We revealed that the three TβRI kinase inhibitors tested suppressed the intracellular signals of TGF-β1, activin, and myostatin in A204 human rhabdomyosarcoma cells and HEK293 embryonic kidney cells. However, the inhibition profile of one inhibitor, Ki26894, against the TGF-β1 and activin signal, differed between A204 human rhabdomyosarcoma cells and HEK293 embryonic kidney cells. Previous studies have shown that individual TβRI kinase inhibitors have different half-maximal inhibitory concentration values or different dissociation constant values for each receptor.[12,19,20] Such an inhibition profile may be due to pharmacological differences between each compound, as well as differences in receptor expression in the assayed cells. Similarly to the results of A204 cells, Ki26894 broadly suppressed the upregulation of p21 induced by myostatin, activin, and TGF-β1 in HaCaT cells. These results suggest that TβRI kinase inhibitors can suppress multiple intracellular TGF-β signaling pathways at their membrane receptor levels.

We also demonstrated that Ki26894 enhanced C2C12 myoblast fusion and differentiation into myotubes under low-serum conditions. Previous studies showed that myostatin, activin, or TGF-β1 impaired myogenic differentiation in cultured myogenic cells from several species.[27,34–36] Using an efficient retroviral gene expression system, we confirmed that these three molecules independently suppressed mouse C2C12 myoblast differentiation. Conversely, the addition of Ki26894 almost completely restored the myotube formation that was impaired by the TGF-β family members. Taken together, Ki26894 enhances myoblast differentiation into myotubes *in vitro* by suppressing the multiple intracellular anti-myogenic TGF-β signaling pathways. Ki26894 similarly restored the impaired C2C12 myoblast differentiation induced by forced expression of the P104L mutant caveolin 3.

Consistent with our *in-vitro* results, oral administration of Ki26894 increased myofiber size and muscle mass in wild-type mice, suggesting enhanced *in-vivo* myogenesis in normal adult muscles. As expected, Ki26894 prevented muscle atrophy in TGF-β-activated muscles in a P104L mutant caveolin 3 model mouse. Besides myostatin, other members of the TGF-β family have recently been demonstrated to suppress myogenesis *in vivo*.[37–40] Transgenic overexpression of follistatin, an inhibitory binding protein of myostatin and activin, enhanced muscularity in mice lacking myostatin.[37] Additionally, conditional deletion of an activin gene increased muscle mass, indicating that activin has a role in suppressing myogenesis *in vivo*.[38] Moreover, activated intramuscular TGF-β signaling was observed in atrophic muscles from dystrophin-deficient muscular dystrophy patients with impaired muscle regeneration and increased fibrosis.[39,40] Therefore, Ki26894 may block the intramuscular signaling of activin and TGF-β1, as well as myostatin. Consistent with these *in-vitro* observations, Ki26894 reduced the increase in the levels of p-Smad2, a TGF-β effector, and reduced upregulation of *p21*, a target gene of TGF-β family members in treated muscles.

Satellite cells are the resident stem cells of adult skeletal muscles. The caveolin 3-deficient muscles in these mice showed a reduced number of satellite cells in both skeletal muscle sections and isolated single myofibers, compared with wild-type mice. Conversely, the oral TβRI kinase inhibitor restored the reduced number of satellite cells in caveolin 3-deficient atrophic myofibers, in which p21 expression was upregulated. This inhibitor also increased the number of satellite cells in wild-type mouse muscles. TGF-β1-induced p21 upregulation in satellite cells contributed to myofiber hypotrophy in sarcopenia and impaired muscle regeneration in aged mouse muscles.[41] Thus, TβRI kinase inhibitors may increase satellite cells both in atrophic and in normal myofiber by suppressing the TGF-β–p21 axis. However, the effects of myostatin on satellite cells are controversial, because one study revealed an increased number of satellite cells in mice lacking myostatin,[42] whereas another study showed no increase in the number of satellite cells in the same mice.[43] Thus, TβRI kinase inhibitors may increase the number of satellite cells by suppressing members of the TGF-β family other than myostatin, which show similar but not identical intramuscular signaling pathways involving p21. Taken together with the *in-vitro* results, these observations indicate that oral TβRI inhibitors enhance myogenesis *via* satellite cells and myoblasts through the caveolin–TGF-β–p21 axis.

In a phase I/II clinical trial, the anti-myostatin neutralizing antibody MYO-029 was reported to dose dependently increase myofiber diameter, but did not improve muscular weakness in patients with muscular dystrophy.[44] Additionally, myostatin-null mice did not exhibit an increase in muscle strength despite an increase in muscle mass.[45] However, administration of Ki26894 enhanced muscle performance in wild-type mice and ameliorated muscular weakness in caveolin 3-deficient mice, as revealed by an increase in grip strength and muscle-specific forces. Thus, pharmacological intervention strategies based on TβRI kinase inhibitors that can suppress the broad TGF-β signaling pathways would be expected to prevent the progression of muscle weakness better than strategies employing other inhibitors that suppress myostatin signaling alone. To evaluate the effect on muscle performance, TβRI kinase inhibitors should be tested in other, larger animal models of muscular atrophy.[46]

Long-term administration of Ki26894 had no adverse effects in caveolin 3-deficient mice based on pathological examination of the major organs. Additionally, there were no adverse effects of TβRI kinase inhibitors in animal models of cancers, lung fibrosis, or renal fibrosis.[15–18,47,48] However, it is necessary to define the optimal, but non-toxic dosage of these drugs for the treatment of individual patients with muscular atrophy. In this respect, our *ex-vivo* myostatin activity assay is a convenient real-time biomonitoring system to determine how myostatin signaling is affected by the administration of

these drugs in individual patients. Both an increase in the number of satellite cells and the enhancement of myoblast differentiation provide a new therapeutic avenue for this type of inhibitor as an off-label drug for the treatment of muscle atrophy in various clinical settings, including muscular dystrophies, sarcopenia, and possibly cancer cachexia.

In agreement with our first report,[5] two other groups have recently shown that caveolin 3 deficiency causes muscle atrophy through intramuscular TGF-β activation.[29,49] Caveolin 3-deficient myoblasts formed thin myotubes upon activation of the TGF-β effector, Smad2.[29] Activated Smad2 was also observed in skeletal muscle from patients with muscular dystrophy with generalized lipodystrophy caused by deficiency of cavin, an assembly molecule for caveolin 3.[49] To confirm the novel concept of the caveolin 3–TGF-β–p21 axis in the pathobiology of muscle atrophy, further characterization of both satellite cells and myoblasts will be required in skeletal muscle samples from patients with LGMD1C and cavin 1 deficiency.

Supplementary Information accompanies the paper on the Laboratory Investigation website (http://www.laboratoryinvestigation.org)

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DISCLOSURE/CONFLICT OF INTEREST

The authors declare no conflict of interest.

