neuro-imaging studies are controversial and are difficult to evaluate, there are several specific findings which may lead to the pathophysiology of stroke-like episodes in MELAS. We have to elucidate what is the trigger of stroke-like episodes in MELAS in future. Currently L-arginine therapy, to cure the symptoms of stroke-like episodes at acute phase, and to prevent or decrease the severity of stroke-like episodes at interictal phase of MELAS, is the most promising therapy for this incurable disorder. Global clinical trial of L-arginine on MELAS using randomized double blind placebo control protocol may be done in the nearer future.

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MELAS: A nationwide prospective cohort study of 96 patients in Japan ☆

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ABSTRACT

Background: MELAS (mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes) (OMIM 540000) is the most dominant subtype of mitochondrial myopathy. The aim of this study was to determine the prevalence, natural course, and severity of MELAS.
Methods: A prospective cohort study of 96 Japanese patients with MELAS was followed between June 2003 and April 2008. Patients with MELAS were identified and enrolled based on questionnaires administered to neurologists in Japan. MELAS was defined using the Japanese diagnostic criteria for MELAS. Two follow-up questionnaires were administered to neurologists managing MELAS patients at an interval of 5 years.
Results: A prevalence of at least 0.58 (95% confidential interval (CI), 0.54–0.62)/100,000 was calculated for mitochondrial myopathy, whereas the prevalence of MELAS was 0.18 (95%CI, 0.02–0.34)/100,000 in the total population. MELAS patients were divided into two sub-groups: juvenile form and adult form. Stroke-like episodes, seizure and headache were the most frequent symptoms seen in both forms of MELAS. Short stature was significantly more frequent in the juvenile form, whereas hearing loss, cortical blindness and diabetes mellitus were significantly more frequent in the adult form. According to the Japanese mitochondrial disease rating scale, MELAS patients showed rapidly increasing scores (mean ± standard deviation, 12.8 ± 8.7) within 5 years from onset of the disease. According to a Kaplan–Meier analysis, the juvenile form was associated with a higher risk of death than the adult form (hazard ratio, 3.29; 95%CI, 1.32–8.20; $p = 0.0105$).
Conclusions and General Significance: We confirmed that MELAS shows a rapid degenerative progression within a 5-year interval and that this occurs in both the juvenile and the adult forms of MELAS and follows different natural courses. This article is part of a Special Issue entitled: Biochemistry of Mitochondria.

1. Introduction

Mitochondrial dysfunction increases the risk of developing various human diseases, including degenerative neuromuscular disorders, diabetic or metabolic conditions, and cancer; it also affects the aging process [1]. The classical clinical entity in this category is the so-called mitochondrial myopathy, in which mitochondrial dysfunction is caused by mitochondrial or nuclear genetic abnormalities. The disease, which encompasses mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (OMIM 540000), is characterized by the early onset of stroke-like episodes and was first described by Pavlakis and colleagues in 1984 [2]; it is thought to be the most dominant subtype of mitochondrial dysfunction. At least 39 distinct mitochondrial DNA mutations have been associated with MELAS [3]; however, approximately 80% of MELAS patients have an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene (OMIM 590050) [4] and [5]. Because this mutation was also found to be a major genetic abnormality in diabetes mellitus, it may be a particularly common genetic variant in human populations [6]. Although more than 26 years have passed since the clinical and pathological definition of MELAS, there are few reports on its prevalence and epidemiology, and no reports exist on the natural course, survival rate or severity of the disease in a cohort study, meta-analysis, or nationwide survey [7] and [8]. In this study, we determined the prevalence, clinical symptoms, natural course, severity, and survival rate of MELAS patients in a nationwide Japanese

Abbreviations: JMDRS, Japanese mitochondrial disease rating scale; NPMDS, Newcastle pediatric mitochondrial disease scale; NMDAS, Newcastle mitochondrial disease adult scale

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cohort study. Additionally, we also evaluated the clinical rating scale that may be a very useful tool for the assessment of efficacy of therapeutic approach for mitochondrial myopathy.

2. Materials and methods

2.1. Study design, patients, and data collection for the Japanese cohort study

The cohort study was performed using questionnaires. To determine the prevalence of mitochondrial myopathies throughout the country, the first questionnaire was mailed in 2001 to 2236 neurology departments within Japan (1474 departments with pediatric neurologists and 762 departments with adult neurologists, including governmental, public, private and university hospitals with 50 beds or more). Patients' medical records were evaluated using MELAS diagnostic criteria (Table 1) and adequately screened. In 2003, after compiling the results of the first questionnaire, we mailed a second questionnaire to the neurologists who had examined MELAS patients in 2001. In 2008, we mailed a third questionnaire to the same group of neurologists. The second and third questionnaires included a Japanese mitochondrial disease rating scale (JMDRS) (Supplemental Table 1). Relevant information from the medical records of eligible patients was transcribed onto case report forms by neurologists, who were later interviewed by telephone if ambiguous data or unsatisfactory descriptions were found in the case report forms. Detailed documentation of the patients' clinical status was compiled by the same neurologists. The case report form was originally constructed according to the JMDRS and was updated whenever the scores were altered. Written informed consent was obtained from the patients or their legal guardians. The study protocol was approved by the Institutional Review Board (Kurume University #9715).

2.2. Diagnostic criteria for MELAS

The nationwide survey of MELAS in this study is based on the definitive diagnosis of MELAS presented in Table 1.

Table 1
Diagnostic criteria for MELAS (MELAS study committee in Japan).

Category A. Clinical findings of stroke-like episodes
1. Headache with vomiting
2. Seizure
3. Hemiplegia
4. Cortical blindness or hemianopsia
5. Acute focal lesion observed via brain imaging[a]
Category B. Evidence of mitochondrial dysfunction
1. High lactate levels in plasma and/or cerebral spinal fluid or deficiency of mitochondrial-related enzyme activities[b]
2. Mitochondrial abnormalities in muscle biopsy[c]
3. Definitive gene mutation related to MELAS[d]
Definitive MELAS
Two items of Category A and two items of Category B (four items or more)
Suspicion of MELAS
One item of Category A and two items of Category B (at least three items)

[a] Focal brain abnormalities in CT and/or MRI.

[b] 2 mmol/L (18mg/dl) or more lactate in plasma at rest or in cerebral spinal fluid and/or deficiency of electron transport chain enzyme, pyruvate-related, TCA cycle-related enzymes or lipid metabolism-related enzymes in somatic cells (desirable for muscle cells).

[c] RRF (ragged-red fiber) in modified Gomori's trichrome stain and/or SSV (strongly SDH-reactive blood vessels) in succinate dehydrogenase stain, cytochrome *c* oxidase-deficient fibers or abnormal mitochondria in electron microscopy.

[d] Definitive mitochondrial gene mutations reported in the literature (G583A, G1642A, G1644A, A3243G, A3243T, A3252G, C3256T, A3260G, T3271C, T3291C, G3481A, G3697A, T3949C, G4332A, G5521A, A5814G, G7023A, T7512C, A8296G, T8316C, T9957C, A12299C, A12770G, G13042A, A13084T, G13513A, A13514G, A13528G, and G14453A) as of 2010 [3].

2.3. Japanese Mitochondrial Disease Rating Scale (JMDRS)

We prospectively analyzed the clinical progress of MELAS using the JMDRS (Supplementary Table 1), which was revised following the European NeuroMuscular Conference (ENMC) in 2003 [9]. The second and the third questionnaires were also based on the JMDRS and enabled longitudinal analysis of disease progression. We established a rating score for each patient in 2003 and 2008, and these values were used to analyze the clinical severity of MELAS.

2.4. Statistical analysis

Demographic and clinical data for the juvenile and adult forms of MELAS were summarized using descriptive statistics. An unpaired *t*-test was used to test for any differences in the death rates of juvenile and adult forms. Differences between the juvenile and adult forms in the symptoms at onset and throughout the entire follow-up period were evaluated by chi-square tests or Fisher's exacts test when the criteria for the chi-square test were not fulfilled. Alterations in the JMDRS scores between 2003 and 2008 were evaluated using unpaired *t*-tests alone or combined with a Welch correction when variances were significantly different. Survival rates were compared between juvenile and adult forms using the log-rank test. Statistical analyses were performed with the SPSS 11.0 J software package for Windows. $p<0.05$ was considered statistically significant.

3. Results

3.1. Questionnaire responses from the Japanese cohort

We received 1051 responses to the first questionnaire (total 47.0% response rate, 1051/2236); among them, 756 were from pediatric neurology departments(51.3% of responses) and 295 were from adult neurology departments(38.7% of responses). We identified 741 patients with mitochondrial myopathies and of these, 233 were MELAS patients (31.4% of total mitochondrial myopathy patients, 233/741), as described by 105 pediatric neurologists and 29 adult neurologists. We received 64 responses to the second questionnaire (total 47.8% response rate, 64/134): 36 from pediatric neurologists (34.3% response rate, 36/105) and 28 from adult neurologists (96.6% response rate, 28/29). We received 64 responses to the third questionnaire (100% response rate, 64/64); only 96 MELAS patients completed the 5-year cohort study.

3.2. Prevalence of MELAS in Japan

We found 741 cases of mitochondrial myopathy in our cohort study. Based on the MELAS diagnostic criteria (Table 1), we found 233 MELAS patients (juvenile/adult = 111/122) among the Japanese population of approximately 127,434,000 (approximately 22,275,000 under 18 years of age and approximately 105,159,000 over 18 years of age, adult form, according to census data from 2001). The prevalence of mitochondrial myopathy in Japan is therefore at least 0.58 (95% confidence interval (CI), 0.54–0.62)/100,000 in the total population. The prevalence of MELAS is at least 0.18 (95%CI, 0.17–0.19)/100,000 in the total population, 0.50 (95%CI, 0.41–0.59)/100,000 in children under 18 years of age, and 0.12 (95%CI, 0.10–0.14)/100,000 in the population over 18 years of age.

