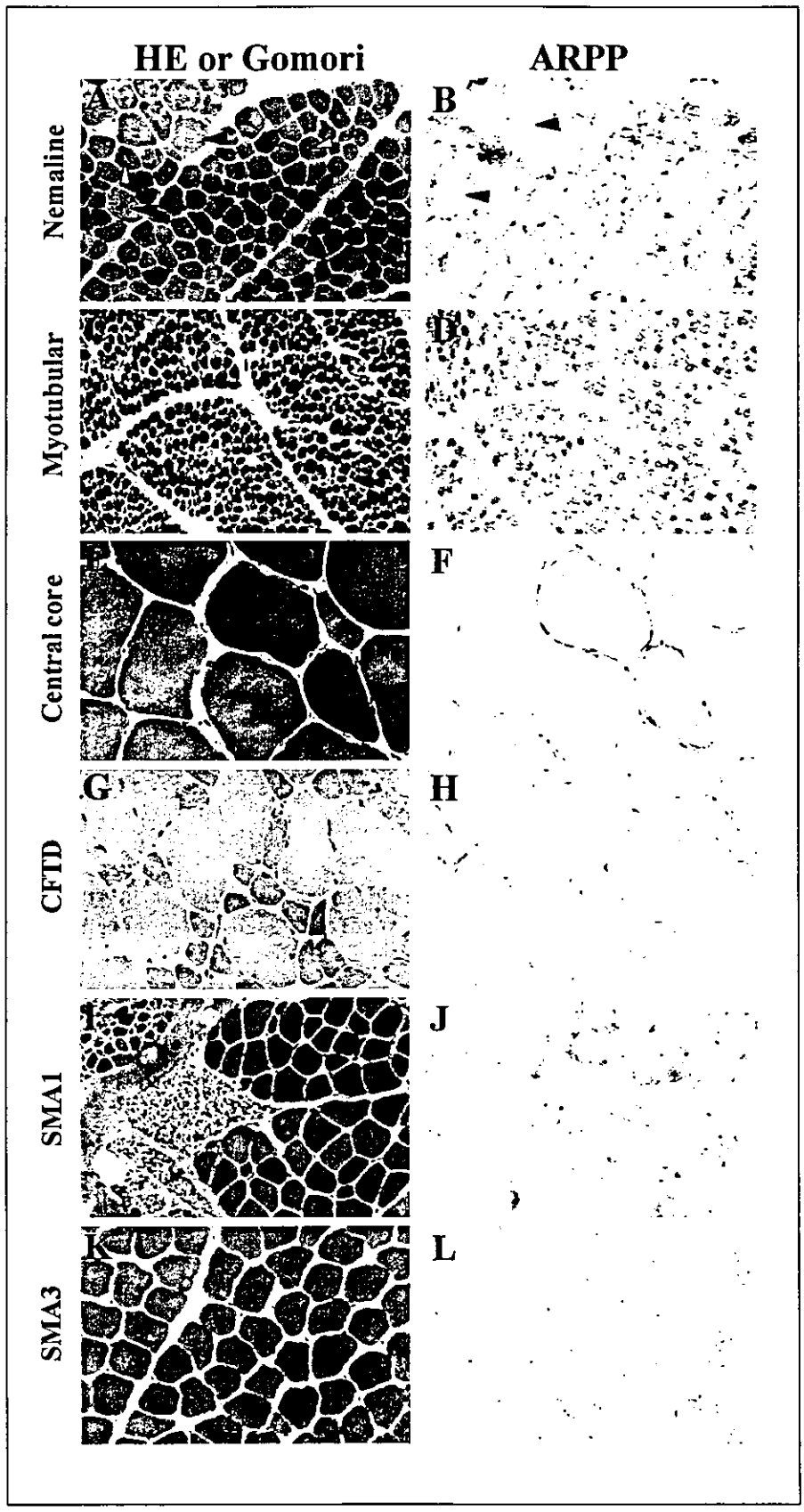

Fig. 2. ARPP expression in patients with congenital myopathies and SMAs. Frozen skeletal muscle tissue sections from patients with congenital myopathies and SMAs were analyzed by immunohistochemical staining with the α-ARPP(FL) Ab (**B**, **D**, **F**, **H**, **J** and **L**), Gomori trichrome staining (**A**) and hematoxylin-eosin (HE) staining (**C**, **E**, **G**, **I** and **K**). Original magnification × 100. Nemaline rods are shown by open arrowheads (**A**). ARPP-negative myofibers without nemaline rods are shown by solid arrowheads (**A** and **B**).

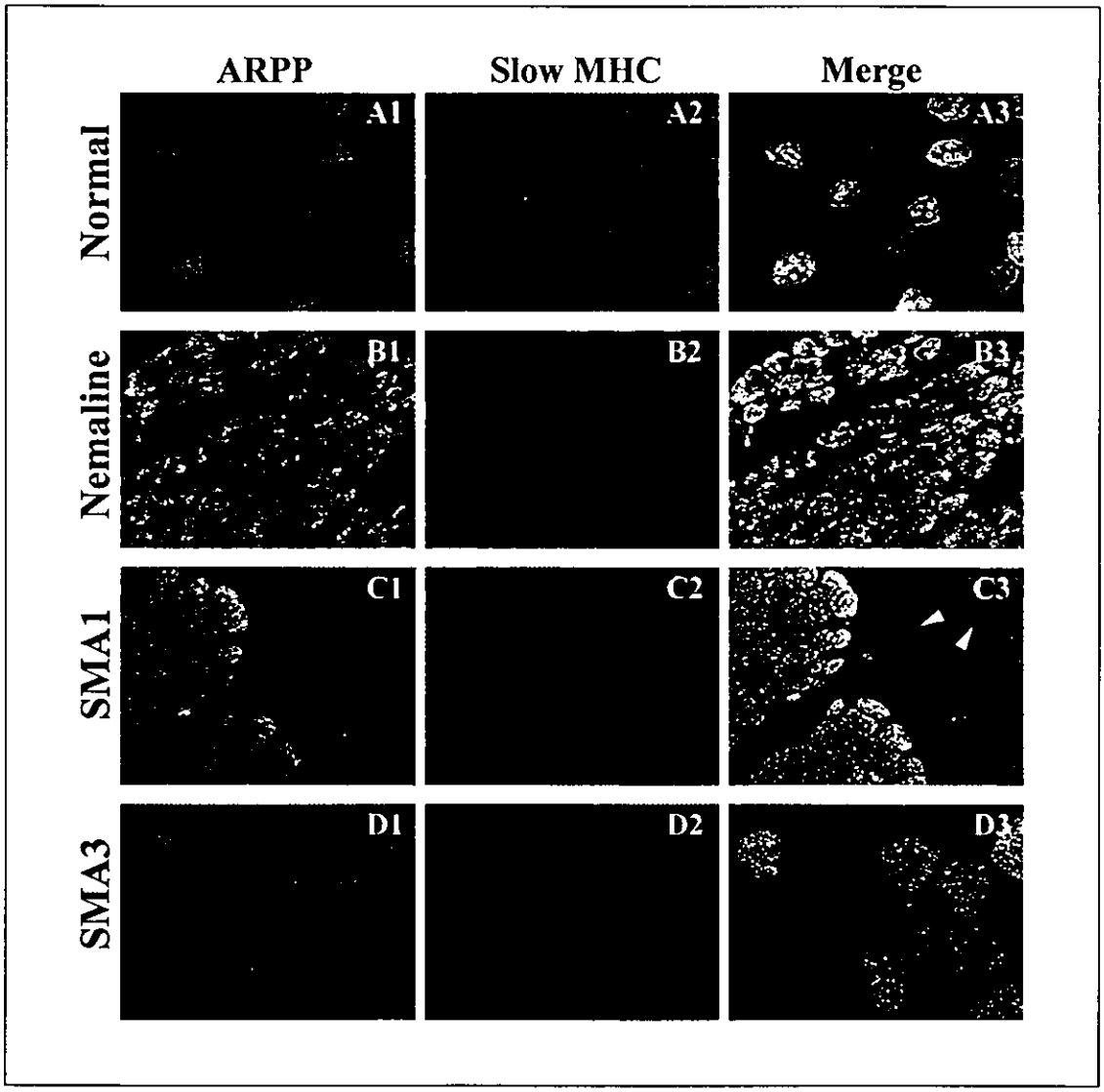

Fig. 3. Double-labeled immunohistochemistry of patients with nemaline myopathy and SMA1 and SMA2 with the α-ARPP(FL) and α-slow-MHC Abs. To evaluate whether ARPP and slow MHC were co-expressed in patients with nemaline myopathy and SMA1 and SMA2, muscle specimens were subjected to double-labeled immunohistochemistry with the α-ARPP(FL) and α-slow-MHC Abs and analyzed using confocal microscopy. The secondary antibodies used were a mixture of Alexa fluor-546-conjugated goat antimouse and Alexa fluor-488-conjugated goat antirabbit antibody. ARPP and slow MHC are shown as green (**A1**, **B1**, **C1** and **D1**) and red (**A2**, **B2**, **C2** and **D2**) signals, respectively, and the merged signals are yellow (**A3**, **B3**, **C3** and **D3**). The arrowheads in **C3** show the slow-MHC-positive atrophic myofibers that did not express ARPP. Original magnification × 200.