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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

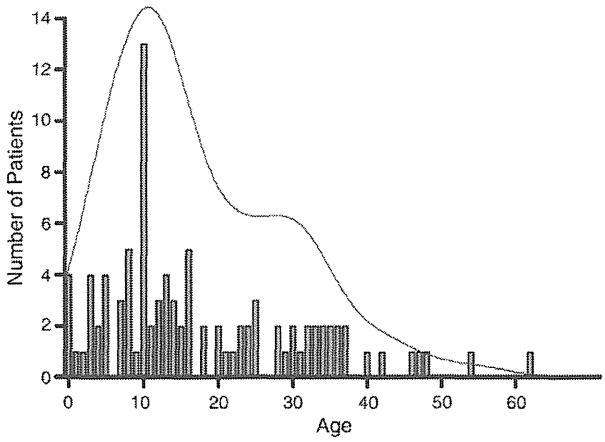

Fig. 1. Histogram detailing age of onset. A histogram and density plot of the various ages of MELAS onset is shown. In total, 96 Japanese patients were identified as having definitive MELAS as determined by diagnostic criteria (Table 1). Given the approximately bimodal distribution of patient age, MELAS 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 (%) ($n = 96$)	Juvenile (%) ($n = 58$)	Adult (%) ($n = 38$)
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.

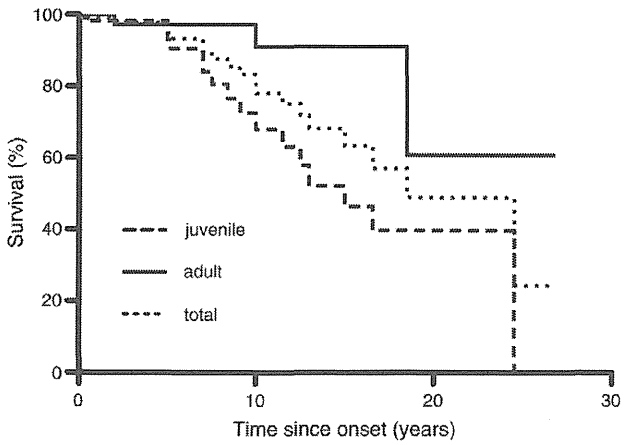

Fig. 2. MELAS survival curve. A Kaplan–Meier survival curve is shown. The dashed line indicates the juvenile form and the solid line indicates the adult form. The results of the log-rank analysis were significant. The juvenile form was associated with a higher risk of mortality than the adult form (hazard 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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Review

Molecular pathology of MELAS and L-arginine effects☆,☆☆

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ABSTRACT

Background: The pathogenic mechanism of stroke-like episodes seen in mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) has not been clarified yet. About 80% of MELAS patients have an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene, which is the base change at position 14 in the consensus structure of $tRNA^{Leu(UUR)}$ gene.

Scope of review: This review aims to give an overview on the actual knowledge about the pathogenic mechanism of mitochondrial cytopathy at the molecular levels, the possible pathogenic mechanism of mitochondrial angiopathy to cause stroke-like episodes at the clinical and pathophysiological levels, and the proposed site of action of L-arginine therapy on MELAS.

Major conclusions: Molecular pathogenesis is mainly demonstrated using ρ^0 cybrid system. The mutation creates the protein synthesis defects caused by 1) decreased life span of steady state amount of $tRNA^{Leu(UUR)}$ molecules; 2) decreased ratio of aminoacyl-$tRNA^{Leu(UUR)}$ versus uncharged $tRNA^{Leu(UUR)}$ molecules; 3) the accumulation of aminoacylation with leucine without any misacylation; 4) accumulation of processing intermediates such as RNA 19, 5) wobble modification defects. All of these loss of function abnormalities are created by the threshold effects of cell or organ to the mitochondrial energy requirement when they establish the phenotype. Mitochondrial angiopathy demonstrated by muscle or brain pathology, as SSV (SDH strongly stained vessels), and by vascular physiology using FMD (flow mediated dilation). MELAS patients show decreased capacity of NO dependent vasodilation because of the low plasma levels of L-arginine and/or of respiratory chain dysfunction. Although the underlying mechanisms are not completely understood in stroke-like episodes in MELAS, L-arginine therapy improved endothelial dysfunction.

General significance: Though the molecular pathogenesis of an A3243G or T3271C mutation of mitochondrial $tRNA^{Leu(UUR)}$ gene has been clarified as a mitochondrial cytopathy, the underlying mechanisms of stroke-like episodes in MELAS are not completely understood. At this point, L-arginine therapy showed promise in treating of the stroke-like episodes in MELAS. This article is part of a Special Issue entitled Biochemistry of Mitochondria.

1. Introduction

Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (OMIM 540000), characterized by an early onset of stroke-like episodes, was first described by Pavlakis and colleagues in 1984 [1]. At least 39 distinct mitochondrial DNA mutations have been associated with MELAS [2], about 80% of MELAS patients have an A3243G mutation in the mitochondrial $tRNA^{Leu(UUR)}$ gene (OMIM 590050) [3–5]. Although more than 25 years have passed since MELAS was first defined clinically and pathologically, the pathogenesis of the stroke-like episodes is still uncertain. Mitochondrial angiopathy with degenerative changes in small arteries and arterioles, which has been reported in many MELAS patients [6,7], is suggested by the observation of strong succinate dehydrogenase activity in the wall of blood vessels (SSVs) [8]. In spite of the fact that many therapeutic trials have been conducted to cure mitochondrial disorders, no trial has been successful, though several clinical trials are still on-going. Based on the hypothesis that stroke-like episodes in MELAS are caused by segmental impairment of vasodilatation in intracerebral arteries, we use L-arginine in MELAS patients during the acute phase to cure the symptoms or to

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decrease the frequency and/or the severity of the stroke-like episodes [9,10,11]. This review aims to give an overview on the actual knowledge about the pathogenic mechanism of mitochondrial cytopathy at the molecular levels, the possible pathogenic mechanism of mitochondrial angiopathy to cause stroke-like episodes in the clinical and pathophysiological levels, and the proposed site of action of L-arginine therapy on MELAS.

2. Molecular pathophysiology of mitochondrial cytopathy in MELAS

2.1. Characteristics of $tRNA^{Leu(UUR)}$ gene and structure stabilization of mutant

A point mutation in the structural gene for a tRNA may be expected to result in a deficiency in translation. However, inhibition of translation due to a mutated tRNA gene may occur at several levels. The base change at position 14 in the consensus structure of $tRNA^{Leu(UUR)}$ is an invariant A in bacterial and cytosolic eukaryotic tRNAs and is typically involved in the tertiary folding of classical tRNAs (Fig. 1A) [12]. Because of above reason, the A3243G mutation is primarily thought to disrupt the tertiary interaction between the highly conserved np A14 (>90% for adenine) and U8, a binding that stabilizes the L-shaped tertiary fold [13,14], which results in partially folded tRNA transcripts into the L-shaped structure with an acceptor branch but with a floppy anticodon branch [15]. The mutant tRNA is able to adapt to the synthetase, but results in incorrect tRNA processing and enzyme maturation and accordingly defects in a variety of biochemical pathways. The mutation may directly affect the mitochondrial tRNA function in translation, such as structure stabilization, methylation, amino-acylation, and codon recognition, or alternatively, may affect recognition of the tRNA by an enzyme not directly involved in translation, such as the enzymes which process the large polycistronic transcripts of the mtDNA.