3.3. Demographic and pathological findings of MELAS in the cohort study

Our cohort study included 96 MELAS patients who were followed prospectively for 5 years. A histogram and a density plot showing the various ages of onset in MELAS in these patients indicate an approximately bimodal distribution (Fig. 1). We therefore divided the MELAS patients into two sub-groups to determine whether MELAS

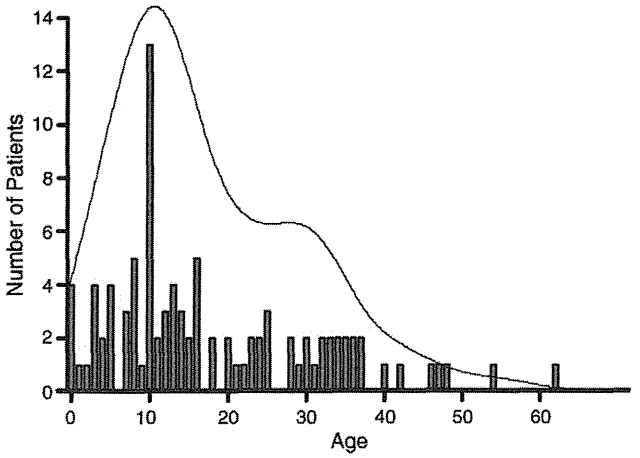

Fig. 1. Histogram detailing age of onset. A histogram and density plot of the various ages of MELAS onset is shown. In total, 96 Japanese patients were identified as having definitive MELAS as determined by diagnostic criteria (Table 1). Given the approximately bimodal distribution of patient age, MELAS patients were divided into two subgroups by the age of onset. Patients with an age of onset less than 18 years old were defined as having the juvenile form of MELAS, and patients with an age of onset greater than 18 years old were defined as having the adult form of MELAS.

has different features depending on the age of onset. Patients with an age of onset under 18 years were defined as having the juvenile form of MELAS, whereas patients with an age of onset above 18 years were defined as having the adult form of MELAS. A summary of the indexed MELAS patients is shown (Table 2). The ages of onset, diagnosis, and death were determined for both juvenile and adult forms. During this study, 17 of the 20 deceased MELAS patients presented with the juvenile form. Causes of death were cardiac insufficiency (7), severe respiratory infection (6), multiple organ insufficiency (3), and unknown causes (4).

Seventy-eight patients received muscle biopsies and 71 patients (91.0%) showed positive findings, including ragged-red fibers (RRF), SDH strongly reactive blood vessels (SSV), or both. However, seven patients presented normal features in the muscle biopsy.

3.4. Symptoms at onset and during the entire course

We evaluated the symptoms at onset in 96 MELAS patients (Table 3). The first sign of any symptoms or events such as seizure, stroke-like episode, or severe headache, which were associated with a neuroimaging-abnormality, was defined as the onset of MELAS. Symptoms at onset and during the entire course were similar as follows; seizure, stroke-like episode, and headache were the most frequent symptoms. Short stature and failure to thrive were significantly more prevalent in the juvenile form than in the adult form. However, hearing loss, diabetes mellitus and hemiplegia were significantly more frequent in the adult form than in the juvenile form.

Table 2
Demographic findings for MELAS cases.

	All form	Juvenile form	Adult
Patient[b] (male/female)	96 (52/44)	58 (35/23)	38 (17/21)
Age of onset, years[a]	17.7 ± 13.6	9.0 ± 4.7	32.2 ± 10.0
Age of diagnosis, years[a]	19.9 ± 13.5	11.0 ± 5.0	33.6 ± 10.6
Age of death, years[a]	18.8 ± 11.5	15.0 ± 7.9	40.0 ± 3.6
Death (%)[b,c]	20 (20.8 %)	17 (29.3 %)	3 (7.9 %)
Time from diagnosis to death[a]	7.3 ± 5.0	6.4 ± 4.5	10.2 ± 8.3
Positive family history[b] (%)	23 (24.0)	13 (22.4)	10 (26.3)
Muscle biopsy examination[b]	78	42	36
Positive findings[b] (%)	71 (91.0)	36 (85.7)	35 (97.2)
RRF[b]	56	24	32
SSV[b]	2	2	0
RRF + SSV[b]	13	10	3
A3243G mutation positive[b] (%)	75 (78.1)	46 (79.3)	29 (76.3)
Other mutation found in mtDNA[b]	4	4	0
Mutation not found[b]	17	8	9

[a] Mean ± SD.
[b] Number.
[c] Death ratio was higher for the juvenile form than for the adult form ($p = 0.0115$).

Table 3
Symptoms.

	Total (%)	Juvenile (%)	Adult (%)
	($n = 96$)	($n = 58$)	($n = 38$)
Symptoms at onset			
Seizure	54/96 (56.3)	36/58 (62.1)	18/38 (47.4)
Stroke-like episode	53/96 (55.2)	29/58 (50.0)	24/38 (63.2)
Headache	48/96 (50.0)	27/58 (46.6)	21/38 (55.3)
Short stature[a]	46/96 (47.9)	35/58 (60.3)	11/38 (28.9)
Muscle weakness	36/96 (37.5)	26/58 (44.8)	10/38 (26.3)
General fatigue	30/96 (31.3)	20/58 (34.5)	10/38 (26.3)
Cortical blindness	26/96 (27.1)	15/58 (25.9)	11/38 (28.9)
Failure to thrive[a]	25/96 (26.0)	23/58 (39.7)	2/38 (5.3)
Vomiting/nausea	23/96 (24.0)	17/58 (29.3)	6/38 (15.8)
Hearing loss[a]	21/96 (21.9)	6/58 (10.3)	15/38 (39.5)
Unconsciousness	19/96 (19.8)	10/58 (17.2)	9/38 (23.7)
Teichopsia	18/96 (18.8)	12/58 (20.7)	6/38 (15.8)
Diabetes mellitus[a]	12/96 (12.5)	2/58 (3.4)	10/38 (26.3)
Symptoms in the entire course			
Stroke-like episode	81/96 (84.4)	49/58 (84.5)	32/38 (84.2)
Seizure	68/96 (70.8)	42/58 (72.4)	26/38 (68.4)
Short stature[a]	53/96 (55.2)	37/58 (63.8)	16/38 (42.1)
Headache	52/96 (54.2)	30/58 (51.7)	22/38 (57.9)
Cortical blindness[a]	43/96 (44.8)	21/58 (36.2)	22/38 (57.9)
Muscle weakness	40/96 (41.7)	27/58 (46.6)	13/38 (34.2)
General fatigue	38/96 (39.6)	26/58 (44.8)	12/38 (31.6)
Mental regression	38/96 (39.6)	20/58 (34.5)	18/38 (47.4)
Gait disturbance	37/96 (38.5)	23/58 (39.7)	14/38 (36.8)
Unconsciousness	36/96 (37.5)	20/58 (34.5)	16/38 (42.1)
Teichopsia	31/96 (32.3)	20/58 (34.5)	11/38 (28.9)
Cardiac dysfunction	29/96 (30.2)	18/58 (31.0)	11/38 (28.9)
Failure to thrive[a]	27/96 (28.1)	24/58 (41.4)	3/38 (7.9)
Speech disturbance	22/96 (22.9)	16/58 (27.6)	6/38 (15.8)
Memory loss	20/96 (20.8)	12/58 (20.7)	8/38 (21.1)
Diabetes mellitus[a]	20/96 (20.8)	5/58 (8.6)	15/38 (39.5)

[a] Significant difference between juvenile and adult forms. $p<0.05$ was considered statistically significant. At onset: short stature ($p = 0.0026$), failure to thrive ($p = 0.0001$), hearing loss ($p = 0.0007$), diabetes mellitus ($p = 0.0014$). During follow-up: short stature ($p = 0.0366$), hearing loss ($p = 0.0012$), cortical blindness ($p = 0.0366$), failure to thrive ($p = 0.0004$), diabetes mellitus ($p = 0.0006$).

3.5. Disease progression monitored with the JMDRS

MELAS was monitored in 2003 and 2008 with the JMDRS, which covers (1) activities of daily living, (2) motor functions, (3) special sensory functions, (4) endocrine functions, (5) cardiac functions, (6) renal functions, and (7) cognitive functions. Though JMDRS has not yet been validated, all MELAS patients had a significantly higher JMDRS score in 2008 than in 2003 (Table 4). Although no differences in the 2003 JMDRS scores were observed between the juvenile and adult forms, the 2008 scores revealed that the juvenile form was associated with a more aggressive deterioration than the adult form. The variation in scores between 2003 and 2008 was much larger in the juvenile form than in the adult form (Table 4).

3.6. Survival curve

Fig. 2 shows the Kaplan–Meier survival curve for MELAS patients. The log-rank analysis showed significant differences in survival between the juvenile and adult forms ($p = 0.0105$). The juvenile

Table 4
Variations of the JMDRS score in the 5-year interval.

	2003	2008	*p* Value
Raw score (minimum = 0, maximum = 81)			
Total ($n=96$)*	4.4 ± 3.2	16.1 ± 9.2	0.0001
Juvenile onset ($n=58$)*	4.9 ± 3.0	19.1 ± 9.7[a]	0.0001
Adult onset ($n=38$)*	3.6 ± 3.5	11.5 ± 6.1[a]	0.006
Score variances between 2003 and 2008			
Total ($n=96$)*	11.8 ± 8.3		
Juvenile onset ($n=58$)*	14.5 ± 8.8[b]		
Adult onset ($n=38$) *	7.8 ± 5.6[b]	*Mean ± SD.	

[a] $p=0.0001$, raw scores between the juvenile and adult forms in 2008.
[b] $p=0.0001$, score variances between the juvenile and adult forms.

form had a higher rate of mortality than the adult form (hazard ratio, 3.29; 95%CI, 1.32–8.20).

4. Discussion

In this nationwide, multicenter, 5-year prospective Japanese cohort study, we determined the prevalence of mitochondrial myopathies such as Kearns Sayre syndrome (KSS)/progressive external ophthalmoplegia (PEO), Leigh syndrome, and MELAS. In our study, the prevalence of mitochondrial myopathy was at least 0.58/100,000 in the total population, with MELAS as the most common subtype (data not shown). Although the reported prevalence of mitochondrial disease varies depending on methodology, geography, ethnic group, and subject group, the population-based prevalence of mitochondrial disease risk was 9.18 to 12.48/100,000 in the total population of northeast England [10,11] and [12], 16.5/100,000 in the pediatric population of northeast England [10], 4.7 (95%CI, 2.8–7.6)/100,000 in the pediatric population of western Sweden [13], and 5.0 (95%CI, 4.0–6.2)/100,000 to 13.1/100,000 at birth in Victoria, Australia [14] and [15]. In general, epidemiological studies have estimated that the minimum prevalence of mitochondrial disease is 1/5000 in the general population [16]. The aforementioned prevalence estimates are approximately 10- to 34-fold higher than our estimate (0.58 (95%CI, 0.54–0.62)/100,000 in the total population).