Myotubular Myopathy. All 3 specimens were ARPP positive and exhibited characteristic morphological features of markedly atrophic myofibers with centrally placed nuclei. Similarly to normal control muscle, ARPP-positive and negative myofibers were randomly admixed (fig. 2C and D). Both the proportions of ARPP-positive myofibers and the ARPP expression level of each myofiber were higher than those observed with the other subtypes of congenital myopathy (cases 5–7 in table 1).

Central Core Disease. In 1 patient (case 9; fig. 2E and F), ARPP-positive and negative fibers were arranged in a checkerboard-like pattern similar to that of normal muscle. But, in the other 2, almost all the fibers were positively immunostained and their expression levels were relative-

Table 2. Expression of ARPP protein in SMAs

Case No.	Histology	Age	Sex	Positive rates	Intensities of immunoreactivity
1	SMA1	1 year	M	+	++
2	SMA1	3 months	M	+	+
3	SMA1	3 months	F	++	++
4	SMA1	4 months	F	++	++
5	SMA2	2 years	F	+++	+
6	SMA2	2 years	F	+++	+
7	SMA2	3 years	F	+++	+
8	SMA3	6 years	M	++	+
9	SMA3	14 years	F	+++	+
10	SMA3	52 years	M	+++	+
11	SMA3	10 years	F	+	+

Positive rates were classified based on the proportions of the positive fibers: +++ = >90%; ++ = 90–50%; + = 50–5%; – = 5–0%. Intensities of immunoreactivity were classified into three groups: +++ = strong; ++ = weak; ± = borderline (not shown).

ly low (cases 8 and 10 in table 1), indicating that the checkerboard-like pattern had been lost in these cases.

Congenital Fiber Type Disproportion. All 3 specimens expressed ARPP (cases 11–13 in table 1). In 1 patient (case 11), almost all the myofibers showed diffuse ARPP expression, but in the other 2 (cases 12 and 13), ARPP-positive and negative myofibers were admixed (fig. 2G and H). In the latter 2 patients, large and small fibers were admixed, and ARPP was preferentially expressed in the small myofibers (fig. 2G and H).

ARPP Is Induced in Hypertrophic Myofibers of SMAs

To evaluate ARPP expression in SMA patients, 11 skeletal muscle tissue biopsy specimens from SMA patients were subjected to immunohistochemical analysis. ARPP was expressed in all 11 samples (table 2). In patients with SMA1, in which muscle atrophy is severe, and those with SMA2, in which muscle atrophy is less severe than that associated with SMA1, we found that the ARPP-positive and negative fibers were arranged in groups, respectively. All the grouped ARPP-positive myofibers expressed ARPP uniformly (fig. 2I and J). In 2 of the patients with SMA3, in which muscle atrophy is milder than that associated with SMA1 and SMA2 [12–14], the ARPP-positive myofibers were arranged in a checkerboard-like pattern similar to that of normal muscle (fig. 2K and L), but in the other 2, the ARPP-positive myofibers were arranged in groups (fig. 3D1). Double-labeled immunohistochemistry of samples from patients with SMA1 and SMA2 with the α-ARPP(FL) and α-slow-MHC Abs revealed that the ARPP-positive myofibers did not necessarily coincide with the slow-MHC-positive myofibers (fig. 3C1–3). As shown in figure 3C1–3, there were ARPP-positive hypertrophic and ARPP-negative atrophic myofibers among the slow-MHC-positive myofibers, whereas, in the SMA3 patients, the ARPP- and slow-MHC-positive myofibers coincided (fig. 3D1–3).

Discussion

MD is a genetically inherited muscle disease characterized histopathologically by the presence of necrosis and concurrent regeneration of myofibers, resulting in progressive destruction of myofibers [12, 14–18]. DMD is the most common subtype of MD and is believed to occur due to lack of the DMD gene product, dystrophin. BMD, LGMD and FSHMD have a later onset, are less severe clinically and progress more slowly than DMD [15]. In this study, we found that ARPP expression was apparently down-regulated in patients with every subtype of MD. However, in patients with congenital myopathies, ARPP expression was not down-regulated in atrophic myofibers but tended to be up-regulated in atrophic or damaged myofibers. This result suggests that such a down-regulation of ARPP may be a phenomenon specific to MDs but not to other muscle diseases. However, the down-regulation of ARPP might be due to reduced numbers of type 1 myofibers in patients with MDs. Therefore, we subjected muscle samples from patients with MDs to immunohistochemical analysis to detect expression of slow fiber-specific MHC, fast fiber-specific MHC and embryonic fiber-

specific MHC and found that slow- and fast-MHC-positive myofibers were arranged in a checkerboard-like pattern similar to that of normal muscle [Nakada et al., unpubl. observation]. Furthermore, embryonic MHC expression was restricted to regenerating myofibers [Nakada et al., unpubl. observation]. Thus, it is evident that ARPP expression was significantly down-regulated in slow-MHC-positive type 1 myofibers. It remains to be determined why ARPP was down-regulated in patients with MDs. Furthermore, an interesting question to be resolved is whether down-regulation of ARPP in muscles of MD patients could be useful for monitoring the clinical status of the disease. Further studies with larger numbers of patients are required to explore these possibilities.

The congenital myopathies are a heterogeneous group of disorders each of which is characterized by marked structural abnormalities [13]. In this study, we found that ARPP expression was induced in the skeletal muscles of patients with congenital myopathies. Both the numbers of ARPP-positive myofibers and their ARPP expression levels in the muscle tissue were apparently increased in comparison with those in normal muscle. The mechanisms responsible for the induction of ARPP expression in patients with congenital myopathies are not known at this stage. It has been reported that type 1 fibers predominate in patients with congenital myopathies, including nemaline myopathy, central core disease, CFTD and myotubular myopathy [14, 19]. In view of our previous finding that ARPP is selectively expressed in type 1 myofibers, we suggest that ARPP expression in patients with congenital myopathies might be associated with the type of myofiber. Indeed, we found that ARPP-positive myofibers co-expressed slow MHC (fig. 3B1–3), which supports this idea. Type 1 fibers have cytochemical features that indicate a mainly aerobic-oxidative metabolic profile, whereas type 2B fibers are mainly endowed with an anaerobic-glycolytic metabolic machinery [20]. Thus, it is an interesting speculation that metabolic disorder may occur in these diseased muscles and that altered expression of ARPP in congenital myopathies may be involved in their pathogenesis. Further studies are required to address this hypothesis. Furthermore, in patients with nemaline myopathy, we found that ARPP-positive fibers corresponded well to nemaline-rod-containing fibers, which led us to speculate that ARPP expression might be associated with nemaline rod formation, although the pathogenesis of these rods is still unknown.