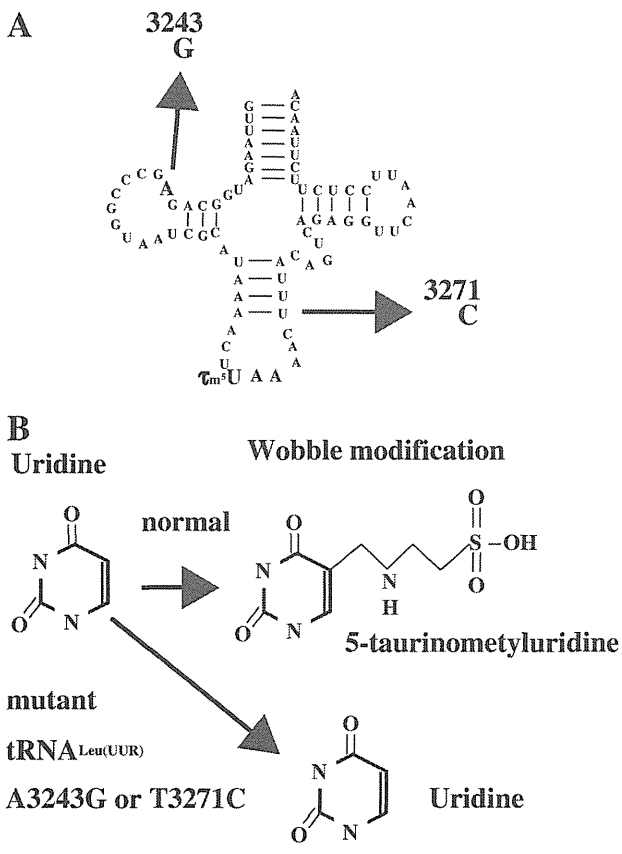

Fig. 1. $tRNA^{Leu(UUR)}$ structure and wobble modification. $tRNA^{Leu(UUR)}$ structure. An A to G change at position 14 in the consensus structure of $tRNA^{Leu(UUR)}$, which is thought to disrupt the tertiary folding of classical tRNAs [12], results in partially folded tRNA transcripts into the L-shaped structure with an acceptor branch but with a floppy anticodon branch [14,15]. B. Wobble modification The wild-type $tRNA^{Leu(UUR)}$ contains an unknown modified uridine at the wobble position and that this modification occurs at the uracil base [35], however its modification is absent in the $tRNA^{Leu(UUR)}$ with a mutation at either np A3243G or T3271C. The wobble modified uridine in the wild-type $tRNA^{Leu(UUR)}$ is 5-taurinomethyluridine (sm5U). The U on the bold indicates the unmodified uridine present in the mutant tRNAs.

2.2. ρ^0 cybrid system in MELAS

King et al. developed the technologies whereby the mitochondria from cells derived from patients are transferred to a cell line lacking mtDNA (so called ρ^0 cybrid system), which allowed to conduct the study of the genotype-phenotype relationships in mitochondrial function [16,17]. In this manner, it is possible to create trans-mitochondrial cell lines containing different proportions of mutated mtDNA from 0% to 100%, and to study the effects of a given mutant load on the activity of respiratory chain complexes, mitochondrial respiration and cell growth, as well as mitochondrial tRNA stability, methylation, aminoacylation, codon recognition and threshold effects. First application of this technique to an A3243G mutation related to molecular basis of MELAS, has been reported by Chomyn et al. [18], and King et al. [19] independently. Mutant transformants showed protein synthesis defects clearly, and demonstrated that there was the direct evidence between single nucleotide change at 14th position of an A to G transition in the mitochondrial $tRNA^{Leu(UUR)}$ gene and mitochondrial dysfunction. However, the reduction in labeling of the various mitochondrial translation products in mutant was not correlated with their UUR-encoded leucine content. King also reported the similar effects in transformants having a T3271C mutation [19]. This ρ^0 cybrid system becomes the orthodox and powerful tool when one evaluates the pathogenicity of any nucleotide changes in the mitochondrial DNA.

2.3. Transcription termination of mitochondrial RNAs in MELAS

The mammalian mitochondrial tRNAs are transcribed as part of larger polycistronic RNAs, in which the tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and the protein-coding sequences (Fig. 2). The ribosomal gene region appears to be transcribed 50–100 times more frequently than the other H-strand genes [20]. In these polycistronic molecules, the tRNA structures are believed to act as recognition signals for the processing enzymes which make precise endonucleolytic cleavages at the 5' and 3' ends of the tRNA sequences in the primary transcripts, yielding the mature rRNAs, mRNAs, and tRNAs [21]. The ribosomal DNA transcription unit, one of three polycistronic transcription units of human mtDNA, terminates at the 3'-end of the 16S rRNA gene just before the $tRNA^{Leu(UUR)}$ gene. This transcript, corresponding to the ribosomal genes, is processed to yield the mature rRNAs and, due to its very high rate of synthesis, is responsible for the bulk of the rRNA formation [22]. Transcription termination is mediated by a protein factor (mTERF: mitochondrial termination factor) which specifically binds within the $tRNA^{Leu(UUR)}$ gene, and which promotes termination of transcription (Fig. 3A) [22,23]. Since this mutation is located exactly in the middle of termination protein binding domain, the A3243G mutation in the $tRNA^{Leu(UUR)}$ gene has been shown in vitro to impair the binding of this protein factor and to affect the efficiency of transcription termination at the end of the 16S rRNA gene [23]. However, in vivo analysis using ρ^0 cybrid system provided no evidence to support above data. There were no alterations of size of the $tRNA^{Leu(UUR)}$ or of the immediately downstream-encoded ND1 mRNA or of the 16S rRNA, as detectable by changes in their electrophoretic mobility [18]. The steady-state amounts of mitochondrial rRNAs, mRNAs, and $tRNA^{Leu(UUR)}$ are not significantly affected by the MELAS mutation in ρ^0 cybrid system. The discrepancy of the data described above may be explained by the possibility that the

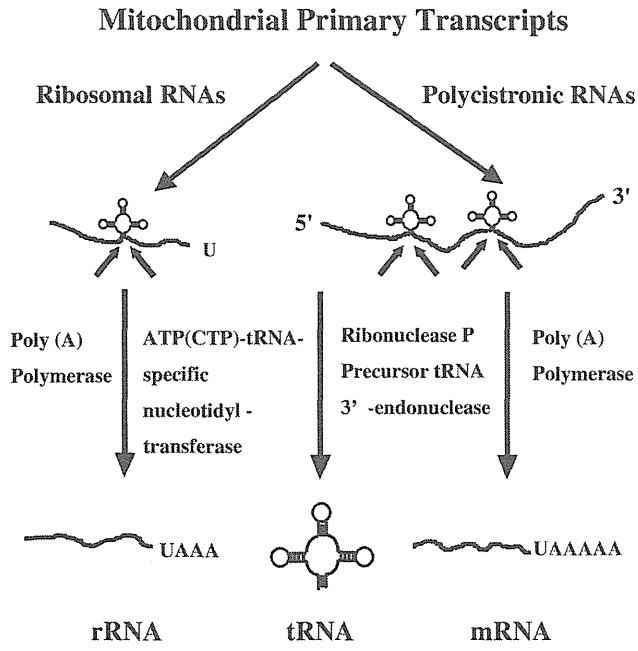

Fig. 2. Mammalian mitochondrial transcription system. The human mitochondrial RNAs are transcribed as a larger polycistronic RNAs, in which the tRNA sequences are contiguous or nearly contiguous to the rRNA sequences and the protein-coding sequences.