This discrepancy can be explained partly by methodological differences. Because all previously reported prevalence data are based on estimations of risk of mitochondrial diseases extrapolated from the mutation or disease frequency in a limited population or region with regional mitochondrial research institutes or mitochondrial specialists, these values are likely to overestimate the prevalence in the entire population. Whether carriers of pathogenic mitochondrial DNA develop severe mitochondrial disorders depends on the degree and distribution of the mutation in important somatic organs. Although all prevalence studies can contain methodological bias, the prevalence of mitochondrial myopathy should be confirmed by a meta-analysis or nationwide cohort studies in other countries. The discrepancy between our prevalence estimate and previously reported data might also be attributable to a number of additional factors. First, we might have missed some patients due to the imperfect response rate (47%), even though our study included almost all of the main hospitals and institutes in Japan. There was no tendency with respect to region for the lack of responses. However, the response rate for the second questionnaire was significantly different between pediatric and adult neurologists. Pediatric neurologists may not have examined MELAS patients in 2003 although they had examined MELAS patients in 2001; because juvenile MELAS develops at a faster rate than adult MELAS, patients may have died or been referred to an inpatient hospital during 2-year interval. All non-responsive hospitals had less than 300 beds. Generally in Japan, MELAS that is very rare and multi-systematic diseases are monitored in large hospitals that have many departments and beds. Second, it is possible that the mitochondrial myopathies may have been misdiagnosed due to their rarity. Finally, given that most of the prior reports described Caucasian populations, it is possible that the disparity may derive from racial differences. In 2010, the Ministry of Health, Welfare and Labour, Japan has newly approved the mitochondrial myopathy as a supported disorders for their medical expenses, and started to collect the application for such privilege in entire Japan. In above situation, only 100 applications have been collected, to date (personal communication). Because our first questionnaire is also including death case of mitochondrial myopathies, our result come from disease-based prevalence study may be more realistic date at least in Japan.

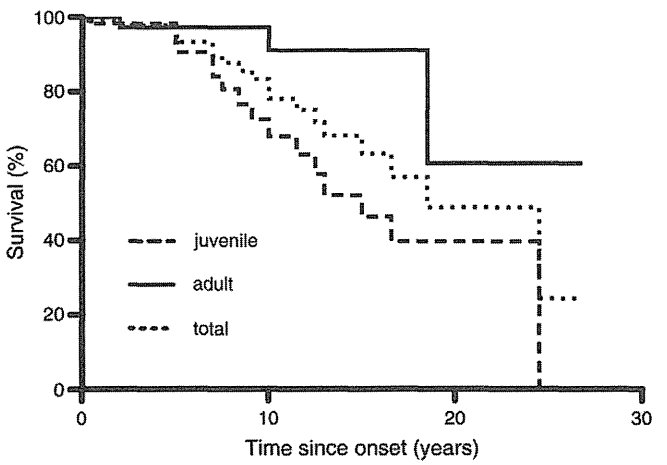

Fig. 2. MELAS survival curve. A Kaplan–Meier survival curve is shown. The dashed line indicates the juvenile form and the solid line indicates the adult form. The results of the log-rank analysis were significant. The juvenile form was associated with a higher risk of mortality than the adult form (hazard ratio, 3.29; 95%CI, 1.32–8.20).

Because MELAS is the most dominant subtype of mitochondrial myopathy and has been associated with an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene, several studies have reported the prevalence of the A3243G mutation. The absolute prevalence of this mutation has been estimated to be as high as 1.41 (95%CI, 0.83–1.20)/100,000 individuals in northern England [12] and [17], 16.3 (95%CI, 11.3–21.4)/100,000 in the adult population of northern Finland [18], and 18.4 (95%CI, 10.9–29.1)/100,000 in the Finnish pediatric population [19]. With the exception of a report from Australia in a large Caucasian population that showed the highest prevalence of 236/100,000 [20], the prevalence of MELAS in Japan (0.18 (95%CI, 0.17–0.19)/100,000) seems to be quite reasonable, given the previously reported prevalence of the A3243G mutation. Because this mutation has been found in association with various clinical conditions, including subclinical asymptomatic carriers and patients with short stature, diabetes mellitus, migraine headache, PEO, MELAS, and/or Leigh syndrome with cardiomyopathy, only individuals whose mutation load in important organs is 50% or more may present with MELAS or a more severe phenotype [21]. Multiple medical problems, including various neurological, cardiological, endocrinological, gastroenterological, and psychiatric symptoms, were reported in 45 families with 45 MELAS patients and 78 carrier relatives in a regional cohort study in the USA [22]. Accordingly, the actual prevalence of the A3243G mutation in human populations may be much higher than previously thought, if we take into account not only the individuals who showed full symptoms of mitochondrial disorders, but also those who show minimal symptoms, even when they were not followed up at a hospital. Nevertheless, MELAS is a clinically and histopathologically defined entity, and the prevalence of MELAS (0.18/100,000 in the total population) in Japan is unlikely to change drastically given more information.

To identify the various symptoms associated with MELAS, we defined the diagnostic criteria for MELAS in Japanese patients. The diagnostic criteria were constructed on the basis of the information provided by Hirano [23] and Hirano and Pavlakis [7]. Category A contains clinical findings of stroke-like episodes, while category B contains evidence of mitochondrial dysfunction. We evaluated only 96 out of 233 MELAS patients. The other patients were excluded for the following reasons; 1) non-response, 2) failure to receive informed consent, 3) patients/neurologists moved to other regions, and 4) other unknown reasons.

According to our data, MELAS can be divided into two subgroups: juvenile and adult forms. This distinction is warranted because of an approximately bimodal distribution of the age of onset, different manifestations of MELAS symptoms in pediatric and adult patients, and differences in the progression of disease as monitored by JMDRS scores. The juvenile form is more severe than the adult form (Tables 2 and 3). No differences in family history were noted between these two forms. However, the juvenile form was associated with significantly higher mortality and a more rapid disease progression than the adult form. We believe that this discrepancy arises because (1) children require more energy to complete their development and maintain their physicality and (2) juvenile patients may have a higher mutation load in mitochondrial genes than in adult patients. Almost all patients with the adult form have a normal life until onset despite having some kind of mitochondrial dysfunction. Therefore, it appears that the adult form requires a longer time for significant symptoms to develop and for the disease to worsen. Because the juvenile form has a greater mutation load than the adult form, it can present more severe complications such as the cardiac and/or renal failure, and patients with the juvenile form are at increased risk of multiple organ failure. Patients with the adult form of MELAS are more likely to have diabetes mellitus and to have a more gradual disease progression. Given these differences in disease progression, our 5-year cohort study may not have provided sufficient time to identify the chronic negative effects of diabetes mellitus especially in the adult form.

Among the clinical symptoms at onset or during the entire course of the disease, seizure and headache were very common and associated with stroke-like episodes in both the juvenile and the adult form. However, of the symptoms present at onset, short stature and failure to thrive were significantly more common in the juvenile form than in the adult form. In contrast, patients with the adult form presented with symptoms such as diabetes mellitus and hearing loss significantly more often than patients with the juvenile form, perhaps because these symptoms are more chronic and maturity (age)-related and can be induced by the accumulation of abnormal mitochondria in low-turnover environments such as pancreatic beta-cells or hearing organs. Of the symptoms encountered during the entire course of the disease, stroke-like episodes were noted in more than 84% of juvenile and adult form patients. Seizure and headache, which are the main symptoms associated with stroke-like episodes, were also common in both juvenile and adult forms. Interestingly, hearing loss, cortical blindness and diabetes mellitus, which are not recognized as main symptoms, were seen significantly more often in the adult form than in the juvenile form. The symptoms listed in our study are consistent with those of previous reports, including the American cohort study [7,8] and [24], the Finnish cohort study [19], and the Japanese muscle biopsy registry of MELAS [5].

We used JMDRS scores to evaluate the progression of MELAS over a 5-year interval. The validated mitochondrial disease rating scale was published in 2006 [25] and [26]. This scale has four classifications, which are age group classification of 0–24 months, 2–11 years, and 12–18 years from the Newcastle pediatric mitochondrial disease scale (NPMDS) [25], and an adult age group classification from the Newcastle mitochondrial disease adult scale (NMDAS) [26]. We had to use the JMDRS although it had not yet been validated because this study started in 2001, and the rating scale was initially mailed to the neurologists in 2003. Contents and indexed factors are similar between NPMDS, NMDAS, and JMDRS. However, NPMDS and NMDAS include contents from patient interviews. This feature is quite different between the Newcastle scales and the JMDRS. In all other respect, the JMDRS is thought to be a comprehensive, quantitative, reproducible, and sensitive monitoring system to detect the progression of disease severity in MELAS. We aimed to use and analyze JMDRS as a pilot study in the present work. According to this analysis, all MELAS patients (both juvenile and adult forms) showed an increased score and worsening of their condition during the 5-year interval. The progression of dysfunction in section 1 (activity of daily living), section 2 (motor activity) and section 7 (cognition and impairment) occurred more rapidly than that in other sections, and it was more pronounced in the juvenile form than in the adult form (data not shown). Patients with more rapidly increasing scores were more commonly found in the group with the juvenile form and had a higher risk of death than those with a more mild disease. This result indicates that the juvenile form progresses more rapidly and is more severe than the adult form. Despite the lack of validation, in this study the JMDRS produced findings that were consistent with a previous study [27] and we believe that the JMDRS is a useful scoring system that allows sensitive and reproducible monitoring of the progression of MELAS. In the future, we will more explicitly validate the JMDRS scoring system for MELAS.

In conclusion, given that no drugs have yet been approved for MELAS, we believe it is important to develop efficacious treatments for MELAS. L-arginine therapy, which is currently in development for MEALS [28], might be a promising drug for the future, and we believe that the results from this study will be helpful for the development of new therapeutic interventions aimed at MELAS.

5. Conclusions

We determined that MELAS occurs into two forms; adult and juvenile, and that the juvenile form is more severe than the adult form. Although our results may contain several biases, including limited information from neurologists, our data highlight new and important information for both pediatric and adult neurologists who are assessing MELAS patients. JMDRS is a useful scoring system for evaluating disease progression in MELAS.

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Preface

Biochemistry of mitochondria, life and intervention 2010

Mitochondrial research and medicine have been continuously expanded for the last 40 years. Since mitochondria play a central role in the metabolism of carbohydrates, lipids, and amino acids, alterations of mitochondrial functions have been implicated in various human disorders, such as mitochondrial myopathy, diabetes mellitus, aging-process, Alzheimer's disease, Parkinsonism, cancer, atherosclerosis, obesity, and metabolic syndrome.

Recent developments of clinical research and medicine indicate that the many human disorders have a link to mitochondrial function and possible to indicate the therapeutic application for cure of the disorders. ROS production from the respiratory chain plays pivotal roles not only in the control of proliferation and differentiation of cells but also in the regulation of mitochondrial mass in the cell. ROS is also related with aging and cartinogenesis. Molecular phathophysiology of maintenance of mitohondria is also discovered as fission and fusion mechanism, which are related to the quality control of mitochondria (mitophagy) seen in Parkinsonism. Many animal models are created by KO mice and are investigated the pathophysiology of disorders. Therapeutic clinical approaches are also investigated such as L-arginine on MELAS, sodium pyruvate for lactic acidosis, and hydrogen water for mitochondrial disorders. Assisted reproductive technology for mitochondrial disease patients is well developed in the fields to apply the clinical application. Such fundamental studies of mitochondrial bioenergetics could apply the new therapeutic indication for mitochondrial disorders.