SMA is an inherited denervating muscle disease, in which the anterior horn motor neurons of the spinal cord are affected, resulting in neurogenic muscle atrophy [13, 14, 21, 22]. Clinically, SMAs have been subclassified into three subtypes: SMA1, or Werdnig-Hoffmann disease, with an onset during infancy and a poor clinical prognosis; SMA2, the intermediate type with a later onset in the first year of life and longer survival without respiratory failure during infancy, and SMA3, or Kugelberg-Welander disease, with an onset in late childhood and considerable clinical heterogeneity [13, 14, 21, 22]. In this study, we found that, in patients with SMAs, ARPP-positive myofibers tended to be distributed in groups. In all those with SMA1 and 2, which are categorized as severe and intermediate subtypes, respectively, the ARPP-positive myofibers were arranged in groups. In half of the patients with SMA3, which is categorized as a milder subtype, the ARPP-positive myofibers were arranged in groups but, in the other half, the ARPP-positive and negative fibers were arranged in a checkerboard-like pattern similar to that of normal muscle. As the grouped myofibers were reported to result from the process of denervation, innervation and subsequent denervation of re-innervated myofibers [12], we suggest that the grouped arrangement of ARPP-positive myofibers in SMA patients may also result from denervation of the motor units. Furthermore, the ARPP expression levels were higher in patients with severe disease than in those with mild disease, which suggests that ARPP expression in SMA patients is associated with disease severity. If this proves to be the case, evaluation of ARPP in denervated muscle may be useful for monitoring the status of this disease. Further studies on larger numbers of patients are required to explore this possibility.

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Clinical Report

Girl With Monosomy 1p36 and Angelman Syndrome due to Unbalanced der(1) Transmission of a Maternal Translocation t(1;15)(p36.3;q13.1)

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We report on a girl with monosomy 1p36.3 and Angelman syndrome due to an unbalanced transmission of a maternal balanced chromosomal translocation. She manifested monosomy 1p36 and Angelman syndrome including generalized hypopigmentation, ataxic movements, intractable seizures with characteristic electroencephalographic (EEG) abnormality compatible with Angelman syndrome, and other minor anomalies, large anterior fontanelle, severe psychomotor retardation, and seizures due to monosomy 1p36. Her karyotype was 45, XX, der(1) t(1;15)(p36.31;q13.1), −15, derived from maternal translocation. Molecular analysis determined a breakpoint of 1p between D1S243 and D1S468, which suggested that most genes contributing to the common phenotype are in the distal region.

KEY WORDS: **Angelman syndrome; chromosomal abnormality; chromosome 1; monosomy 1p36**

INTRODUCTION

Recently, we studied a girl with partial monosomy 1p36.3 and 15q13 derived from a maternal balanced translocation. Some of her manifestations were compatible with Angelman syndrome, due to deletion of a maternal allele on 15q [Angelman, 1965]. She also showed manifestations not seen in patients with Angelman syndrome due to haploinsufficiency of the 1p36 region.

In recent years, subtelomeric rearrangements have been identified as a major cause of mental retardation and/or malformation syndromes [de Vries et al., 2003]. With the advance of molecular and cytogenetic methods, a specific phenotype of partial monosomy 1p36 has been characterized by moderate to severe psychomotor retardation, seizures, growth delay, and minor anomalies [Keppler-Noreuil et al., 1995; Reish et al., 1995; Giraudeau et al., 1997; Shapira et al., 1997; Fan et al., 1999; Riegel et al., 1999; Slavotinek et al., 1999; Wu et al., 1999; Knight-Jones et al., 2000; Giraudeau et al., 2001; Angle et al., 2002; Okamoto et al., 2002; Heilstedt et al., 2003]. A phenotype/genotype study showed that this condition is a contiguous gene syndrome and that most genes contributing to the phenotype might be in the distal region of 1p36 [Shapira et al., 1997; Slavotinek et al., 1999; Wu et al., 1999; Knight-Jones et al., 2000; Heilstedt et al., 2003].

CLINICAL REPORT

A female infant (Fig. 1) was referred for seizures at the age of 7 months. She was the first child of a healthy non-consanguineous 34-year-old mother and a 28-year-old father. Her mother did not have any history of abortions or gestational difficulties.

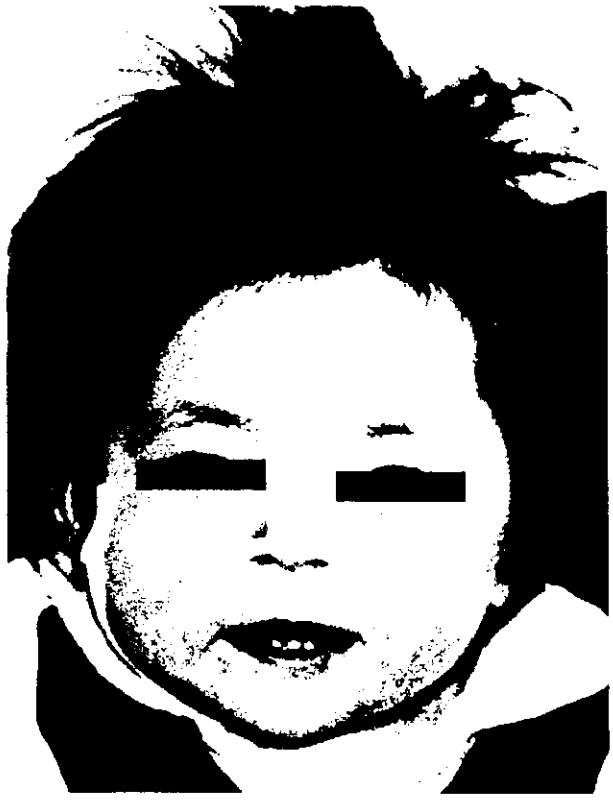

Fig. 1. The patient at age of 14 months.

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She was delivered spontaneously at 41 weeks of gestation, weighing 2,738 g. Before her first epileptic attack comprising a series of tonic spasms, she attained almost complete head control by the age of 3 months. However, after this event, she showed deterioration, and electroencephalographic (EEG) abnormalities were noted.

On admission, she weighed 7.55 kg (−0.41 SD), was 66.5 cm (−0.19 SD) in length, and had a 42 cm (−0.67 SD) head circumference. Her hands and feet were small, the palmar and plantar lengths being 7.3 cm (−5.0 SD) and 7.8 cm (−3.7 SD), respectively. She had an unusual facial appearance with frontal bossing, hypertelorism, a flat nasal root, a pointed chin, apparently low-set ears with asymmetry and cupping, and a preauriclar tag on right side. Generalized hypopigmentation of the skin, hair, and irises was noted. There were no anomalies of eyes, fundi, external genitalia, or limbs.

She had severe psychomotor developmental delay, postural hypotonia with head lag, and did not smile or pay any attention to her surroundings. Cranial nerve function was normal, but she had ataxic movements of the upper limbs, like an intentional tremor, especially when she brought her hands to her mouth. Her seizures consisted of infrequent generalized tonic spasms and frequent atypical absences. These clinical findings are summarized in Table I.

Results of laboratory examinations including urinalysis, complete blood count, total protein, urea nitrogen, liver enzyme, serum electrolyte, creatine kinase, uric acid, and total cholesterol levels were showed normal. Metabolic screening including blood gas analysis, ammonia, lactate, and pyruvate in blood and cerebrospinal fluid, and an amino acid profile in blood and urine, also showed normal results. No anomaly of heart and other visceral organs was documented by ultrasonographic examination. She had a large anterior fontanelle. Brain magnetic resonance imaging (MRI) disclosed mild cerebral atrophy with thin corpus callosum and large cortical sulci. Her interictal EEG findings consisted of continuous generalized 1–2 Hz, over 300 μV, and theta waves with frequent spikes in the occipital areas during sleep.

High-resolution chromosome study of her peripheral lymphocytes documented a karyotype 45, XX, der(1) t(1;15)(p36.31;q13.1), −15 (Fig. 2A). Deletion of the critical region for Angelman syndrome was also confirmed by fluorescence in situ hybridization (FISH) (Fig. 2B). Her mother had the balanced translocation 46, XX, t(1;15)(p36.31;q13.1), and her father had normal chromosomes.

After admission, a tube feeding was required because of inadequate oral intake. Although the seizures ceased with 200 mg/day of valproate and 0.9 mg/day of clonazepam, she did not catch up in development and had severe psychomotor retardation.