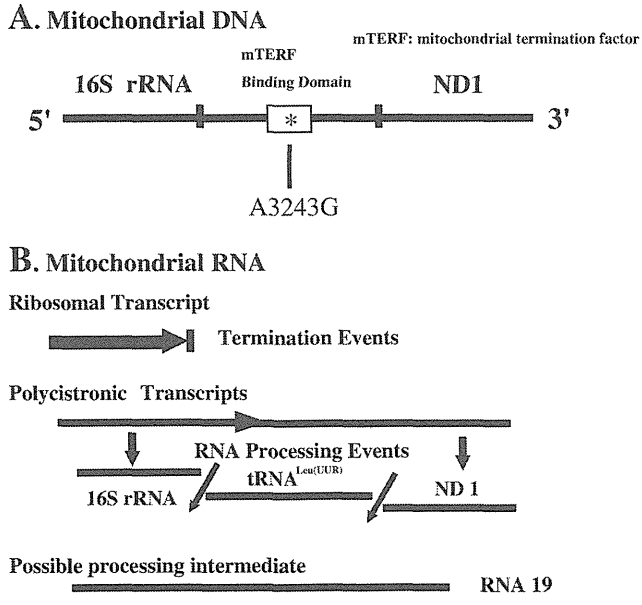

Fig. 3. Post-transcriptional modification. A. Transcription termination. The ribosomal gene region appears to be transcribed 50–100 times more frequently than the other H-strand genes [20]. Transcription termination is mediated by a protein factor (mTERF: mitochondrial termination factor) which specifically binds within the $tRNA^{Leu(UUR)}$ gene, and which promotes termination of ribosomal transcription. B. Post-transcriptional modification and RNA 19 The increase of RNA 19, corresponding to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND 1 genes, found in mutant $tRNA^{Leu(UUR)}$ cybrids clearly demonstrate that RNA processing is *not occurring in mutant cybrids as efficiently as in wild-type cybrids* [19]. RNA 19 is also accumulated in muscle specimens from 8 MELAS patients [26]. The proportion of mutated RNA in RNA 19 fraction is always higher than those in the percentage of mutation in mitochondrial DNA, suggesting that the A3243G mutation exhibited dominant negative effects on the mitochondrial RNA processing events, resulting in the accumulation of RNA 19 transcripts in these patients [28–30].

reduction in affinity of mTERF for the mutated target sequence is compensated by hyper-expression of the protein. Anyway, using genetic, biochemical, and morphological techniques, it was found that the mutant, but not wild-type cybrids, displayed quantitative deficiencies in cell growth, protein synthesis, and respiratory chain activity [19].

2.4. Processing of polycistronic transcripts in MELAS

It was found that there was an accumulation of a previously unidentified RNA transcript in mutant cybrids (A3243G or T3271C), designated as RNA 19, corresponds to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND1 genes, which are contiguous in the mtDNA (Fig. 3B) [19]. The ratios of mtDNA-encoded rRNAs to mRNAs were not found to be altered in these in vitro experiments. In order to analyze whether the MELAS mutation is associated with errors in transcription termination and processing of the polycistronic transcripts in the region of the mutation, it was performed fine mapping of the mature transcripts derived from the 16S rRNA, $tRNA^{Leu(UUR)}$, and ND 1 genes in both wild-type and mutant cybrids. It was also analyzed the steady-state levels of $tRNA^{Leu(UUR)}$ by high-resolution RNA transfer hybridizations. It was found that mutation has no effect in vivo on the accuracy of transcription termination at the end of the ribosomal RNA genes, on the precise endonucleolytic cleavage of the polycistronic RNA at $tRNA^{Leu(UUR)}$, or on the post transcriptional addition of -CCA at the 3′ end of $tRNA^{Leu(UUR)}$ [24]. On the other hand, the experiments using plasmids carrying $tRNA^{Leu(UUR)}$ inserts (wild type, as well as A3243G) which designated to evaluate the endonucleolytic 3′-end processing and CCA addition at the tRNA 3′ terminus, showed that A3243G mutation reduced 2.2 fold of the efficiency of 3′-end cleavage, and almost has no abnormal effects on CCA addition [25].

2.5. Accumulation of RNA 19 in MELAS cybrids and organs from patients

The increased amounts of the transcript corresponding to the 16S rRNA + $tRNA^{Leu(UUR)}$ + ND 1 genes, designated as RNA 19, found in mutant $tRNA^{Leu(UUR)}$ cybrids clearly demonstrate that RNA processing is not occurring in mutant cybrids (A3243G or T3271C) as efficiently as in wild-type cybrids [19]. It was demonstrated that RNA 19 is accumulated in muscle specimens from 8 MELAS patients who have a heterogeneous percentage of mutation (58% to 99%) in the A3243G of $tRNA^{Leu(UUR)}$ gene [26]. An increase in the levels of RNA 19 was observed in nearly all tissues examined from these patients, which do not provide evidence for tissue-specific differences in mitochondrial RNA processing. The elevation of steady-state levels of RNA 19 have also reported in skeletal muscle and fibroblasts of a patient with mitochondrial myopathy and a complex I deficiency who harbored an A to G transition in $tRNA^{Leu(UUR)}$ gene at position 3302 [27]. Thus, altered RNA processing may be associated with other point mutations in $tRNA^{Leu(UUR)}$ gene associated with MELAS. It also analyzed a mutated proportion of RNA 19 in an RNA fraction obtained from sampled skeletal muscles from 6 unrelated patients with MELAS. The proportion of mutated RNA in RNA 19 fraction exceeded 95% in all patients, although the percentage of mutation in mitochondrial DNA ranged from 54 to 92, suggesting that the A3243G mutation exhibited dominant negative effects on the mitochondrial RNA processing events, resulting in the accumulation of RNA 19 transcripts in these patients [28–30]. The protein synthesis defect has been proposed to be due to stalling of translation by pseudoribosomes that have incorporated RNA 19, an incompletely processed transcript reported to accumulate in A3243G, T3271C and A3302G mutant cells, in place of 16 S rRNA, or possibly to defective posttranscriptional modification of the $tRNA^{Leu(UUR)}$ (Fig. 4) [31]. Though the reason why RNA 19 was elevated in patients who have the point mutation of $tRNA^{Leu(UUR)}$ gene is unknown, we believe that elevated levels of RNA 19 may play an important role in the pathogenesis of this disorder.

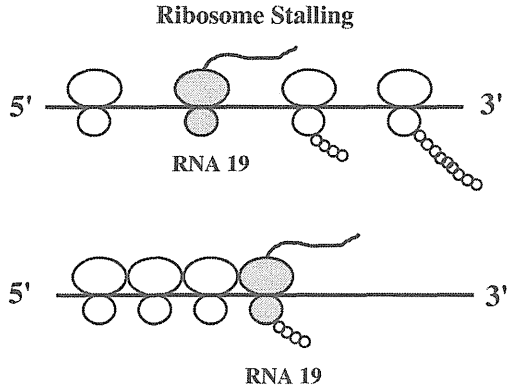

Fig. 4. xRibosomal stalling. The protein synthesis defect has been proposed to be due to stalling of translation by pseudoribosomes that have incorporated RNA 19, an incompletely processed transcript reported to accumulate in A3243 mutant cells, in place of 16 S rRNA, or possibly to defective posttranscriptional modification of the $tRNA^{Leu(UUR)}$ [31].