In this special issue of BBA-general on " Biochemistry of Mitochondria, Life and Intervention 2010" which contains selected papers from 7th annual meeting of Asian Society for Mitochondrial Research and Medicine and 10th J-mit (Japanese Society of Mitochondrial Research and Medicine), we discuss the new aspect of mitochondrial functions relating to human disorders, and possible and on-going therapeutic approach of human disorders. This issue is organized in five chapters as follows: (i) Update mitochondrial research field, (ii) Mitophagy (fission and fusion), (iii) Animal model of mitochondrial disorders, (iv) Therapeutic approach of mitochondrial disorders, and (v) Mitochondrial pathophysiology in atherosclerosis, cancer, and aging.

Dr. Yasutoshi Koga is a professor of Pediatrics and Child Health, Kurume University Graduate School of Medicine, Japan. After he completed the MD and PhD, he joined the Mitochondrial Research Group in 1990 as a post doctoral research fellow granted by Muscular Dystrophy Association at the Department of Neurology, College of Physicians and Surgeons of Columbia University (Profs. DiMauro and Schon EA), where he directed his research to mitochondrial genetics especially pathogenic mechanism of MELAS. This led to the development of rho-zero cybrid system in mitochondrial research in 1992. He is the vice-president of Asian Society of Mitochondrial Research and Medicine and is organizing the Joint Symposium of 7th Asian Society of Mitochondrial Research and Medicine, and 10th Japanese Society for Mitochondrial Research and Medicine in 2010 at Fukuoka, Japan. He pioneered the development of a novel therapeutic procedure for MELAS and has completed the investigator-mediated clinical trial of L-arginine on MELAS. He received the Kelsey Wright Award from United Mitochondrial Disease Association (USA) in 2008. He now become a core committee member of International Mitochondrial Research and Medicine especially therapeutic division.

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Evaluation of Systemic Redox States in Patients Carrying the MELAS A3243G Mutation in Mitochondrial DNA

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Key Words
MELAS · A3243G mutation · Mitochondrial DNA · Oxidative stress · Antioxidant activity · Redox states · d-ROMs test · BAP test

Abstract
Background/Aims: To clarify the change of systemic redox states in patients carrying the A3243G mutation in mitochondrial DNA (A3243G), we evaluated oxidative stress and antioxidant activity in the serum of patients. ***Methods:*** Oxidative stress and antioxidant activity in the serum samples obtained from 14 patients carrying A3243G and from 34 healthy controls were analyzed using the diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests, respectively. ***Results:*** The mean d-ROMs level of all patients was significantly greater than that of the controls ($p < 0.005$), and the mean BAP/d-ROMs ratio of all patients was significantly lower than that of the controls ($p < 0.02$). In the patients with a history of stroke-like episodes ($n = 10$), both mean d-ROMs and BAP levels were increased compared with those of the controls (both $p < 0.01$). The mean BAP level of the patients without a history of stroke-like episodes ($n = 4$) was significantly decreased compared with that of the controls ($p < 0.001$), but the mean d-ROMs levels were not significantly different. ***Conclusion:*** d-ROMs and BAP tests indicated that patients carrying A3243G are always exposed to underlying oxidative stress, even at a remission state of stroke-like episodes.

Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome is the most common type of mitochondrial disease, and is mainly caused by an A-to-G transition mutation at nucleotide position 3243 (A3243G) in mitochondrial DNA (mtDNA) [1]. MELAS is characterized by stroke-like episodes that occur repeatedly and provoke neurological symptoms (e.g. headache, epilepsy, hemiparesis, and dementia) due to 'stroke-like' brain lesions [2]. In other words, stroke-like episodes are diagnostic symptoms of MELAS, and are crucial factors determining the prognosis of patients with this syndrome [2].

In addition, A3243G is responsible for not only stroke-like episodes but also mitochondrial cardiomyopathy or diabetes mellitus (DM) [1, 3–6]. Conversely, some patients carrying A3243G present with typical MELAS syn-

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Table 1. Demographic characteristics of patients and controls

Subject	Patients			Normal controls
	all	'stroke type'	'non-stroke type'	
Number	14	10	4	34
Gender (males/females)	7/7	5/5	2/2	20/14
Mean age at examination, years	32.1 ± 14.7	27.8 ± 12.5	42.8 ± 15.8	34.6 ± 7.4
Clinical features				
Stroke-like episodes, n	10	10	0	0
Cardiomyopathy, n	6	2	4	0
Diabetes, n	3	0	3	0
Under antioxidant therapy, n	11	10	1	0

Values are mean ± SD.

dromes with stroke-like episodes, and others present with only cardiomyopathy or DM without stroke-like episodes. However, the pathophysiological difference of phenotypes between the presence and absence of stroke-like episodes in patients carrying A3243G remains obscure.

Recent studies using cells cultured in vitro demonstrated increased oxidative stress in cells with impaired mitochondria due to A3243G [7–10]. Oxidative stress is provoked by reactive oxygen species (ROS) generation exceeding antioxidant defenses, such as manganese superoxide dismutase and glutathione peroxidase, and damages nucleic acids, proteins and lipids, which leads to cellular dysfunction. Indeed, previous pathological or imaging studies demonstrated enhanced regional oxidative stress in lesions of both stroke-like episodes and cardiomyopathy in patients carrying A3243G [11–13]. Therefore, there is a high possibility that oxidative stress participates in the pathogenesis caused by A3243G, and influences the phenotypic diversity. In other words, redox (reduction-oxidation) states should be evaluated in patients carrying A3243G both with and without a history of stroke-like episodes to clarify the role of oxidative stress in the emergence of stroke-like episodes.

To perform such an investigation, a rapid and reliable method of evaluating redox states in patients carrying A3243G is needed. Direct measurement of oxidative stress and antioxidant activity in living humans has been difficult; redox states have thus not been clearly evaluated in patients carrying A3243G to date. Recently, the diacron-reactive oxygen metabolites (d-ROMs) and biological antioxidant potential (BAP) tests have been used to evaluate redox states in serum. The d-ROMs level reflects the intensity of oxidative stress, and the BAP level indicates the activity of endogenous antioxidants [14, 15]. Their effectiveness as clinical markers has been reported in various diseases [16–22]. We evaluated redox states in fresh serum of both patients carrying A3243G and healthy volunteers using d-ROMs and BAP tests, and clarified the change of redox states due to A3243G and the pathophysiological difference in phenotypes with or without stroke-like episodes.

Subjects and Methods

Subjects

Fourteen Japanese patients (7 men and 7 women; mean age 32.1 ± 14.7 years) carrying A3243G were recruited at the University of Fukui Hospital, Fukui, and at the Kurume University Hospital, Fukuoka, Japan (table 1). Patients were classified by the presence or absence of stroke-like episodes into 'stroke type' and 'non-stroke type'. Ten patients with a history of stroke-like episodes were categorized as 'stroke type', and the other 4 patients who presented with mainly cardiomyopathy without a history of stroke-like episodes were categorized as 'non-stroke type'. Eleven patients were treated by antioxidant therapy such as coenzyme Q_{10} (CoQ_{10}; daily dose 30–90 mg) and/or vitamin E (daily dose 100 mg) administration; 10 of these patients were 'stroke type', and the other patient was 'non-stroke type'. Eight patients categorized as 'stroke type' were also treated with an oral administration of L-arginine (daily dose 14–21 g). All patients were in remission, free from exacerbation of symptoms or acute stroke-like episodes, when they were examined. Functional status was evaluated using the performance status rating (mean rating 1.3 ± 1.0). In 'stroke type' patients, the mean age of the first stroke-like episode was 21.1 ± 15.2 years, and the mean duration between the examination and the last stroke-like episode was 14.2 ± 9.2 months. 'Stroke type' patients had headaches and/or vomiting on average twice a month, but almost none had convulsions. Thirty-four Jap-

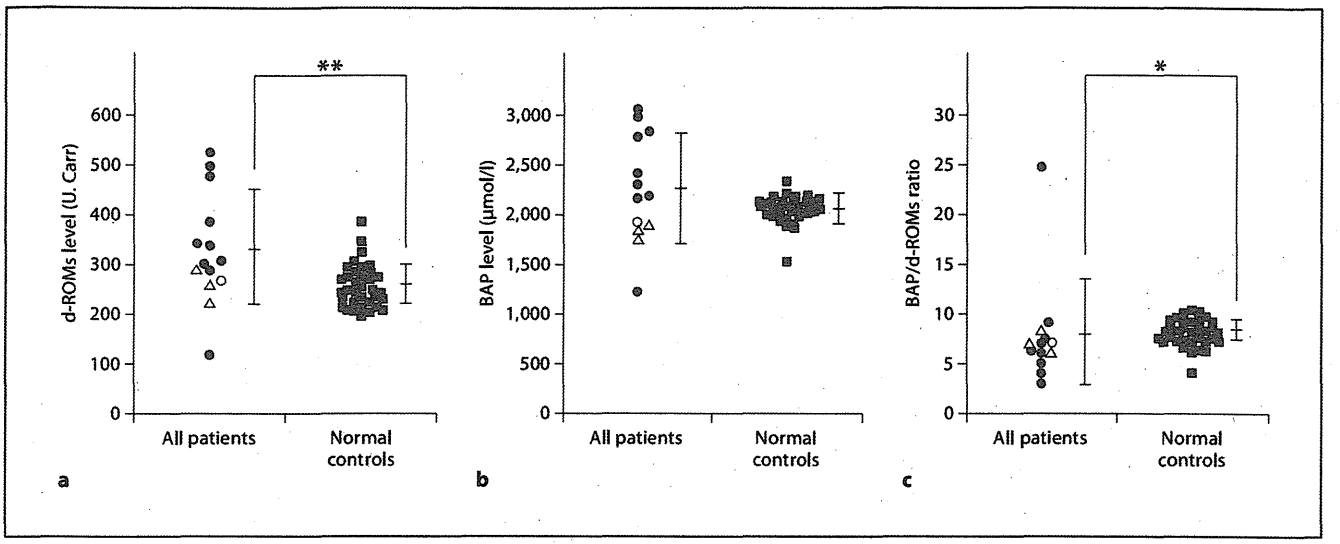

Fig. 1. Scatter plots portraying the levels of d-ROMs (**a**) and BAP (**b**) and BAP/d-ROMs ratios (**c**) in all the patients and controls. Circles and triangles correspond to the patients with and without antioxidant administration, respectively. In addition, closed and open diagrams correspond to the 'stroke type' patients and 'non-stroke type' patients, respectively. * $p<0.02$, ** $p<0.005$, according to the two-tailed Mann-Whitney U test. Bars indicate mean ± SD.

anese healthy volunteers (20 men and 14 women; mean age 34.6 ± 7.4 years) were also recruited as normal controls from the local community (table 1). This study was approved by the Ethics Committee of the University of Fukui. All subjects provided written informed consent to participate in the study.