MOLECULAR INVESTIGATIONS

After informed consent, we investigated the breakpoints of chromosomes 1 and 15 by molecular methodology. Genomic DNAs from peripheral blood lymphocytes of the patient and her parents were obtained, and then analyzed by PCR amplification of polymorphic microsatellite markers located on the 1p36 region (D1S207, D1S214, D1S243, D1S244, D1S450, D1S468, D1S2633, D1S2660, D1S2667, D1S2736,

TABLE I. Manifestations in Our Patient and Those With Angelman Syndrome (Deletion Type) and Monosomy 1p36 Syndrome

	Angelman syndrome [Smith et al., 1996]			1p36 deletion syndrome [Shapira et al., 1997]	
Findings	n	%	Our case	n	%
Minor anomalies					
Large/wide mouth/large chin	24/26	92	−		
Minor anomalies at birth	0/27		+		
Pointed chin			+	12/15	80
Flat nasal bridge			+	11/17	65
Low-set ear(s)			+	10/17	59
Ear asymmetry			+	8/14	57
Deep-set eyes			−	7/14	50
Physical abnormalities					
Microcephaly	8/15	53	−	5/13	38
Growth delay	21/26	81	−	11/13	85
Hypopigmentated	19/26	73	+		
Large anterior fontanelle			+	11/11	100
Fifth finger short/clinodactyly			+	9/14	64
Small hands/feet			+	3/15	20
Congenital heart defect			−	3/18	17
Neurological abnormalities					
Severe mental retardation	27/27	100	+	12/13	92
Motor delay/hypotonia	15/22	68	+	12/13	92
Seizures	26/27	96	+	13/18	72
Ataxic movements	27/27	100	+		
Sleeping problems	18/21	86	+		
Eye/vision problems			−	9/12	75
Hearing deficits			−	5/9	56
Happy disposition in infancy	21/22	95	−		
Paroxysmal laughter	21/23	91	−		
Abusive behavior			−	5/9	56
Examination					
Abnormal EEG	25/25	100	+	8/14	57
CT/MRI anomaly			+	2/11	18
Infantile cardiomyophathy			−	4/9	44

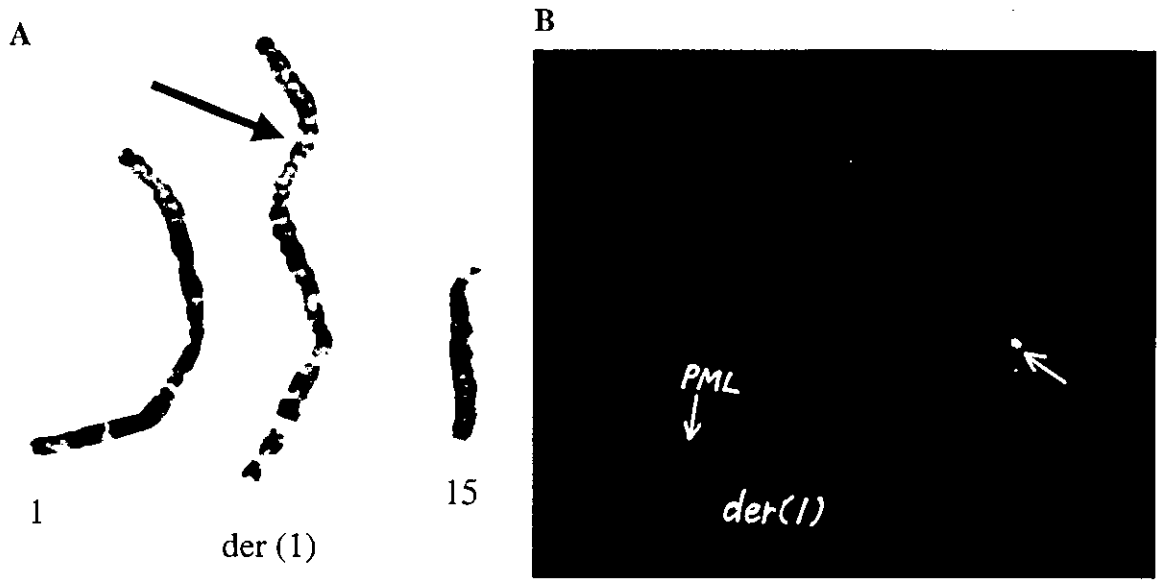

Fig. 2. A: Partial karyotype obtained from a phytohemagglutinin blood culture using trypsin (GTG) banding and schematic demonstration. The arrow indicates chromosome 15, which is translocated to the short arm of chromosome 1. The karyotype is 45, XX, der(1) t(1;15)(p36.31;q13.1),−15. B: Photograph of FISH. We used a red probe (D15S10: 15q11.2), for the region responsible for Angelman syndrome; and a green probe (D15Z1: 15p11.2) and another red probe (PML: 15q22) as control. The arrow indicates the normal chromosome 15, in which all of the probes were identified. "der(1)" indicates the derivative chromosome, in which the PML probe could be identified, whereas the red probe responsible for Angelman syndrome could not be identified. FISH utilizing a DNA probe confirmed the deletion of 15q13. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

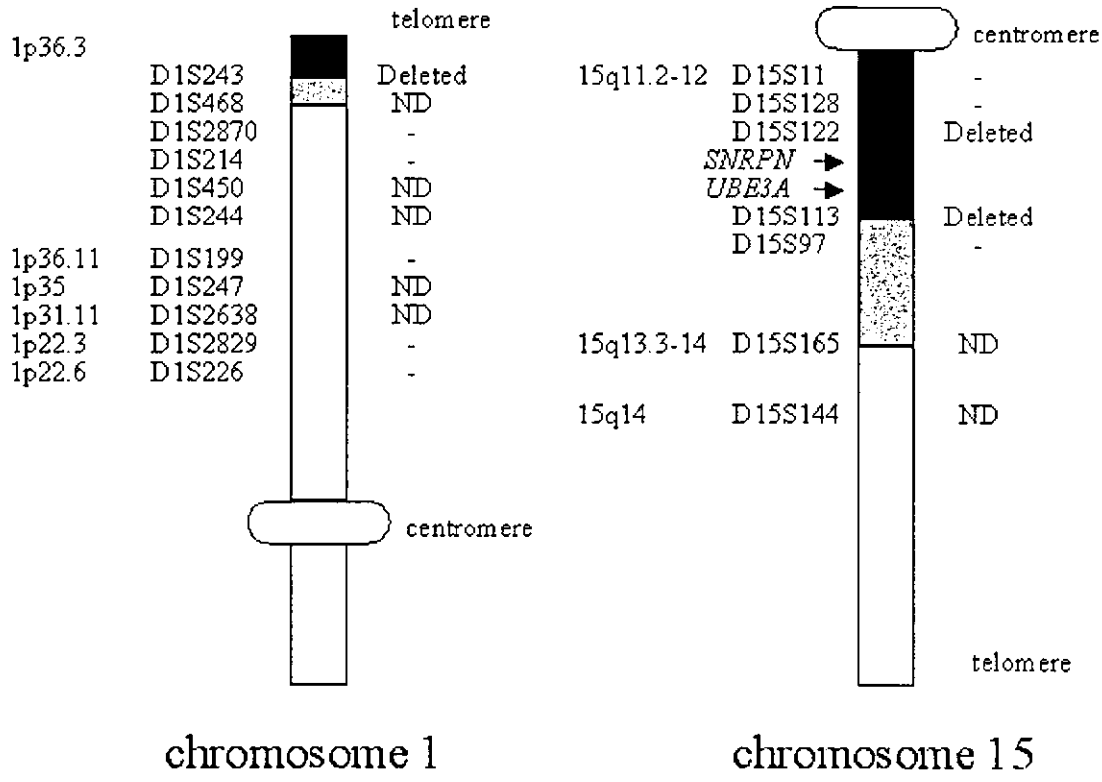

Fig. 3. Schematic presentation of the deleted regions of chromosome 1 and 15. The determination of the deleted region was based solely on the presence or absence of the parental alleles. The areas with black and white indicate the identified deleted region and non-deleted region, respectively. The gray area is that which we could not identify as deleted or not. ND, not deleted; −, not informative. Italic characters indicate the existence of well-known genes responsible for Angelman syndrome.

D1S2845, and D1S2870) and the 15q11-13 region (D15S11, D15S128, D15S122, D15S113, D15S97, and D15S165), according to methods described elsewhere [Yamamoto et al., 2001]. The primer sequences of the markers were obtained from the Genome Data Base (http://www.genethon.fr).

On chromosome 1, the breakpoint was determined to be between D1S243 and D1S468, because we could not identify the maternal allele of D1S243 in the patient. In the same way, the breakpoint on chromosome 15 was determined to be between D15S113 and D15S165 (Fig. 3).