2.6. Aminoacylation

The decrease in level of total $tRNA^{Leu(UUR)}$ observed in the mutant cell lines (46–62% of the control values) could arise either from decreased rate of formation from the corresponding primary heavy strand transcript or from a decreased metabolic stability [32]. The increased amount of RNA 19, which may be a precursor of $tRNA^{Leu(UUR)}$, was demonstrated in ρ^0 cybrid system as well as somatic tissues in MELAS patients. RNA 19 may suggest the former possibility. On the other hand, the A3243G mutation could perfectly destabilize the tertiary structure of the molecule, and mutant $tRNA^{Leu(UUR)}$ becomes more susceptible to nucleolytic attack [13,33]. It is proposed that mutant tRNA induces the misincorporation of amino acids in mitochondrial DNA encoded polypeptides. However, the demonstration of aminoacylation by mutant tRNA has been little pursued because a chemical amount of the mutant tRNA has not been purified, probably due to technical difficulties. In 2000, Yasukawa succeeded in purifying the mutant $tRNA^{Leu(UUR)}$ molecules in a chemical amount by taking advantage of the solid phase probing method [34], and clearly demonstrated that the mutant $tRNA^{Leu(UUR)}$ is aminoacylated with leucine only. However the extent of aminoacylation of the mutant tRNAs was relatively low. The total amounts of leucyl-$tRNA^{Leu(UUR)}$ with the mutations were estimated to be less than 30% that of the wild-type counterpart [35]. To determine if the decreased fraction of aminoacylated $tRNA^{Leu(UUR)}$ in mutant cells was due to a defect in the ability of mutant tRNA to be aminoacylated by the human mitochondrial leucyl-tRNA synthetase, Park et al. examined the aminoacylation kinetics of wild-type and mutant $tRNA^{Leu(UUR)}$, using both native and in vitro transcribed $tRNA^{Leu(UUR)}$ [36]. An A3243G mutant $tRNA^{Leu(UUR)}$ was 25-fold less efficiently aminoacylated in vitro, compared to the wild-type $tRNA^{Leu(UUR)}$. There are many evidences that aminoacylation capacities in $tRNA^{Leu(UUR)}$ gene mutations are reduced [37]. The reduced amount of aminoacyl-$tRNA^{Leu(UUR)}$ with the A3243G mutation could explain the reduction in protein synthesis.

2.7. Modified defects at wobble position in mitochondrial tRNA gene

A number of reports suggest that a decrease of protein synthesis cannot explain the decline in respiratory enzyme activity or in oxygen consumption [38,39]. Even when the mitochondrial protein synthesis rate was normal, the enzymatic activity of complex I was observed to be significantly affected in cybrid clones containing 60% to 95% mutant mtDNA. The muscle form of Complex I deficiency turned out to be MELAS clinically and was confirmed to have an A3243G mutation in all patients [40]. Thus, the decrease in protein synthesis may not itself contribute directly to the pathogenesis caused by mitochondrial dysfunction. Some unusual mobilities of proteins in SDS-polyacrylamide gel electrophoresis have been reported [18,38], which strongly suggest that amino acids were misincorporated into the proteins synthesized in the mitochondria with the mutant mtDNA. The steady-state amounts of $tRNA^{Leu(UUR)}$ with the A3243G or the T3271C mutation in the respective cybrid clones were about 30% that of the wild-type in the control cybrid clones with wild type mtDNA [35]. In contrast, the steady-state amounts of $tRNA^{Phe}$ and $tRNA^{Ile}$ (encoded upstream and downstream of the $tRNA^{Leu(UUR)}$gene) remained unchanged in both the mutant and control cybrid cells. The life span of the mutant $tRNA^{Leu(UUR)}$ is significantly shortened. The half-life of the wild-type $tRNA^{Leu(UUR)}$ was estimated to be about 56 h, whereas those of the A3243G and T3271C mutants were only about 6 and 12 h. Therefore the reduced steady-state levels were due to the shortened life spans of the mutant tRNAs. Yasukawa found that the wild-type $tRNA^{Leu(UUR)}$ contains an unknown modified uridine at the wobble position and that this modification occurs at the uracil base (Fig. 1B). In contrast, this uridine modification is absent in the $tRNA^{Leu(UUR)}$ with a mutation at either np A3243G or T3271C. It is interesting to note that both of the mutant $tRNA^{Leu(UUR)}$ are deficient in the modification at the wobble position despite having mutations at different positions. Modified defects at wobble position in mitochondrial tRNA gene are also demonstrated by primer extension methods [41]. The deficiency in uridine modification at the wobble position in the mutant $tRNA^{Leu(UUR)}$ strongly suggests mistranslation by these mutant tRNAs according to the mitochondrial wobble rule, which is also demonstrated in other tRNA mutation in MERRF (myoclonus epilepsy with ragged-red fibers) [42–45]. Although mutant $tRNA^{Leu(UUR)}$ does not follow the wobble rule, the mutant $tRNA^{Leu(UUR)}$ is aminoacylated with only leucine, not with other aminoacids. The stability and aminoacylation of the mutant $tRNA^{Leu(UUR)}$ were found to be decreased, suggesting that the molecular pathogenesis of MELAS could be a combination of a lowered availability of aminoacyl $tRNA^{Leu(UUR)}$ and defective translation. This is the first observation of a common modification defect affected by different point mutations within a single tRNA gene.

2.8. Threshold effects in various steps in the cell or in the organs

The phenotypic threshold effect observed at the single-cell level could arise when the products of the wild-type mtDNA can no longer "complement" the effects of the mutated ones [46,47]. For instance, a heteroplasmic mutation in mtDNA will result in the co-existence of mutated mRNAs, mutated tRNAs and defective respiratory chain subunits along with their wild-type homologues. These wild-type molecules may be sufficient to support normal function of the organelle until their levels fall below a critical value (threshold), at which point they can no longer compensate for the effect of the mutation, leading to impairment of mitochondrial function. The phenotypic threshold effect is based on this reserve of different macromolecules (mRNAs, tRNAs, subunits), and can then be considered as a protective mechanism providing a safety margin against the effects of deleterious mutations. Above complementation can occur at different levels of mitochondrial gene expression, such as 1) gene transcription, 2) structural stability of the tRNAs, 3) maturation process of the tRNAs, ribosomal RNA, and mRNAs, 4) wobble modification of tRNAs, 5) aminoacylation, 6) translation, 7) molecular assembly of the active form of enzyme complexes in harmony with mitochondrial and nuclear-encoded polypeptides, 8) locate to the mitochondrial inner membrane, 9) biochemical overall function of mitochondria in the cell, 10) biochemical overall function of mitochondria in the organ, 11) original threshold of organ to the mitochondrial energy requirement. The cells which require high energy states, such as neurons, muscles, heart, and kidneys, may be more severely affected by the threshold level of mutation than cell that require low energy levels. The phenotypes in the severity of the disease may influence various factors listed above and are more complicated to elucidate.