Measurement of Oxidative Stress Levels

The oxidative stress levels were evaluated by measuring the quantity of hydroperoxides (R-OOH) in fresh serum samples using the d-ROMs test with the Free Radical Analytical System 4 (FRAS4^R; H&D srl, Parma, Italy) automatically [14]. Blood sampling was performed at fasting and at rest. Hydroperoxides consist of dehydrogenized and peroxidized proteins, lipids and fatty acids produced by ROS. In the d-ROMs test, hydroperoxides are turned to radicals by the Fenton reaction in an acid medium, and these generated radicals oxidize *N,N*-diethyl-paraphenylenediamine (DEPPD). Oxidized DEPPD quantity is determined by an absorbency measurement (white light 505 nm). The sequence of these methods is automated, and oxidative stress levels can be evaluated easily and quickly. The values are expressed as U. Carr, where 1 U. Carr corresponds to 0.8 mg/l H_2O_2.

Measurement of Antioxidant Activity Levels

The antioxidant activity levels were evaluated by measuring the quantity of molecules with antioxidative potency in fresh serum samples using the BAP test in the FRAS4^R automatically [15, 17]. Blood sampling was performed at fasting and at rest. In the BAP test, serum molecules with antioxidative potency reduce and decompose compounds of ferric chloride ($FeCl_3$) and thiocyanate derivative (AT) to $FeCl_2$ and free AT. Free AT is achromatized and dissociates from compounds, and is quantified by an absorbency measurement (white light 505 nm). The sequence of these methods is automated, and antioxidant activity levels can be evaluated easily and quickly. The results are expressed as μmol/l.

Statistical Analysis

The BAP-to-d-ROMs ratio (BAP/d-ROMs ratio) was calculated from the ratio of the BAP levels and d-ROMs levels for each subject. Data are presented as means ± standard deviations (SD). The resultant differences between normal controls and all patients were analyzed by means of a two-tailed Mann-Whitney U test. Since the subject number of each group was small, a non-parametric Kruskal-Wallis test was used for multiple data comparison and a post hoc Dunn test was performed to evaluate differences among normal controls, 'stroke type' patients and 'non-stroke type' patients. All statistical analyses were performed in SPSS Statistics Version 17.0 (SPSS Japan Inc., Tokyo, Japan), and $p<0.05$ was considered significant.

Results

The levels of serum d-ROMs and BAP, and BAP/d-ROMs ratios of all the patients and controls are shown in figure 1, and those of the 'stroke type' patients, 'non-stroke type' patients and controls are shown in figure 2. The mean age of each group demonstrated no significant differences.

The mean d-ROMs level of all patients (332.6 ± 110.7 U. Carr) was significantly higher than that of the controls

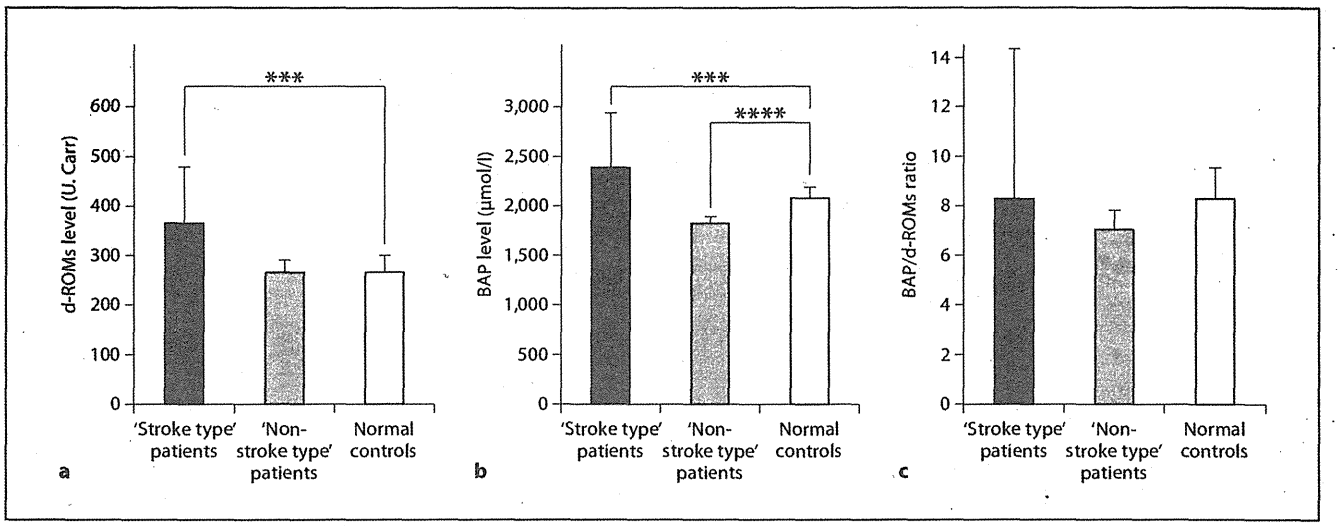

Fig. 2. The mean d-ROMs (**a**) and BAP (**b**) levels and mean BAP/d-ROMs ratio (**c**) in the 'stroke type' patients (black), 'non-stroke type' patients (grey) and controls (white). *** $p < 0.01$, **** $p < 0.001$, according to the Dunn test. Bars indicate mean ± SD.

(259.1 ± 42.0 U. Carr; $p < 0.005$) (fig. 1a). In particular, the mean d-ROMs level of the 'stroke type' patients (361.0 ± 119.6 U. Carr) was significantly greater than that of the controls ($p < 0.01$) (fig. 2a). Meanwhile, the mean d-ROMs level of 'non-stroke type' patients (261.5 ± 28.0 U.Carr) demonstrated no significant differences compared with those of the controls and 'stroke type' patients (fig. 2a).

The mean BAP level of all patients (2,258.9 ± 517.7 μmol/l) was not significantly different compared with that of the controls (2,057.6 ± 149.5 μmol/l) (fig. 1b). However, compared with the controls, 'stroke type' patients (2,428.9 ± 523.1 μmol/l) demonstrated significantly high BAP levels ($p < 0.01$), and 'non-stroke type' patients (1,834.0 ± 59.2 μmol/l) demonstrated significantly low BAP levels ($p < 0.001$) (fig. 2b). There was no significant difference between 'stroke type' patients and 'non-stroke type' patients in terms of the mean BAP levels.

The mean BAP/d-ROMs ratio of all patients (7.87 ± 5.05) was significantly lower than that of the controls (8.13 ± 1.30; $p < 0.02$) (fig. 1c). However, there were no significant differences among the controls and patient groups (fig. 2c).

There was no relationship between the functional status evaluated by performance status rating and the d-ROMs level or BAP level or BAP/d-ROMs ratio.

Discussion

In the present study, the d-ROMs and BAP tests were applied to evaluate the redox states in serum of patients carrying A3243G. These tests demonstrated that oxidative stress represented by the d-ROMs levels was increased and redox balance represented by the BAP/d-ROMs ratios was decreased (tendency for oxidation) in the patients compared with those of the controls (fig. 1). These findings suggested that an imbalance of redox states due to mitochondrial dysfunction affects the pathogenesis in patients carrying A3243G.

In the 'stroke type' patients in particular, both d-ROMs levels (oxidative stress) and BAP levels (antioxidant activity) were increased compared with those of the controls (fig. 2a, b). In vitro studies previously demonstrated that A3243G enhances ROS generation leading to oxidative stress [7–10], and enhanced oxidative stress is proportional to mitochondrial dysfunction [7, 23]. In the present study, all of the 'stroke type' patients have been treated with antioxidants, and 8 out of 10 patients were also treated with an oral administration of L-arginine. Although serum antioxidant activity may be increased by antioxidants and L-arginine therapy, serum oxidative stress was still increased in 'stroke type' patients. Increased oxidative stress even with increased antioxidant activity suggested a severe deterioration of mitochondrial function in patients with a history of stroke-like epi-

sodes, and that oxidative stress plays a crucial role not only in the brain lesions of stroke-like episodes [11, 12] but also systemically in these patients. In other words, a history of stroke-like episodes indicates that patients who have these episodes are exposed to underlying oxidative stress.

In the 'non-stroke type' patients, the mean d-ROMs level (oxidative stress) was not significantly different compared with that of the controls (fig. 2a). Meanwhile, the BAP levels (antioxidant activity) were significantly decreased (fig. 2b). Only 1 of 4 patients was treated with antioxidants, and antioxidant therapy may not affect antioxidant activity in 'non-stroke type' patients. These findings may reflect that antioxidants are consumed in order to prevent increase of oxidative stress in these patients. In addition, the difference of profiles in redox states between 'stroke type' and 'non-stroke type' suggested phenotypic diversity in patients carrying A3243G.

In the present study, we presented redox states in the serum of patients carrying A3243G using the d-ROMs and BAP tests. Rapid evaluation of redox states in serum has been difficult to date. To assay oxidative stress in serum, the spin trap method using electron spin resonance (ESR) has been the most reliable method [24]. However, performing ESR is cumbersome, thus it is difficult to apply this method in clinical practice. The d-ROMs test can evaluate oxidative stress in serum by measuring oxides due to hydroperoxides, and this test has been validated by ESR [25]. Likewise, each endogenous antioxidant can be measured, but there has been no method estimating the whole activity of endogenous antioxidants in serum to date. The BAP test provides a reliable indicator of the antioxidant activity in serum by measuring the ability to reduce ferric to ferrous ions [15]. Moreover, the d-ROMs and BAP tests only need a small amount of blood, and require only 15 min for measurement. Therefore, these methods are prompt and reliable, and suitable for evaluating redox states in patients.

Previous studies using postmortem organs or positron emission tomography imaging have demonstrated regional enhancement of oxidative stress in the brain lesions of stroke-like episodes and the heart lesions of cardiomyopathy in patients carrying A3243G [11–13]. Although enhanced oxidative stress due to A3243G has been proven in these lesions, systemic oxidative stress in patients carrying A3243G has not been evaluated to date. The present study demonstrated a systemic and underlying imbalance of redox states in these patients.

The present study has some limitations. (1) The 'non-stroke type' group included only 4 patients. (2) The mean age of 'non-stroke type' patients was likely older than that of 'stroke type' patients. (3) The 'stroke type' group included only 2 of 10 patients with cardiomyopathy or diabetes, which might affect the systemic redox states. (4) All of the 10 'stroke type' patients received antioxidant therapy, but only 1 of the 4 'non-stroke type' patients received antioxidant therapy. (5) This study did not show any significant difference in either value of oxidative stress or antioxidant activity between the 'stroke type' and 'non-stroke type' groups. (6) The possibility that the 'non-stroke type' patients in this study will also subsequently develop stroke-like episodes cannot be ruled out. Further studies are necessary to confirm our preliminary results.