DISCUSSION

The breakpoints of the chromosomes were confirmed to be in the telomeric region from 1p36.3 and the centromeric region from 15q13. The deleted region in chromosome 15 includes small nuclear ribonucleoprotein-associated polypeptide N (*SNPRN*) and the ubiquitin protein ligase E3A gene (*UBE3A*) which was major gene causing Angelman syndrome (Fig. 3) [Kishino et al., 1997; Matsuura et al., 1997]. We could not determine whether the deleted region includes the genes between D15S113 and D15S165, such as the gamma-aminobutyric acid A receptor, beta 3 gene (*GABRB3*), and the oculocutaneous albinism II gene (*OCA2*), which could associate with intractable epilepsy and hypopigmentation. However, the deleted region probably includes the two genes, because, as for deletion type of Angelman syndrome, there is one common breakpoint cluster at distal end, which is distal to the characteristic genes [Kuwano et al., 1992], and because specific findings in our patient are mostly compatible with Angelman syndrome [Buoni et al., 1999].

She had a distinct facial appearance with marked floppiness and a profound mental deficit, which appeared to be exceptionally severe for Angelman syndrome. Thus, these findings were probably due to partial monosomy 1p36. So far, over 50 patients with partial monosomy 1p36 have been reported and it is thought to be more common than other deletion syndromes [Keppler-Noreuil et al., 1995; Reish et al., 1995; Giraudeau et al., 1997; Shapira et al., 1997; Fan et al., 1999; Riegel et al., 1999; Slavotinek et al., 1999; Wu et al., 1999; Knight-Jones et al., 2000; Giraudeau et al., 2001; Angle et al., 2002; Okamoto et al., 2002; de Vries et al., 2003; Heilstedt et al., 2003]. Partial monosomy 1p36 is a contiguous gene deletion syndrome comprising, hypotonia, developmental delay (usually severe), and growth abnormalities (growth retardation, microcephaly, and obesity), craniofacial anomalies with large anterior fontanelle, prominent forehead, deep-set eyes, flat nasal bridge, midface hypoplasia, ear asymmetry, a pointed chin, and orofacial clefting. Our patient had almost all of these findings (Table I).

In comparison with previously reported patients, the deleted region in our patient is limited to only the terminal region of 1p and its size is one of the smallest [Keppler-Noreuil et al., 1995; Reish et al., 1995; Giraudeau et al., 1997; Shapira et al., 1997; Fan et al., 1999; Riegel et al., 1999; Slavotinek et al., 1999; Wu et al., 1999; Knight-Jones et al., 2000; Heilstedt et al., 2003]. A previous genotype/phenotype study showed that most genes contributing to the common phenotype are in the distal region [Shapira et al., 1997; Slavotinek et al., 1999; Wu et al., 1999; Knight-Jones et al., 2000]. The study of our patient supported this notion, suggesting that there is a critical region for this syndrome between D1S468 and pter [Wu et al., 1999; Heilstedt et al., 2003].

The potassium voltage-gated channel, shaker-related subfamily, beta member 2 gene, *KCNAB2*, is located in 1p36, and deletion of this gene may be responsible for the seizures in patients with monosomy 1p36 syndrome [Heilstedt et al., 2001]. Our patient had a severe seizure phenotype including infantile spasms, although the *KCNAB2* locus was not deleted, which resides proximal to the not-deleted microsatellite marker D1S468.

The gamma-aminobutyric acid A receptor, delta gene (*GABRD*) is another gene assigned to the 1p36 region [Emberger et al., 2000; Windpassinger et al., 2002]. This gene has been reported to represent an integral mechanism for the functioning of the central nervous system, and a locus for the action of many mood- and emotion-altering agents. Although further investigation is required to elucidate whether this gene is included in the deleted region or not, *GABRD* may be involved in the psychomotor developmental delay in patients with partial monosomy 1p36.

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Genetics

A non-NF2 case of schwannomas of vestibular and trigeminal nerves with different genetic alterations of NF2 gene: case report

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Abstract

Background: We report a patient with 2 separate schwannomas, a vestibular schwannoma and a trigeminal schwannoma, that were attached to each other and appeared to be a single tumor on imaging studies.

Case Description: The patient, without any family history of neurofibromatosis, presented with a progressive hearing loss and mild left facial nerve palsy. Magnetic resonance imaging showed a snowman-like tumor in the left cerebellopontine angle. Surgical exposure revealed that the tumor consisted of 2 "kissing" schwannomas, a trigeminal and vestibular schwannoma. Molecular genetic analysis detected a 1-base pair deletion at exon 10 of the neurofibromatosis type 2 (NF2) gene in the trigeminal schwannoma, but not in the acoustic schwannoma. However, loss of heterozygosity at chromosome 22q (D22S282 and D22S929) was detected in both tumors, losing the same allele.

Conclusion: Multiple schwannomas in non-NF2 patients are extremely rare, and possible causes include simple coincidence or germline genetic alteration of adjacent gene on chromosome 22q, similar to the cause recently suggested in familial schwannomatosis. Although not always possible, molecular genetic examination may help to understand the underlying mechanism and would be warranted in such cases.

Keywords: Trigeminal schwannoma; Vestibular schwannoma; NF2 gene; Mutations

1. Introduction

Intracranial schwannomas account for approximately 8% of intracranial tumors [4,5,7] and most frequently arise from the vestibular nerve followed by the trigeminal nerve. However, it is rare to find 2 independent schwannomas simultaneously in the same patient, unless the patient is affected by neurofibromatosis type 2 (NF2), an autosomal dominant disorder caused by a germline mutation of the NF2 gene. Here we report a patient with 2 simultaneous schwannomas, in which we performed molecular genetic analysis to investigate the possible cause.

2. Case report

A 46-year-old man without any family history of neurofibromatosis noticed partial hearing loss (70 dB) and mild left facial nerve palsy (House and Brackmann grade 2). There was no trigeminal dysfunction. Magnetic resonance imaging (MRI) of the brain demonstrated a heterogeneous mass with a size of 2.0 × 1.5 × 1.8 cm, showing a snowman-like configuration. The tumor mass was enhanced homogeneously by gadolinium except the cystic compo-

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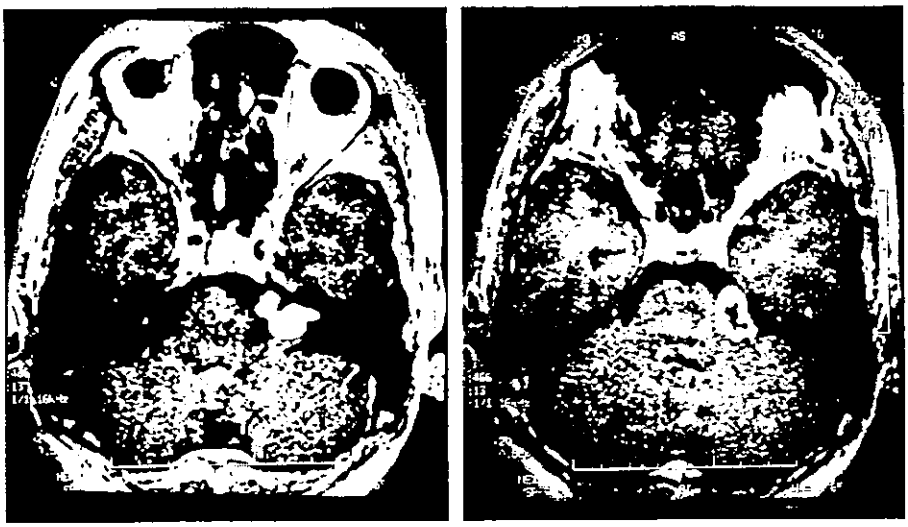

Fig. 1. Preoperative gadolinium-enhanced axial MRI. Left, Preoperative gadolinium-enhanced axial MRI demonstrating the caudal portion of the mass showing extension into the left auditory canal. Right, Preoperative gadolinium-enhanced axial MRI demonstrating the rostral portion, with cystic components involving the left trigeminal nerve.

nent. On close examination, the posterior portion of the mass showed extension into the left internal auditory canal, whereas the anterior portion with cystic components apparently involved the left trigeminal nerve (Fig. 1). He had no history of previous radiation to any part of the body.

The patient underwent a left retromastoid suboccipital craniotomy. The tumor initially looked like a single mass despite the presence of constricted portion. After internal decompression of the caudal part of the tumor, however, it was found that the rostral and caudal masses were separate tumors that only weakly adhered to each other. The caudal tumor occupying the left internal auditory canal had a typical schwannoma appearance, and the rostral tumor arising from the trigeminal nerve had both solid and cystic components. Therefore, this patient was considered to have 2 separate schwannomas, a vestibular and a trigeminal schwannoma. Both tumors including the portion extending into the left internal auditory canal were totally removed.