2.8.1. Summary of molecular mechanisms of mitochondrial cytopathy

The mutation creates the protein synthesis defects caused by 1) decreased life span of steady state amount of $tRNA^{Leu(UUR)}$ molecules; 2) decreased ratio of aminoacyl-$tRNA^{Leu(UUR)}$ versus uncharged $tRNA^{Leu(UUR)}$ molecules; 3) accumulation of processing intermediates such as RNA 19, 4) wobble modification defects leading to translation defect. The A3243G mutation shows dominant negative effects in the processing system of mitochondrial transcription seen in both trans-mitochondrial cell and muscles in MELAS patients. Molecular mechanisms described above may contribute to respiratory chain enzyme defects, especially complex I, and lead to the mitochondrial cytopathy seen in the MELAS patients. Moreover the A3243G mutation affects the nuclear background [46,47], resulting in a high glycolytic rate, increased lactate production, reduced glucose oxidation, impaired NADH-response, reduced mitochondrial membrane potential, markedly reduced ATP production, deranged cell calcium handling with an increased cytosolic calcium handling with an increased cytosolic calcium load, an increased amount of reactive oxygen species in cybrid cells, reduced insulin secretion, premature aging, and deregulation of genes involved in the metabolism of amino groups and urea genesis. The above mechanism may lead to the cytotoxic edema seen in stroke-like episodes in MELAS.

3. Pathophysiology of mitochondrial angiopathy in MELAS

3.1. Hypotheses of stroke-like episodes in MELAS

The primary cause for stroke-like episodes in young MELAS patients—whether 1) mitochondrial cytopathy, 2) mitochondrial angiopathy, 3) non-ischemic neurovascular cellular mechanism, or combined—remains controversial. Mitochondrial cytopathy is caused by an oxidative phosphorylation defect in neurons, glia, or both as supported by evidence of an oxidative phosphorylation defect described by molecular pathogenesis section. Mitochondrial angiopathy is caused by the endothelial dysfunction evidenced by pathological, vascular physiological [11], or therapeutic findings [9,10]. Finally, the non-ischemic neurovascular cellular mechanism has been recently proposed by the clinical and neuroimaging data by Iizuka et al. [3].

3.2. Mitochondrial angiopathy in MELAS

Mitochondrial angiopathy with degenerative changes in small arteries and arterioles in the brain has been reported in autopsy cases of MELAS patients [6,7]. The mitochondria in the endothelium and smooth muscle cells of cerebral arterioles and capillaries also proliferate in a similar fashion as an area of ragged-red fibers (RRFs). Abnormal accumulation of mitochondria in vascular endothelial cells and smooth muscle cells is responsible for the infarct-like lesions [48]. These blood vessels have been designated as strongly succinate dehydrogenase-reactive vessels (SSVs), since they are rich in abnormal mitochondria [8]. Unlike RRFs and SSVs seen in MERRF and Kearns–Sayre syndrome (KSS), RRFs and SSVs seen in MELAS are typically cytochrome c oxidase (COX) positive, while those seen in MERRF or KSS are mostly COX negative, what is known as the "MELAS paradox"[49]. Since nitric oxide (NO) can bind to the active site of COX and displace heme-bound oxygen, hyperactive COX may decrease the regional NO concentration and lead to the segmental vasodilatation defect in SSV regions. Although infarct-like lesions histopathologically and stroke-like episodes clinically may not be caused simply by occlusion or obliteration of small vessels, this mitochondrial angiopathy, which can be severe in pial arterioles and small arteries, seems to explain the distribution of multiple areas of necrosis [50]. Since MELAS was associated with respiratory dysfunction, accumulated superoxide radical anion may react with nitric oxide to create the powerful oxidant hydroxypernitrite which may induce the neuronal apoptosis or cell damage [51]. All findings, described here, suggest that mitochondrial angiopathy is a unique and common change in all MELAS brains examined. This pathological abnormality, called mitochondrial angiopathy, may lead to the vasogenic edema seen in stroke-like episodes in MELAS.

3.3. Non-ischemic neurovascular cellular mechanism

Iizuka et al. proposed that the stroke-like episodes in MELAS may reflect neuronal hyperexcitability (epileptic activity), which increases energy demand and creates an imbalance between energy requirements and the adequate availability of ATP due to an oxidative phosphorylation defect, particularly in the susceptible neuronal population [3,52]. The generalized cytopathic mechanism and non-ischemic neurovascular cellular mechanism reflect the so-called mitochondrial cytopathy theory.

3.4. Neuro-imaging analysis in stroke-like episodes

Unlike thrombotic or embolic stroke usually seen in adult patients, the stroke-like episodes in MELAS are atypical because they affect young people and are often triggered by febrile illnesses, migraine-like headaches, seizure, psychological stress, and dehydration. Many neuro-imaging studies have been reported at different phases of onset from stroke-like episodes in MELAS through the use of computed tomography (CT), magnetic resonance imaging (MRI), magnetic resonance spectroscopy (MRS), single emission computed tomography (SPECT), and positron emission tomography (PET). Calcification of the basal ganglia is frequently observed in MELAS by CT even before starting the stroke-like episodes. MRI scans of acute stroke-like events show an increased signal on T2-weighted or on fluid attenuation inversion recovery (FLAIR). The regions do not conform to the territories of large cerebral arteries but rather affect the cortex and subjacent white matter with sparing of deeper white matter. Acute changes in these regions may fluctuate, migrate, or even disappear during the time course. Cerebral angiograms in MELAS patients have confirmed absence of large-vessel pathology by demonstrating normal results, increased size of caliber arteries, veins, or capillary blush with early venous filling, with the exception of several case reports [53,54]. MRS studied revealed that the decrease in N-acetylaspartate (NAA), which is thought to be an amino acid specific to neurons, and an increase of lactate, which is reflected of anaerobic metabolism by ^{1}H-magnetic resonance spectroscopy (^{1}H-MRS), were in evidence in the affected areas at acute stroke-like episodes. Kubota et al. reported that L-arginine infusion protect the accumulation of lactate by MRS analysis in stroke-like episodes in MELAS [55]. The increased level of lactate on ^{1}H-MRS is also recognized even in normal appearing regions [56]. Phosphorus MRS studies have shown decreased levels of high-energy phosphate compounds in the brains of MELAS patients [57], showing that mitochondrial cytopathy constantly exists in the MELAS patient. SPECT studies have generally revealed that the increased tracer accumulation was reported in acute (several days) and subacute stage (month) from the onset of stroke-like episodes and lasted for several months. In the chronic stage (several months or years later), the decreased tracer accumulation was reported. However, in the hyperacute stage (3 h after the onset of stroke-like episodes), we observed hypoperfusion by SPM-SPECT analysis [58]. Moreover, the hypoperfusion and the hyperperfusion areas are both demonstrated in the MELAS patients not only at an acute phase but at an interictal phase, showing that MELAS has inappropriate cerebral circulation [54]. Moreover, MELAS showed hypoperfusion in the posterior cingulated cortex by SPM-SPECT, which is the common finding in Alzheimer disease, and may be related to the dementia state usually seen in the progressive stage of MELAS. There are several PET studies using ($rCMRO_2$), [^{62}Cu]-diacetyl bis (N4-methylthiosemicarbazone) (^{62}Cu-ATSM), and [^{18}F]-fluorodeoxyglucose (^{18}FDG) in stroke-like regions [59,60]. All of the PET studies of