Taken together, the d-ROMs and BAP tests clearly demonstrated an abnormality of redox states in patients carrying A3243G. In particular, enhanced oxidative stress in patients with a history of stroke-like episodes may reflect severe mitochondrial dysfunction, which would contribute to the emergence of stroke-like episodes. In addition, in patients without stroke-like episodes, consumption of antioxidant activity may indicate latent oxidative stress. These findings suggested that patients carrying A3243G are always exposed to underlying oxidative stress, and further antioxidant therapy would be beneficial to prevent an intensification of the symptoms.

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Disclosure Statement

The authors report no conflicts of interest.

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Metabolomic profiling rationalized pyruvate efficacy in cybrid cells harboring MELAS mitochondrial DNA mutations

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ABSTRACT

Pyruvate treatment was found to alleviate clinical symptoms of mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome and is highly promising therapeutic. Using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS), we measured time-changes of 161 intracellular and 85 medium metabolites to elucidate metabolic effects of pyruvate treatment on cybrid human 143B osteosarcoma cells harboring normal (2SA) and MELAS mutant (2SD) mitochondria. The results demonstrated dramatic and sustainable effects of pyruvate administration on the energy metabolism of 2SD cells, corroborating pyruvate as a metabolically rational treatment regimen for improving symptoms associated with MELAS and possibly other mitochondrial diseases.

1. Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) syndrome is one of the mitochondrial cytopathies first introduced by Pavlakis et al. (1984). Complex pathologies observed in MELAS patients primarily stem from the adenine-to-guanine transition mutation at position 3243 of the mitochondrial genome (A3243G) located in the mitochondrial $tRNA^{Leu(UUR\ (R=A\ or\ G))}$ gene (Goto et al., 1990), which accounts for about 80% of all cases of MELAS (Koga et al., 2012b) and is estimated to be carried by as high as 0.06% of the general population (Sproule and Kaufmann, 2008). The mutated mitochondrial $tRNA^{Leu(UUR)}$ gene recognizes the UUA but not the UUG codon and causes protein synthesis defects due to a shortened life-span of $tRNA^{Leu(UUR)}$, a lowered ratio of aminoacyl- to uncharged-$tRNA^{Leu(UUR)}$, accumulations of leucine aminoacylation and processing intermediates, and a defect in modifying uridine to 5-taurinomethyluridine at the first position of the anticodon (Koga et al., 2012b). Due to the frequent appearance of the UUG codons in the coding sequence of NADH dehydrogenase subunit 6, mitochondria in the skeletal muscle of MELAS patients often exhibit defects in activities of complex I (Ichiki et al., 1989) and complex IV and others in severe cases (Iizuka and Sakai, 2005; Yoneda et al., 1989). The defects in these respiratory complexes induce impaired oxidative phosphorylation, increased generation of free radicals, and a decreased level of free nitric oxide (Hussein et al., 2009). Disordered ATP production has also been confirmed as a pathogenesis of MELAS by studies using cytoplasmic hybrids, or "cybrids," which are human cell lines containing the patient's mitochondria with mutated genomes (DiMauro and Schon, 2003). Most treatment regimens for mitochondrial diseases including MELAS are designed to mitigate the cellular consequences of dysfunction of the respiratory chain by supplementation with electron acceptors and reactive oxygen species scavengers such as creatine, coenzyme Q10, α-lipoic acid, and vitamins (riboflavin, thiamine, vitamin C, vitamin E, and biotin); however, the clinical efficacy of these supplements remains limited or doubtful (Sproule and Kaufmann, 2008). Recently, pyruvate treatment was found to alleviate muscle impairment in patients with not only MELAS (Tanaka et al., 2007) but also other mitochondrial diseases such as Leigh's syndrome (Koga et al., 2012a; Komaki et al., 2010) and mitochondrial depletion syndrome (Saito et al., 2012) without causing notable side effects, and thus is considered promising as an alternative therapeutic. Metabolic mechanisms of pyruvate efficacy in MELAS mutant cells, however, are not clearly understood. We thus aimed to

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elucidate the metabolic responses of pyruvate-supplemented MELAS mutant cells by performing time-course metabolome analysis using capillary electrophoresis time-of-flight mass spectrometry or CE-TOFMS (Soga et al., 2003, 2006). Metabolomics technologies make it possible to simultaneously identify and quantify hundreds of metabolites contained in cells (Ishii et al., 2007; Ohashi et al., 2008), tissues (Hirayama et al., 2009; Soga et al., 2006) or body fluids (Soga et al., 2006; Sugimoto et al., 2010). Among other analysis platforms frequently used in metabolomics research, such as liquid chromatography or gas chromatography combined with mass spectrometry (LC-MS and GC-MS, respectively) and nuclear magnetic resonance (NMR) analysis, CE-TOFMS specializes in a comprehensive measurement of charged compounds (Soga et al., 2003) and thus is best suited to quantitatively analyze alterations of energy metabolism in cells. In addition, the use of ^{13}C-labeled compounds and quantification of the resulting ^{13}C-labeled isotopomers enable an investigator to trace small amounts of labeled compounds, thus facilitating our understanding of metabolic fluxes. Therefore, we used this approach to measure and quantify time-course changes in 161 intracellular metabolites in, and 85 metabolites released into the medium (hereafter referred to as medium metabolites) by, cybrid human 143B osteosarcoma cells containing normal mitochondria (2SA cells) or MELAS mutant mitochondria (2SD cells) cultured with 10 mM [3-^{13}C] pyruvate. Since most MELAS patients exhibit a symptom of lactic acidosis in addition to seizures and stroke-like events (*e.g.*, 94 of 101 (94%), 97 of 102 (96%), and 106 of 107 (99%) patients, respectively (Hirano and Pavlakis, 1994)), the medium was supplemented with 10 mM [3-^{13}C] lactate as a comparative control condition. The resulting metabolomic profiles highlighted the basal metabolic differences between 2SA and 2SD cells and their metabolic alterations and flux patterns in response to a high dose of lactate or pyruvate. In particular, constantly low ATP levels and poor energy charge characterized the basal metabolism of lactate-supplied 2SD cells and were likely due to impaired oxidative phosphorylation; however, pyruvate administration improved the lactate-to-pyruvate ratio ([Lac]/[Pyr]) and NADH-to-NAD^+ ratio ([NADH]/[NAD]) in 2SD cells, which enhanced glycolysis and replenished TCA cycle intermediates for maintaining the ATP at a level as high as that in 2SA cells. These results demonstrated a dramatic and favorable effect of pyruvate administration on the energy metabolism of 2SD cells, supporting the idea that balancing the [NADH]/[NAD] ratio is crucial for facilitating active glycolysis and replenishing TCA intermediates in MELAS mutant cells for a sufficient and stable energy production.

2. Materials and methods

2.1. Cell culture

The 2SA and 2SD cybrid cell lines carrying 100% wild-type and 94% A3243G mutant mtDNA, respectively, were established by fusion of mtDNA-deficient $\rho^0$206 cells generated from human 143B osteosarcoma cell line with enucleated myoblasts derived from a MELAS patient (Chomyn et al., 1992; Tanaka et al., 2002; Yoneda et al., 1994). The cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, and 0.4 mM uridine and maintained under 5% CO_2 at 37 °C. The experiments were initiated by replacing the medium with DMEM supplemented with 10% fetal bovine serum, 0.4 mM uridine, and 10-mM [3-^{13}C] lactate or 10-mM [3-^{13}C] pyruvate.

2.2. Metabolite extraction

Cell and medium samples were obtained 0, 1, 2, and 4 h after 10 mM [3-^{13}C] lactate or 10 mM [3-^{13}C] pyruvate administration. The sample medium was mixed with methanol containing 50 μM internal standards (3-aminopyrolidine, L-methionine sulfone, trimesate, 2-morpholinoethanesulfonic acid, and D-camphor-10-sulfonic acid), Milli-Q water, and $CHCl_3$ in the ratio of 1:4:2:5. The cells were washed twice with 5% mannitol solution and covered with methanol (1 ml) containing 25 μM internal standards for enzyme inactivation. The methanol and cell mixtures were collected and mixed with Milli-Q water and $CHCl_3$ in the ratio of 2:1:2. Both the medium and cell sample solutions were then centrifuged at 20,000 ×*g* for 15 min, and the aqueous layers were collected for centrifugal filtration through a 5-kDa-cutoff filter at 9,000 ×*g* for 2.5 h. The extracted metabolites were concentrated with a centrifugal concentrator (Tomy, Tokyo, Japan) and stored at −80 °C until analysis could be performed. The appropriate volume of Milli-Q water was added for the dissolution of the concentrated metabolites immediately before the sample injection into the CE-TOFMS.

2.3. Metabolome analysis

Concentrations of all the charged compounds were measured by CE-TOFMS using the methods developed by (Soga et al., 2006, 2003). Briefly, for analyzing cations (Soga et al., 2006), a fused silica capillary (50 μm i.d. × 100 cm total length) was used with 1 M formic acid as both the running and preconditioning buffer. Each sample (approximately 3 nl) was injected by applying a pressure of 50 mbar for 3 s and a continuous voltage of +30 kV. A solution of 5 mM ammonium acetate and 0.5 μM reserpine in 50% (v/v) methanol in water was used as the sheath liquid at a flow rate of 10 μl/min. For analyzing anions (Soga et al., 2006), a commercially available cationic capillary, SMILE(+) (Nacalai Tesque, Kyoto, Japan), was used with 50 mM ammonium acetate solution (pH 8.5) as the running buffer and 50 mM acetic acid (pH 3.4) as the preconditioning buffer. Each sample (approximately 30 nl) was injected by applying a pressure of 50 mbar for 30 s and a continuous voltage of −30 kV. For analyzing nucleotides and coenzyme A compounds (Soga et al., 2007), the fused silica capillary and 50 mM ammonium acetate (pH 7.5) were used. A voltage of −30 kV was applied to the inlet capillary, along with pressure of 50 mbar, to maintain the conductive liquid junction at the capillary outlet.