The patient recovered well after surgery but showed complete loss of hearing in the left ear and mild left trigeminal dysesthesia. The patient did not have any stigmata of neurofibromatosis such as cutaneous neurofibromas, café au lait spots, or posterior subcapsular cataracts. MRI performed 2 months after the surgery showed no residual tumor. Histologic examination showed that both tumors were typical schwannomas without any

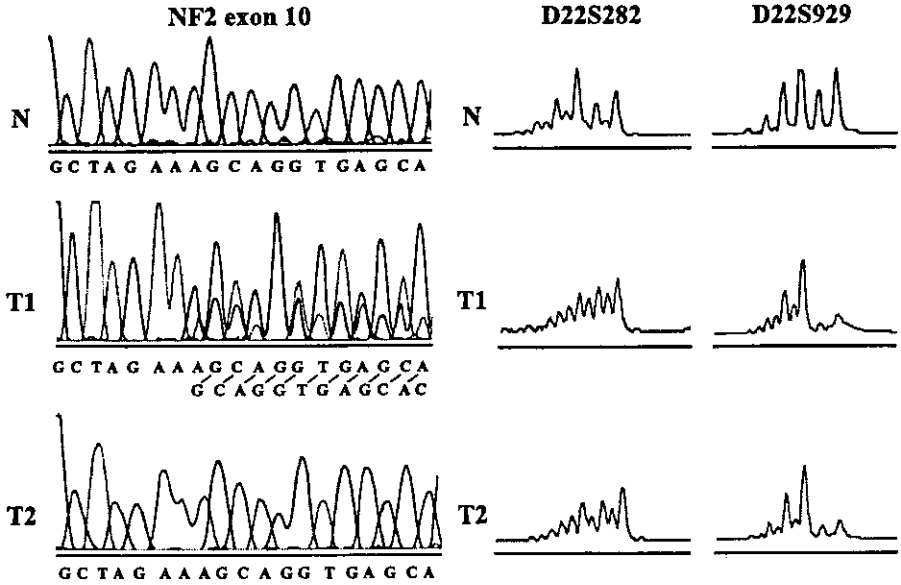

Fig. 2. Molecular genetic analysis on the trigeminal tumor (T1) and the acoustic tumor (T2) in comparison with the blood DNA (N). There was 1-base pair deletion in the NF2 exon 10 in T1, but not in T2 nor in the blood DNA (left). On the other hand, LOH at chromosome 22q12 (D22S282 and D22S929) was evident in both tumors, apparently losing the same allele.

histologic atypia. Molecular genetic analysis of the tumors was performed to investigate the possible influence of the NF2 gene alteration on the formation of the schwannomas.

After obtaining written informed consent, DNA was extracted from each tumor and from peripheral blood lymphocytes of the patient. All 17 exons of the NF2 gene were polymerase chain reaction-amplified from the tumor DNA samples using the previously reported primers and were directly sequenced [6]. In addition, loss of heterozygosity (LOH) analysis of the NF2 locus chromosome 22q12 was performed on 3 microsatellite markers, D22S929, D22S282, and D22S1163, using the constitutional DNA as a reference. There was 1-base pair deletion at exon 10 causing a frame shift in the trigeminal schwannoma, but there was no mutation either in the acoustic schwannoma or in the constitutional DNA (Fig. 2). On the other hand, the microsatellite analysis showed that both tumors had LOH at chromosome 22q (D22S282 and D22S929) (Fig. 2). The pattern of allelic loss indicated that the same allele was lost in both tumors.

3. Discussion

Most intracranial schwannomas present with a solitary lesion, and bilateral occurrence of vestibular schwannomas by itself fulfills the clinical diagnostic criteria for NF2. Furthermore, simultaneous growth of multiple schwannomas in different loci usually indicates high likelihood of NF2 as well. Without underlying NF2, combination of unilateral vestibular and trigeminal schwannomas is a quite rare condition, with only 2 similar cases reported in the literature. Both cases were considered to be forme fruste of NF2 by the authors, although no molecular genetic analysis was performed [1,7].

In the present case, molecular genetic analysis provided interesting information. Although both tumors had loss of 1 allele of chromosome 22q around the NF2 lesion, only 1 had detectable mutation in the NF2 gene, and the other had no detectable NF2 gene alteration. Given that no germline NF2 mutation was detected in the blood lymphocytes while the trigeminal schwannoma had a NF2 mutation, it was unlikely that this patient was affected by NF2. If the patient suffered mosaic type of NF2, the same mutation should have been detected in the vestibular schwannoma as well [2]. Instead, there are other possibilities that would explain the mechanism of the present case. First, the formation of 2 tumors in this patient was a mere coincidence, although it would be an extremely remote possibility considering the incidence of sporadic schwannoma. Second, this patient may have the similar molecular genetic background found in familial schwannomatosis. In a recent study, MacCollin et al demonstrated that most of the tumors found in patients with familial schwannomatosis showed LOH at chromosome 22q and many, but not all, of those also showed typical truncation mutation of the NF2 gene. Based on the data, they proposed that the responsible gene or locus for the schwannomatosis should lie centromeric to NF2, and inherited mutation of this gene/locus contributes to the tumor formation in combination with somatic inactivation of NF2 [3]. In the present case, the molecular genetic event detected is consistent with this mechanism, although other currently unknown mechanisms are not excluded. Existence of vestibular schwannoma automatically excludes our case from clinical definition of schwannomatosis, but the phenomenon itself appears to be very much similar.

Although the present patient apparently does not have NF2, currently available methods or knowledge does not allow us to tell definitely whether this patient has the risk of schwannomatosis-like situation. But, again, considering that the mere coincidence of 2 sporadic tumors could be an extremely remote possibility, it would be safe to monitor patients closely by imaging studies for new tumor formation, when a similar situation is encountered.

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Original article

Dichloroacetate treatment for mitochondrial cytopathy: long-term effects in MELAS

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Abstract

The long-term effects of the sodium salt of dichloroacetic acid (DCA) were evaluated in four patients with mitochondrial encephalomyelopathy with lactic acidosis and stroke-like episodes (MELAS) carrying A3243G mutation. Oral administration of DCA in MELAS patients was followed for an average of 5 years 4 months. Serum levels of lactate and pyruvate were maintained at around 10 and 0.6 mg/dl, respectively. Serum levels of DCA were 40–136 μg/ml. Symptoms responding to treatment included persistent headache, abdominal pain, muscle weakness, and stroke-like episodes. In contrast, no improvements in mental status, deafness, short stature, or neuroelectrophysiological findings were observed. Adverse effects included mild liver dysfunction in all patients, hypocalcemia in three and peripheral neuropathy in one. None of these adverse events was severe enough to require discontinuation of treatment. To determine suitable indications for DCA therapy, analysis of many more patients who have undergone DCA administration is required.

Keywords: Mitochondrial encephalomyelopathy with lactic acidosis and stroke-like episodes; Treatment; Dichloroacetate; Long-term effects

1. Introduction

Mitochondrial diseases remain largely untreatable, despite numerous advances in our understanding of molecular genetics and pathology. Mitochondrial encephalomyelopathy with lactic acidosis and stroke-like episodes (MELAS) represents one of the most common mitochondrial disorders of childhood. The disease presents as a slowly progressive entity associated with deteriorating neuromuscular function. Patients suffer from a wide variety of symptoms, including intractable abdominal pain, headache, stroke-like episodes, dementia, deafness, muscle weakness, and short stature [1]. Among these, stroke-like episodes play an important part in the neurological prognosis of MELAS. Affected patients suffer worsened mental and motor function or visual disturbance, depending upon the severity and location of brain lesions. About 80% of MELAS patients display a point mutation in the tRNA-Leu (UUR) gene of mitochondrial DNA [2].