patients have revealed decreased oxygen consumption relative to glucose utilization, further confirming the impairment of oxidative phosphorylation [61]. The dissociation in PET findings between cerebral glucose and oxygen metabolism may be the characteristic feature of MELAS, suggesting the mitochondrial cytopathy theory or non-ischemic neurovascular cellular mechanism. Diffusion-weighted (DWI) imaging is a new MRI technique for detecting diffusion of water molecules. Using DWI, local water mobility can be assayed as the absolute value of tissue water and expressed as the apparent diffusion coefficient (ADC). It has been shown using a stroke model in rats that ADC (a marker for cytotoxic brain edema) significantly declined within the first 5–10 min after stroke onset, while T2-relaxation time (a marker for vasogenic brain edema) increased as early as at the first T2-imaging time-point (20–35 min after embolization) [62]. The acute phase of stroke-like lesions in MELAS appear as a high signal on DWI with normal or increased ADC values, suggesting vasogenic edema which support the mitochondrial angiopathy theory [63,64]. On the contrary, many case reports found a decrease in ADC, which suggests mitochondrial cytopathy theory [65]. Recently, it was reported that increased and decreased ADC portions are mixed in stroke-like lesions, in which the increased ADC portion showed disappearance of the lesions thereafter, and the decreased portion showed persistent lesions. They suggested that there might be different levels of mitochondrial energetic transport impairment, correlated with cellular dysfunction. Specifically, this would be a mild energy failure resulting in moderate cellular dysfunction, responsible for vasogenic edema (high ADCs) and a severe energy failure resulting in irreversible cellular failure with cytotoxic edema (low ADCs) [66].

3.5. Endothelial dysfunction in MELAS

Physiologically, MELAS patients have a decreased vasodilation capacity in small arteries examined by flow mediated vasodilatation (FMD) methods, sized from 3 to 5 mm in their diameter [11]. MELAS patients have significantly decreased levels of L-arginine at acute phase of stroke-like episodes, which plays an important role in endothelial-dependent vascular relaxation [67], vasodilatation may be more severely affected in MELAS. Since MELAS patients have defective respiratory chain enzyme activities, a high NADH/NAD + ratio inhibits the NO synthetase reaction to cause a decreased production of NO at the endothelial cells or smooth muscle cells in the artery. In addition, ADMA (asymmetrical dimetyl-arginine), a risk factor of ischemic heart disorders, was relatively increased in MELAS patients [10], which may lead to a negative effect on the endothelial NO synthetase activity. If hyperactive COX may decrease the regional NO concentration as described in "MELAS paradox" [49], all of the above scenarios lead to the segmental vasodilatation defect especially in the segment of SSV regions in the cerebral artery or arterioles. The investigator-mediated clinical trial of L-arginine on MELAS (Dr. Koga as a principle investigator) 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 are on-going at 15 institutions of university hospital in Japan.

3.5.1. Summary of mitochondrial angiopathy and L-arginine effects

Pathophysiological mechanisms of mitochondrial angiopathy and the effects of L-arginine are summarized in Fig. 5.

4. Conclusion and future direction

The possible pathogenic mechanism of stroke-like episodes in MELAS may not be simple but complicated as described by the mechanisms in mitochondrial cytopathy and in mitochondrial angiopathy. Mitochondrial cytopathy has been demonstrated clearly as molecular and cellular defects by trans-mitochondrial cellular models. Mitochondrial angiopathy also has been demonstrated in brain and muscle pathology and vascular physiology. Although the results of

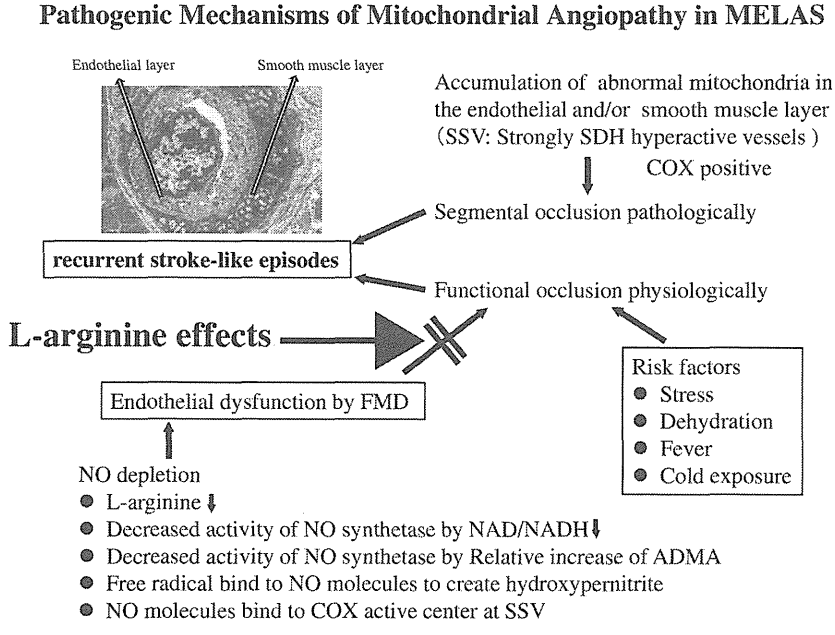

Fig. 5. Pathogenic mechanisms of mitochondrial angiopathy in MELAS. Segmental occlusions of small artery or arteriolen are evident in brain as well as in muscle pathology, in which abnormal accumulations of mitochondria have seen in endothelial and smooth muscle layers [6,7]. This phenomenon is recognized as SSVs in muscle and brain in MELAS [8], whereby mitochondrial function is more profoundly defective than the rest of the vessels, and demonstrated as endothelial dysfunction by FMD physiologically [11]. In MELAS patients, decreased levels of L-arginine is reported at acute phase of stroke-like episodes, a potent donor of NO, is also responsible for NO-dependent vascular dilatation defect. The decreased NAD/NADH ratio and accumulation of superoxide come from respiratory chain deficiency results in the inhibition of NO synthetase at generation process and decrease NO molecules by binding to create hydroxypernitrite, also contribute to the NO-dependent vasodilation abnormality. Since SSVs has usually high COX-positive feature histochemically, high COX activity decrease the residual NO molecules by binding to COX reactive center. The mental stress, dehydration, fever and cold exposure are also very important factors to increase the risk of the stroke-like episodes in MELAS.