2.4. Metabolome data processing

The CE-TOFMS data were preprocessed by our proprietary software, MasterHands, which calculates accurate *m/z*, quantifies peak areas from the electropherogram, and aligns the peaks of multiple datasets. We quantified the concentrations of 161 intracellular and 85 medium metabolites including ^{13}C-labeled isotopomers involved in primary energy metabolism such as glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle, urea cycle, and the metabolism of amino acids and nucleotides. The average amount of each metabolite per cell was evaluated based on the number of viable cells, which was counted at each sampling time by using a Countess Automated Cell Counter (Invitrogen, Carlsbad, California, US). For pyruvate, lactate, phosphoenolpyruvate, Gly, Ala, Ser, Asn, Asp, citrate, isocitrate, 2-oxoglutarate, succinate, fumarate, and malate, intracellular amounts and medium concentrations of their isotopomers were evaluated, taking into account the natural isotope abundance of C, H, and O atoms, according to the method of van Winden et al. (2002). Subsequently, z-values were evaluated for each compound on the basis of the average values of the time-course data and presented as a heat map in relation to the values determined at t = 0 followed by Euclidean distance-based hierarchical clustering using MeV (Saeed et al., 2003). The processed datasets were also comprehensively visualized on metabolic pathways constructed by using VANTED software (Junker et al., 2006).

3. Results

3.1. Heat map representation of time-series metabolome data from 2SA and 2SD cells

The concentrations of 161 intracellular and 85 medium metabolites including ^{13}C-labeled isotopomers were comprehensively mapped onto

metabolic pathways to enhance viewability (Supplementary Fig. 1A and B for the cell and medium metabolome data, respectively). Overall trends of the intracellular metabolomic changes in 2SA and 2SD cells were analyzed by Euclidean-distance-based hierarchical clustering analysis, and the results were presented as a heat map (Fig. 1), which highlighted the following intriguing features: First, the metabolomic profiles of lactate- and pyruvate-supplied 2SA cells were analogous to each other except for the changes in the metabolites in cluster 2 including glycolytic and PPP intermediates such as sedoheptulose 7-phosphate, dihydroxyacetone phosphate, ribulose 5-phosphate, and glucose 1-phosphate. Second, the metabolomic profiles of the lactate-supplied 2SA and 2SD cells were significantly different, particularly regarding the changes in metabolites in cluster 1, such as essential amino acids and those in cluster 3, such as ATP and 2-oxoglutarate, which showed nearly opposite trends. Third, the trends of the pyruvate-supplied 2SA and 2SD cells, however, significantly resembled each other except for the changes in only a few TCA cycle intermediates and amino acids. This resemblance was made even clearer from the results of the principal component analysis of the time-course metabolome data (Fig. 2A), which illustrates that the metabolomic profiles of lactate-supplied 2SD cells were located in the 3rd quadrant and thus deviated from the other profiles, whereas those of pyruvate-supplied 2SA and 2SD cells were aggregated mostly in the 4th quadrant. Pyruvate administration thus exhibited a dramatic effect on the energy metabolism of the MELAS mutant 2SD cells, redressing their overall metabolomic profile such that it resembled the profile of 2SA cells as a whole.

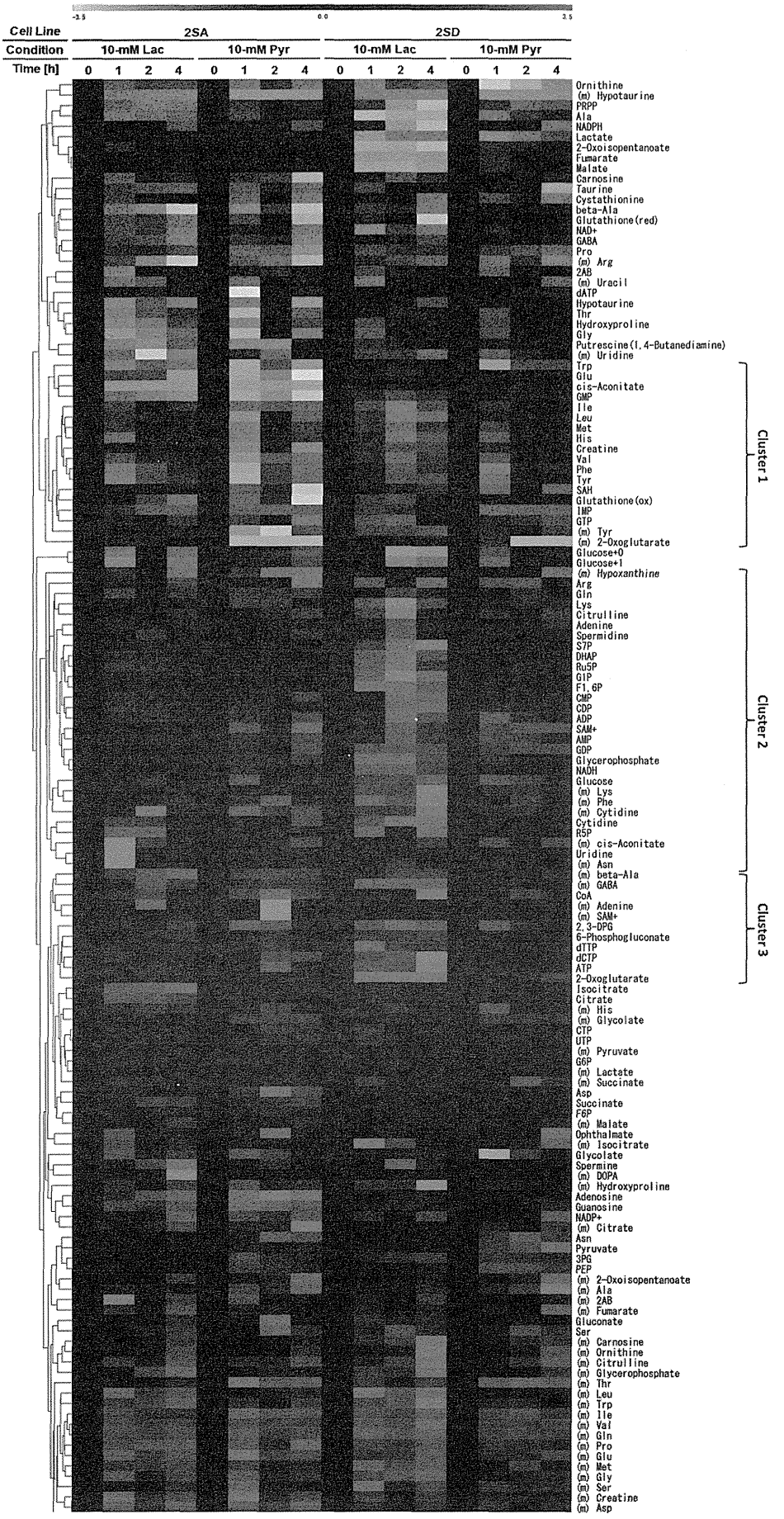
3.2. Energy status and metabolic parameters

The energy charge in the lactate-supplied 2SD cells decreased to 0.92 in 4 h from the initiation of the experiment but was otherwise maintained at ~0.96 under the other conditions (Fig. 2B). In these lactate-supplied 2SD cells, the levels of ATP and resulting total adenylates also dropped but ADP and AMP levels slightly increased (Fig. 2B), which characterizes a defective energy production in 2SD cells when cultured with a high dose of lactate. A low basal ATP level in 2SD cells was also identified in a previous study of ours (Fujita et al., 2007). Since no significant difference was observed in the reduced- to oxidized-glutathione ratio ([GSH]/[GSSG]) or in the NADPH-to-$NADP^+$ ratio ([NADPH]/[NADP]), the redox status of the 2SD cells appeared to be relatively well-maintained even under lactate-supplied condition (Fig. 2B). In contrast, [Lac]/[Pyr] and [NADH]/[NAD] ratios significantly increased only in the lactate-supplied 2SD cells (Fig. 2B), which implies an enhancement of lactic acidosis, NAD^+ shortage, and resulting stagnations of NAD^+-dependent reactions. Indeed, levels of fructose 1,6-bisphosphate and dihydroxyacetone phosphate, metabolites upstream of the glyceraldehyde 3-phosphate dehydrogenase reaction, which requires NAD^+ as a cofactor, significantly increased in level only in the lactate-supplied 2SD cells (Supplementary Fig. 1A). Glycolytic ATP production thus might have been stagnated due to the high [NADH]/[NAD] ratio. With pyruvate treatment, however, the 2SD cells retained [Lac]/[Pyr] and [NADH]/[NAD] ratios as low as those in 2SA cells (Fig. 2B). Taken together, these results indicate that the energy and redox statuses of 2SA cells were relatively robust in response to a high dose of either lactate or pyruvate whereas those of 2SD cells were vulnerable and susceptible to a high dose of lactate but were well-maintained and greatly approximated to those of 2SA cells as a result of pyruvate treatment, which facilitated efficient ATP production and improved the energy status by decreasing the [Lac]/[Pyr] ratio and maintaining the [NADH]/[NAD] ratio.

3.3. Isotopomer distribution and amino acid metabolism

The changes in lactate or pyruvate metabolism of 2SA and 2SD cells were interpreted by examining the distribution of isotopomers in the cells (Fig. 3) and medium (Supplementary Fig. 2), which were derived from the [3-^{13}C] lactate or [3-^{13}C] pyruvate used to supplement the medium. The initial intracellular lactate level in 2SD cells (at $t=0$) was more than twice as high as that in 2SA cells (47.9 and 21.2 fmol/cell, respectively; Fig. 3). The lactate level in 2SD cells abruptly dropped 1 h after the medium replacement but gradually increased afterwards under both lactate- and pyruvate-supplied conditions, whereas that in 2SA cells was almost constant and remained low. The percentage of ^{13}C-labeled lactate to the total lactate in pyruvate-supplied 2SD cells was ~35% and unexpectedly higher than under the other conditions (~25%), which indicates that the proportion of lactate production from the medium-derived [3-^{13}C] pyruvate increased in the pyruvate-supplied 2SD cells. Intracellular pyruvate, in contrast, significantly increased only under the pyruvate-supplied condition (8.7- and 13.2-fold in 4 h in 2SA and 2SD cells, respectively) but was remained low and almost constant in lactate-supplied conditions. Accordingly, the [Lac]/[Pyr] ratio in pyruvate-supplied 2SD cells was lowered not by the decrease in lactate but primarily by the significant increase in pyruvate, which thus boosted the flux from pyruvate to lactate and thereby balanced the [NADH]/[NAD] ratio. In addition, the significant increase in unlabeled pyruvate in pyruvate-supplied 2SD cells implies that pyruvate treatment enhanced not only the influx of pyruvate from the medium into the cells but also the pyruvate production from other routes such as glycolysis. Similar to the trend of the pyruvate level, 2-oxoglutarate, fumarate, and malate levels in 2SD cells were also significantly higher under pyruvate-supplied condition than under the lactate-supplied condition, as were the proportions of ^{13}C-labeled isotopomers. Pyruvate administration thus enhanced the replenishment of these TCA cycle intermediates, which were produced partly from [3-^{13}C] pyruvate (~37% and ~3% in 2SA and 2SD cells, respectively, for 2-oxoglutarate and ~40% and ~18% in 2SA and 2SD cells, respectively, for both fumarate and malate) and largely from other metabolites. Nevertheless, this finding does not necessarily imply that the TCA cycle activity of 2SD cells was enhanced by pyruvate treatment, since $^{13}C_2$-isotopomers of most TCA cycle-intermediates, which were supposedly produced from [3-^{13}C] pyruvate after the 2nd or more cycles of the TCA cycle, were quantifiable in 2SA cells but were largely below the detection limits in 2SD cells, probably due to the impaired oxidation of NADH by the respiratory chain in the 2SD cells. Intracellular and medium levels of Ala under the pyruvate-supplied condition were also higher than those under the lactate-supplied one, with a high proportion of $^{13}C_1$-labeled isotopomers in both cell lines (~51% and ~50% in 2SA and 2SD cells, respectively). The increase in the concentrations of both labeled and unlabeled medium Ala in the pyruvate-supplied 2SD cells (Supplementary Fig. 2) is of particular significance among other changes of medium amino acids (Supplementary Fig. 3). These data imply that pyruvate treatment enhanced conversion of [3-^{13}C] pyruvate to $^{13}C_1$-alanine coupled with the glutamate to 2-oxoglutarate conversion by alanine aminotransferase, which might have contributed to the increase in 2-oxoglutarate (Fig. 3) while excreting Ala into the medium as a byproduct, especially in the pyruvate-supplied 2SD cells. Pyruvate treatment appears also to have enhanced Asp production in both cell lines, but the basal level of Asp was higher in 2SA cells than in 2SD ones. Although the levels of both ^{13}C-labeled and unlabeled Asp isotopomers increased in both cell lines, the $^{13}C_2$-labeled Asp isotopomer, which would have been produced from $^{13}C_2$-labeled