In contrast to the molecular genetics, relatively little progress has been reported in treatment regimens for these conditions. Intravenous administration of cytochrome *c*, or oral administration of CoQ10 with or without vitamin supplementation reportedly shows some clinical effect in MELAS patients [3–8]. These reagents are thought to act as substrates, electron carriers in the respiratory chain, or antioxidants. The sodium salt of dichloroacetic acid (DCA) is effective in reducing circulating lactate by increasing pyruvate dehydrogenase activity, but has been trialed in the treatment of congenital lactic acidosis and Leigh syndrome without obvious clinical success [9–15]. DCA has been widely used and studied for its beneficial effect in the improvement of lactic acidosis and in the metabolism of ischemic tissues [16–20]. Despite numerous studies examining DCA administration in animals and humans, the effects in mitochondrial disorders remain controversial [21]. We have previously reported on the short-term results of DCA administration in three MELAS patients in 1998, with all three patients exhibiting beneficial effects directly related to the treatment [22]. To assess the long-term effects of DCA administration in patients with mitochondrial disorders, we report herein on four MELAS patients treated with DCA for up to 5 years. We discuss the application of DCA therapy in patients with mitochondrial disorders, including adverse effects.

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Table 1
Clinical findings and laboratory data before and after treatment with DCA

	Patient 1			Patient 2			Patient 3			Patient 4		
	Before	After 1 year	After 6 years	Before	After 1 year	After 5 years	Before	After 1 year	After 5 years	Before	After 1 year	After 4 years
Headache	Everyday	1–2/month	None	Everyday	1–2/week	3/month	1/week	0–1/month	None	1/week	0–1/month	None
Stroke-like episodes	1–3/year	None	None	−	−	+	1/month	None	None	1/month	None	+
Seizure	1–2/month	1–2/year	None	−	−	−	1–2/month	1–2/week	1–2/week	1–2/month	1–2/year	1–2/year
Weakness	−	−	−	+++	++	+	+	+	+	+	+	+
Hearing loss	+	+	+	−	−	+	+	−	−	+	+	++
Intelligence	5years 3 months	5years 9 months	5 years 3 months	IQ 97	IQ 98	IQ 101	6 years 8 months	7 years 11 months	7 years 10 months	IQ 58	IQ 46	IQ < 40
EEG	Spikes at Rt T	No change	No change	Spike and waves	No change	No change	Spikes at Lt O	No change	Spike and waves	Spikes at Rt O	No change	Spike and waves
ABR (threshold) (dB)	65	65	75–85	Normal	Normal	Elongation of latency	65	25	25	35–45	ND	ND
Cranial CT calcification/low density area	+/Rt O	No change	ND	−/−	No change	ND	+/Lt O	No change	No change	+/Rt O	No change	No change
Liver dysfunction	ND	+	+	ND	+	+	ND	+	+	ND	+	+
Abdominal echography	ND	Hypertrophy of liver/polyps of gallbladder	Hypertrophy of liver/polyps of gallbladder	ND	Hypertrophy of liver	NP	ND	Hypertrophy of liver	Polyps of gallbladder	ND	Hypertrophy of liver	Hypertrophy of liver
NCV	ND	Normal	Normal	ND	Normal	Normal	ND	Decreased at Sural N.	No change	ND	ND	ND

−, not detected; +, detected, able to walk more than 1 km; ++, unable to walk more than 1 km, unable to hear small voice; +++, unable to walk more than 100 m; Rt, right; Lt, left; T, temporal area; O, occipital area; ND, not done; NP, nothing in particular; EEG, electroencephalography; ABR, auditory brainstem response; NCV, nerve conduction velocity. Intelligence, patients 1 and 3 undertook the Kaufmann Assessment Battery for Children. Patients 2 and 4 undertook the Wechsler Intelligence Scale for Children-III.

2. Cases

Four patients with MELAS were enrolled in the DCA treatment program. All four MELAS patients displayed the A3243G mutation. Clinical course for three of the four MELAS patients has been reported elsewhere [22]. Additional data from a 9-year-old female patient who joined the program at a later date are included. She displayed mental retardation, short stature and mild hearing disturbance, and had been treated with DCA since an episode of intractable status epilepticus 4 years previously. All four patients with MELAS were female. Ages of patients 1–4 at the start of treatment were 16 years 11 months, 9 years 5 months, 13 years 11 months and 9 years 2 months, respectively. Duration of treatment ranged from 4 years 2 months to 6 years 1 month. The major clinical features of patients are listed in Table 1.

3. Treatment program

The ethics committee of Jichi Medical School approved the DCA treatment program for patients with mitochondrial encephalomyopathy. Informed consent was obtained from patients when possible, or from their parents. DCA is a prime class reagent manufactured by Tokyo Kasei Co., Ltd (Tokyo, Japan). Patients were initially given three separate oral 50 mg/kg doses of DCA at 12-h intervals, followed by 25 mg/kg doses twice a day. DCA administration was supplemented with 100 mg/day of vitamin B1. Doses were adjusted relative to serum and CSF levels of lactate and pyruvate. Serum lactate levels were maintained between 10 and 20 mg/dl to prevent depletion of serum lactate.

Laboratory studies included routine blood and urine tests, electroencephalography, auditory brain stem responses, abdominal and cardiac ultrasonography, and neuroimaging. The Kaufmann Assessment Battery for Children (K-ABC) or Wechsler Intelligence Scale for Children III (WISC-III) was used to assess mental function.

4. Results

Clinical and laboratory findings of MELAS patients before and after treatment are listed in Table 1. Persistent headache was observed in all four patients, with complete resolution in three cases and partial improvement in the other case after DCA treatment. Stroke-like episodes were documented in three patients before treatment. Two patients experienced stroke-like episodes during the treatment period. Lactate and pyruvate levels in CSF were higher than those in serum. DCA doses were increased following these episodes, with no subsequent attacks. Epileptic seizures were controllable in three of the patients. Patient 2 displayed epileptic seizure during the treatment period, at which time serum lactate levels were around 30 mg/dl. Due to mild liver dysfunction, carbamazepine was initiated instead of increasing dose of DCA. Improvements in muscle weakness were observed in patient 2, who was unable to walk 1 km before treatment due to weakness and fatigability, but over the course of treatment became capable of walking as far as she wanted. Effect on hearing acuity varied, but was partial, if evident at all.

Mental tests revealed no worsening of dementia in two patients, decreased scores in one, and normal intelligence in one. No changes were observed on electroencephalography. Auditory brain stem response studies in three patients revealed elevated threshold and prolonged latencies. Absent nerve potential was detected in the sural nerve in one patient who developed weakness and paresthesia of the lower limbs after 5 months of treatment. Symptoms in the lower limbs did not change during the remainder of the 5-year treatment period. No prominent changes were apparent on neuroimaging. Serum lactate levels were maintained at around 10 mg/dl (Fig. 1a), and pyruvate at around 0.6 mg/dl (Fig. 1b). Levels of lactate and pyruvate in CSF ranged from 25 to 42, and from 0.8 to 1.4 mg/dl, respectively (Fig. 2a and b).

Dosage of DCA was adjusted according to the clinical condition of patients and serum levels of lactate (Fig. 3). A daily dose of around 30 mg/kg appeared to represent the optimal dose for obtaining stable and constant amelioration of symptoms.

With regard to possible adverse effects, serum transaminases were slightly elevated, although not in a progressive

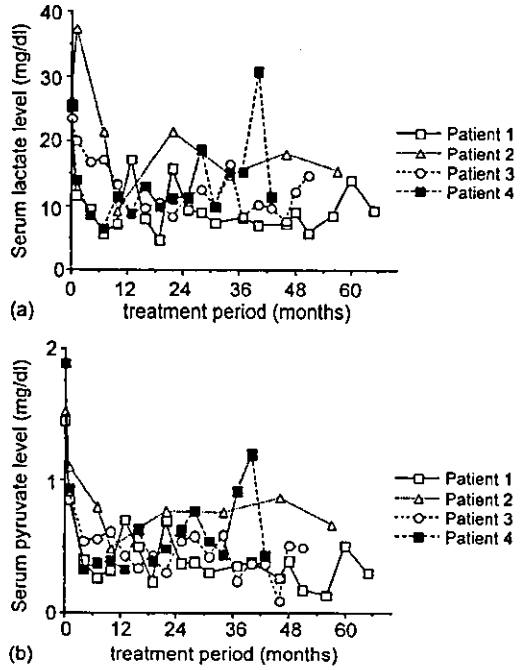

Fig. 1. Time course for levels of (a) serum lactate and (b) pyruvate. After DCA treatment was started, serum lactate and pyruvate levels were maintained within almost normal ranges.