Fig. 1. Metabolomic profiles of 2SA and 2SD cells supplemented with 10-mM lactate or 10-mM pyruvate for 161 intracellular metabolites and 85 metabolites in the medium. The metabolite names preceded by "(m)" indicate the medium levels of the corresponding metabolites and the others indicate the intracellular levels. The total concentration of ^{13}C-labeled and -unlabeled isotopomers was used for pyruvate, lactate, phosphoenolpyruvate, Gly, Ala, Ser, Asn, Asp, citrate, isocitrate, 2-oxoglutarate, succinate, fumarate, and malate. Features of the clusters 1–3 are explained in the text.



oxaloacetate, for example, was barely observed in 2SD cells, representing again their stagnated TCA cycle activity. Among other amino acids (Fig. 4 for cells and Supplementary Fig. 3 for medium), the Pro level was overall higher in 2SA cells than in 2SD cells. Despite our previous finding of the up-regulation of the asparagine synthetase (ASNS) gene in MELAS mutant cells through the elevation of ATF4 expression and its binding to NSRE-1 (Fujita et al., 2007), the change in the Asn level was not significantly different between cell lines but rather was dependent on lactate or pyruvate treatment in this study. Interestingly, the levels of all the essential amino acids except Thr were slightly but consistently higher in lactate-supplied 2SD cells than in those under the other conditions (Fig. 4), and this trend nevertheless disappeared by pyruvate treatment.

4. Discussion

MELAS syndrome is one of the most frequently occurring, maternally inherited mitochondrial disorders that devastatingly affect multiple organs including brain, nervous system, and muscles, as well as cognitive abilities. Although the efficacy of most treatment regimens has remained limited or doubtful (Sproule and Kaufmann, 2008), pyruvate was recently identified as an effective, safe, and affordable therapeutic agent that exhibits favorable effects on symptoms associated with not only MELAS (Tanaka et al., 2007) but also other mitochondrial diseases (Komaki et al., 2010; Saito et al., 2012). In order to elucidate the therapeutic mechanisms of pyruvate treatment from a viewpoint of energy metabolism, we used CE-TOFMS to investigate the metabolic profiles of the 2SA cells or MELAS mutant 2SD cells treated with 10 mM lactate or 10 mM pyruvate. The results revealed significant differences between the metabolomic profiles of 2SA and 2SD cells under the lactate-supplied condition and contrasting remarkable resemblances under the pyruvate-supplied condition. We previously reported that 2SD cells show up-regulated expression of genes associated with growth arrest (GADD45A, GADD45B, and CHOP) and exhibit a slower growth rate than their parental strain (Fujita et al., 2007). A recent study using human cybrid cells harboring the A3243G mutation also showed an increasing susceptibility of the cells to apoptosis and a high level of mutated mtDNA (Liu et al., 2004). Although no difference was observed between the cell viability of 2SA and 2SD cells in 4 h of culturing under the lactate- or pyruvate-supplied condition in this study (Supplementary Fig. 4), an unsound energy status of 2SD cells characterized by consistently low ATP levels and energy charges was observed under the lactate-supplied condition (Fig. 1), rationalizing their low cell viability in long-term cultures. Due to their limited capacity for oxidative phosphorylation-dependent ATP production, the lactate-supplied 2SD cells might have relied more on GTP and GTP → ATP conversion, which can be backed by relatively high GTP levels and significantly higher GTP-to-ATP ratios ([GTP]/[ATP]), than the cells in the other 3 groups (Supplementary Fig. 4). The increased IMP only in the lactate-supplied 2SD cells suggests a possibility of enhanced purine degradation via AMP deaminase. The energy metabolism of 2SD cells treated with lactate was thus not only defective in maintaining stable energy production but also unordinary in terms of their purine turnover.

The metabolomic profiles of the lactate-supplied 2SD cells exemplified the basal metabolism of MELAS patients with the symptom of lactic acidosis. With impaired mitochondrial complexes and a limited capacity for oxidative phosphorylation, 2SD cells are considered to rely inevitably on enhanced anaerobic glycolysis for ATP generation, which leads to increased flux from pyruvate to lactate and thus exacerbates lactic acidosis. ATP production via glycolysis is known to be stagnated when [Lac]/[Pyr] exceeds 25.6 (Voet and Voet, 1995). In both cell lines, the [Lac]/[Pyr] ratio was maintained below 25.6 under the pyruvate-supplied condition but not under the lactate-supplied one, under which the [Lac]/[Pyr] ratio in the normal 2SA cells was almost constant and ~43.1 but that in 2SD cells continuously increased and eventually exceeded 100 in 4 h. The 2SA cells can potentially shift their metabolism to oxidative phosphorylation for energy production when glycolysis is stagnated. In contrast, 2SD cells are left with ineffective glycolysis and intrinsically defective oxidative phosphorylation, and this situation impedes the production of sufficient ATP. Under the lactate-supplied condition, the increasing rate of the total (labeled and unlabeled) lactate concentration in the medium, which can be estimated by assuming a linear change in time and considering only the initial and final concentrations, was 731 and 919 fmol/cell/h in 2SA and 2SD cells, respectively, and that of unlabeled lactate in the medium was 765 and 1133 fmol/cell/h in 2SA and 2SD cells, respectively. Thus, the excretion rate of unlabeled lactate, which is probably the by-product of anaerobic glycolysis, appears to have been much higher in 2SD cells than in 2SA cells. Similarly, the decreasing rate (or incorporation rate into the cells) of $^{13}C_1$-labeled medium lactate was estimated and was again much higher in 2SD cells than in 2SA cells (206 and 32.6 fmol/cell/h, respectively), which thus shows a quick turnover of lactate in the lactate-supplied 2SD cells. Even under the pyruvate-supplied condition, the increasing rate of lactate (the total of labeled and unlabeled) was higher in 2SD cells than in 2SA cells (1310 and 676 fmol/cell/h, respectively). Unexpectedly, this increase in 2SD cells was even higher with pyruvate treatment in comparison with lactate treatment, indicating that pyruvate administration did not lower but rather enhanced lactate production. Although the trend of the total pyruvate concentration in the medium in 2SA and 2SD cells was analogous under the pyruvate-supplied condition, the turnover of medium pyruvate was also significantly higher in 2SD cells than in 2SA cells. The decreasing rate of $^{13}C_1$-labeled medium pyruvate was 353 and 536 fmol/cell/h in pyruvate-supplied 2SA and 2SD cells, respectively, while the increasing rate of unlabeled medium pyruvate was 271 and 424 fmol/cell/h in pyruvate-supplied 2SA and 2SD cells, respectively. Accordingly, pyruvate treatment significantly enhanced the incorporation of [3-^{13}C] pyruvate from and the excretion of lactate into the medium of 2SD cells, resulting in a balanced [NADH]/[NAD] and a sustainable ATP production primarily via anaerobic glycolysis in these cells. The [Lac]/[Pyr] ratio was therefore maintained low primarily by the alteration of pyruvate levels rather than lactate levels.

In lactate-supplied 2SD cells, the levels of essential amino acids increased (cluster 1 in Fig. 1), whereas 2-oxoglutarate and nucleoside triphosphates such as ATP, dTTP, and dCTP decreased (cluster 3 in Fig. 1); however, these characteristics were alleviated by pyruvate treatment, and the profile closely resembled that of the pyruvate-supplied 2SA cells. The differences in these metabolite profiles were rather trivial between lactate- and pyruvate-supplied 2SA cells. Supposedly, lactate was actively converted to pyruvate and then to TCA cycle intermediates for efficient oxidative phosphorylation in the 2SA cells, whereas lactate was hardly converted to pyruvate and rather accumulated in and out of the 2SD cells due to a high [NADH]/[NAD] ratio. In short, pyruvate treatment of 2SD cells facilitated their pyruvate-to-lactate conversion, decreased their [Lac]/[Pyr] ratio, and normalized their [NADH]/[NAD] ratio, and thus geared up their glycolysis for boosting ATP production and energy charge, without significantly changing the intracellular lactate level. This important finding implies that the decrease in lactate level is not necessarily essential to ameliorate the energy status of the MELAS mutant cells but rather that the lactate-to-pyruvate ratio and resulting [NADH]/[NAD] ratio are considered to be crucial. TCA cycle intermediates such as 2-oxoglutarate, fumarate, and malate significantly increased in level in the pyruvate-supplied 2SD cells. This increase might have been due to a potentially high activity of succinate dehydrogenase in 2SD cells, since a strong expression of succinate dehydrogenase in blood vessels is a well-known characteristic of MELAS patients (Hasegawa et al., 1991). The $^{13}C_1$-labeled isotopomers of these TCA cycle intermediates derived from ^{13}C-pyruvate accounted for ~2% of 2-oxoglutarate and ~17% of both fumarate and malate, and these proportions did not change considerably over a 4-h period; whereas the increase in unlabeled isotopomers contributed more to the increase in the total levels of these metabolites. Citrate and succinate, however, did not increase as much as the other TCA cycle intermediates in response to pyruvate treatment. These results imply that the addition of