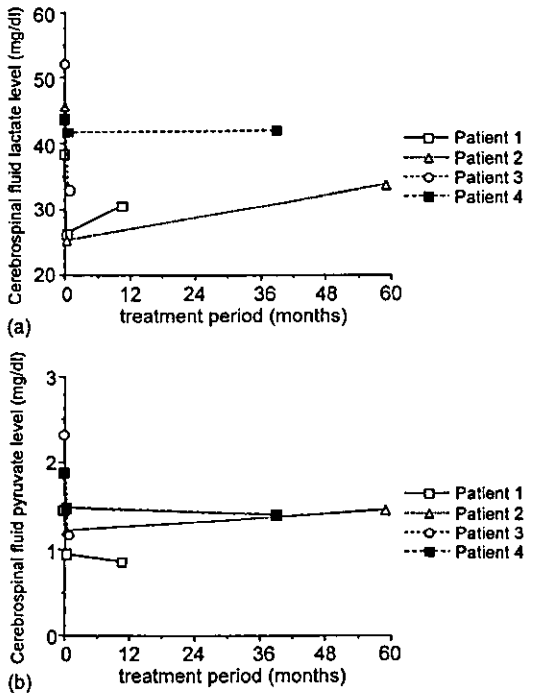

Fig. 2. Time course for cerebrospinal fluid levels of (a) lactate and (b) pyruvate. After DCA treatment was started, cerebrospinal fluid levels of lactate and pyruvate decreased immediately. However, levels remained higher than serum levels.

manner in all patients (Fig. 4). Abdominal ultrasonography revealed enlargement of the liver in all patients. This was improved by changing anticonvulsant in one patient. No space-occupying lesions were detected in the livers. Polyp-like echogenicity was detected in the gallbladders of patients 1 and 3, but no progression was seen. Since development of polyps in patient 3 obviously occurred during treatment, DCA administration may have been responsible. Three patients developed asymptomatic hypocalcemia during treatment. Levels normalized in one patient, but vitamin D supplementation was required in the other two. Peripheral neuropathy presented as weakness

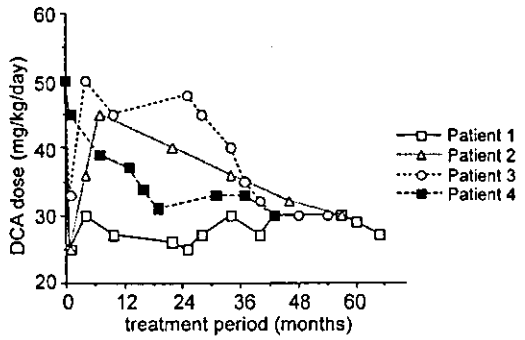

Fig. 3. Dosage of DCA. All patients were treated with about 30 mg/kg per day of DCA.

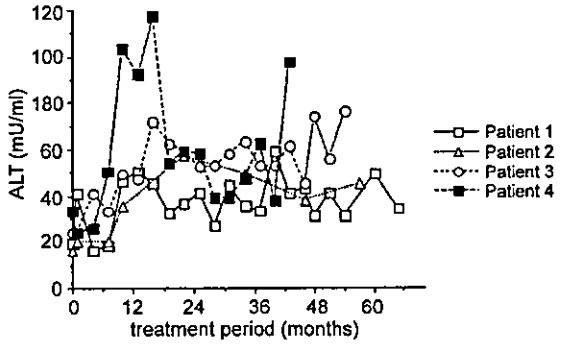

Fig. 4. Time course of transaminase (ALT). Transaminase levels were increased from 6 to 12 months after initiation of DCA treatment. No patients elected to discontinue treatment.

of the lower limbs and drop foot in patient 3. These symptoms remained stable for 3 years.

5. Discussion

Although our knowledge about the genetic mutations and molecular pathologies of mitochondrial disorders is steadily increasing, these conditions remain largely untreatable. Therapeutic drug regimens have included cytochrome *c*, CoQ10, DCA, and vitamins B1, B2, and B6. CoQ10 is reportedly effective for suppressing major symptoms in CoQ10-deficient patients through its function as an antioxidant and electron carrier in the respiratory chain. However, the efficacy of these drugs for other diseases remains controversial [23]. Nakagawa and associates [24] reported that 22 months of co-administrating cytochrome *c* and vitamins B1 and B2 to a patient with Kearns-Sayre syndrome somewhat improved extraocular symptoms, but had no effect on ocular symptoms. The same group reported a 1-year follow-up of cytochrome *c* treatment in nine patients, including four patients with MELAS [3]. Weakness, fatigability, respiratory failure, and nystagmus were resolved, but no improvements in laboratory findings were observed.

DCA is a pyruvate dehydrogenase activator, and has been used to ameliorate lactic acidosis caused by either overproduction or underutilization of lactic acid [21]. So far, the effects of DCA have been described in treatments for shock, sepsis, hypoxia [16,17], diabetes mellitus [18], myocardial ischemia [19], and mitochondrial disorders [9–15,25,26], in both human and animal models. The beneficial effects of DCA are thought to be attributable to either increased oxidative phosphorylation or increased ATP utilization by tissues. If the effects occur through enhancement of respiratory chain activity, then such effects should be limited in mitochondrial disorders in which the respiratory chain is partially affected. Cells or tissues must maintain a certain level of respiratory chain activity in order

to be able accept the enhanced pyruvate dehydrogenase complex (PDHC) product. In addition, serum lactate levels need to be maintained so that substrate can be supplied. Based on data taken from the 4-year average levels seen in MELAS patients, serum lactate levels of around 10 mg/dl may be appropriate.

Our results from four MELAS patients after 4–6 years of treatment showed that DCA is effective in suppressing symptoms of a recurring nature. Headache, abdominal pain and stroke-like episodes appear to be ameliorated by DCA, while persistent symptoms such as dementia, deafness, or short stature are unaffected. Chandy and associates reported that DCA decreased the size of infarcts in a primate model of focal cerebral ischemia. They reported that size of the infarct was reduced to one-third by prior administration of DCA [27]. Their report on the animal model of cerebral ischemia is consistent with the effects we observed for complete suppression of stroke-like episodes by DCA. Taniguchi and associates [28] reported that the effect of DCA on hypoxic heart function was due to increasing efficiency of ATP use by cardiac tissue, and not through an increase in the efficiency of ATP production. We cannot confirm that this is what happened in the tissues of our patients or what was the cause of differences seen in responsiveness to DCA. Several possible factors may define responsiveness to DCA. Remnant activity of the respiratory chain in cells, tissues, or organs should represent the central factor defining responsiveness. Tissue dependence on glycolysis could be another factor.

Mild liver dysfunction was observed in all patients with MELAS. Since hepatocarcinogenicity of DCA has been reported in rodents [29,30], these patients were followed by ultrasonographic studies of the liver. No patients developed abnormal echogenicity in the livers. Walgren and associates [31] reported that humans might not be susceptible to DCA-induced hepatocarcinogenesis, as DCA does not increase DNA synthesis in cultured human hepatocytes. Enlargement of the liver observed in three patients was possibly due to glycogen accumulation, as has been documented in rodent livers [32]. Peripheral neuropathy observed in one patient may be part of the symptoms of mitochondrial disorders, or may represent an adverse effect of DCA administration. The ineffectiveness of vitamin B supplementation in neuropathy seems to support the former. Hypocalcemia observed in three patients was asymptomatic and improved with administration of vitamin D.

DCA treatment was administered for MELAS in four patients, all of whom obtained improved daily quality-of-life (QOL) without experiencing further severe stroke-like episode. However, two patients with lactate levels in CSF exceeding those in serum experienced stroke-like episodes. The mitochondrial heteroplasmy of tissues must be considered when treating patients. No one proposed cessation of treatment, despite the adverse effects described above. To make DCA treatment valid and safe, several problems remain to be solved. Firstly, better protocols for determining optimal dose of DCA for each patient are required. In the present cases, dose was regulated by carefully observing patient condition and serum and CSF lactate levels. However, that was insufficient to avoid overdoses, as required lactate levels differ for each patient. Secondly, whether DCA improves QOL for each patient must be documented. With these considerations in mind, symptoms that do not respond to treatment must be carefully followed for many years, as further degradation could lead to the patient becoming bed-ridden. Thirdly, hepatocarcinogenesis and other potential serious adverse effects of DCA need to be carefully monitored throughout the lifetime of the patient. The high incidences of hypocalcemia and liver dysfunction also require investigation. Further studies with animal models may help to provide answers to these questions.

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