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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Original article

Beneficial effect of pyruvate therapy on Leigh syndrome due to a novel mutation in PDH E1α gene

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Abstract

Leigh syndrome (LS) is a progressive untreatable degenerating mitochondrial disorder caused by either mitochondrial or nuclear DNA mutations. A patient was a second child of unconsanguineous parents. On the third day of birth, he was transferred to neonatal intensive care units because of severe lactic acidosis. Since he was showing continuous lactic acidosis, the oral supplementation of dichloroacetate (DCA) was introduced on 31st day of birth at initial dose of 50 mg/kg, followed by maintenance dose of 25 mg/kg/every 12 h. The patient was diagnosed with LS due to a point mutation of an A–C at nucleotide 599 in exon 6 in the pyruvate dehydrogenase E1α gene, resulting in the substitution of aspartate for threonine at position 200 (N200T). Although the concentrations of lactate and pyruvate in blood were slightly decreased, his clinical conditions were deteriorating progressively. In order to overcome the mitochondrial or cytosolic energy crisis indicated by lactic acidosis as well as clinical symptoms, we terminated the DCA and administered 0.5 g/kg/day TID of sodium pyruvate orally. We analyzed the therapeutic effects of DCA or sodium pyruvate in the patient, and found that pyruvate therapy significantly decreased lactate, pyruvate and alanine levels, showed no adverse effects such as severe neuropathy seen in DCA, and had better clinical response on development and epilepsy. Though the efficacy of pyruvate on LS will be evaluated by randomized double-blind placebo-controlled study design in future, pyruvate therapy is a possible candidate for therapeutic choice for currently incurable mitochondrial disorders such as LS.

Keywords: Leigh syndrome; PDH E1α mutation; Pyruvate; Lactic acidosis; Therapy

1. Introduction

LS, originally reported as subacute necrotizing encephalomyelopathy by Dr. Denis Leigh in 1951 [1], is an early-onset progressive neurodegenerative disorder characterized by developmental delay or regression, lactic acidosis, and bilateral symmetrical lesion in the basal ganglia, thalamus, and brainstem [2]. The clinical presentations of the disease are heterogeneous, due to the severity of biochemical defects caused by mutations in both nuclear and mitochondrial genes involved in energy metabolism. Though many molecular defects are reported to be associated with LS [3], the underlying gene defects remain unidentified in nearly half of the patients [4,5]. Since LS is associated mainly with the respiratory chain deficiency, there is no established treatment except for a limited number of patients such as those with thiamine-responsive pyruvate dehydrogenase deficiency [6], or those with defects in the biosynthetic pathway of coenzyme Q [7]. We have proposed that pyruvate has a therapeutic potential for mitochondrial diseases, because: (a) pyruvate can stimulate the

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glycolytic pathway by reducing the NADH/NAD ratio in the cytoplasm, (b) pyruvate can activate PDHC by inhibiting pyruvate dehydrogenase kinase, and (c) pyruvate can scavenge hydrogen peroxide by non-enzymatic reaction [8]. Recently, we reported that pyruvate produced a slightly favorable change in the plasma lactate and pyruvate levels in LS with cytochrome c oxidase deficiency [9]. In the present report, we describe a clinical experience of pyruvate therapy in a child with LS having PDH deficiency caused by a novel mutation in PDH E1α gene.

2. Patient and methods

2.1. Patient

The 5-years-old boy, presented as severe psychomotor retardation with severe lactic acidosis, was born weighing 1797 g at full term gestational age as the second child of unconsanguineous parents. He was transferred to neonatal intensive care units because of fatal distress with the severe lactic acidosis. The concentrations of lactate and pyruvate in blood were 6–10 times higher than normal range, with normal lactate/pyruvate ratio (Table 1). He was under respiratory care with medication of severe metabolic acidosis. Aminogram of his plasma showed an elevated alanine concentration of 1.82 mM (normal range, 0.21–0.52). Since he was showing continuous lactic acidosis, the oral supplementation of DCA was introduced on 31st day of birth at initial dose of 50 mg/kg, followed by maintenance dose of 25 mg/kg/every 12 h. Though he showed severe floppy infant, his mechanical ventilation has been terminated at the 45th day of birth, and starting oral administration of ingredient nutrient. Although the concentrations of lactate and pyruvate in blood were

Table 1
Biochemical parameters during therapy with none, DCA, or pyruvate.

	None ($n = 8$)	DCA therapy ($n = 12$)	Pyruvate therapy ($n = 10$)
Lactate (mM) (normal: 0.03–0.17)	9.6 ± 0.54	8.6 ± 2.63	5.28 ± 1.73[a,b]
(Range: minimum–maximum)	(8.70–10.10)	(3.56–12.70)	(2.73–7.75)
Pyruvate (mM) (normal: 0.003–0.10)	0.69 ± 0.13	0.61 ± 0.19	0.42 ± 0.13[a,b]
(Range: minimum–maximum)	(0.49–0.82)	(0.31–0.93)	(0.26–0.68)
L/P ratio (normal: 10–15)	14.5 ± 3.10	14.2 ± 2.12	12.6 ± 1.52
(Range: minimum–maximum)	(10.6–18.7)	(11.5–17.9)	(10.5–15.1)
Alanine (mM) (normal: 0.21–0.52)	1.7 ± 0.28	1.13 ± 0.27[a]	0.77 ± 0.38[a]
(Range: minimum–maximum)	(1.11–1.82)	(0.76–1.51)	(0.39–1.42)

All date are presented as mean ± SD during each treatments.
Lactate, pyruvate L/P ratio, and alanine were analyzed the significance between periods of none, DCA and pyruvate therapy using the two-tailed Mann–Whitney *U*-test. *P* value less than 0.05 showed significant.
[a] It showed significance between none and DCA or pyruvate therapy.
[b] It showed significance between DCA and pyruvate therapy. *n*: number of measurements.

Table 2
Entire clinical course and symptoms.

	Clinical course		
	None	DCA	Pyruvate
Study periods	1 month (1 m)	17 months (2–18 m)	58 months (1 year 6 months–6 years 4 months)
Hospitalization (day)	31	124	3
Emergency visit (time)	0	14	4
Diagnosis by EEG	Infantile epilepsy	West syndrome or Lennox–Gastaut syndrome	Lennox–Gastaut syndrome
Convulsion			
Frequency	15 or more/days	18 or more/days	2–3/months
Duration	5–15 s/Epilepsy	5–20 s/Epilepsy	5–10 s/Epilepsy
Series formation	None	Series formation	No series formation
Anticonvulsants	Phenobarbital 20 mg/kg/day	Carbamazepine 10 mg/kg/day Valproate 10–15 mg/kg/day Clobazam 1.0 mg/kg/day Zonisamide 2–4 mg/kg/day	Carbamazepine 10 mg/kg/day Valproate 15 mg/kg/day Clobazam 1.5 mg/kg/day Zonisamide 2–4 mg/kg/day
JMDRS	58	58	57
Developments	Severe floppy infant Respiratory care	Cannot head control Cannot sit alone Cannot rolling over Floppy infant Eating mainly by S-tube	Floppy infant Head control (21 months) Rolling over (42 months) Sit alone (56 months) Eating mainly by mouth

slightly decreased by DCA, his clinical conditions were deteriorating progressively. He could not fix the head control, and roll over at 6 months of age. He was diagnosed with West syndrome at 6 months-old because of his intractable generalized convulsions. Though he received two types of anti-convulsants as shown in Table 2, his convulsion did not stop and showed several seizures a day with series formation. Brain MRI on 7-months-old showed a premature myelination and atrophy in frontal lobe with callosal hypoplasia, and brainstem abnormality. He showed severe floppiness, loose head control, inability to sit alone and roll over, feeding difficulty, and no significant words at the age of 18 months-old. His EEG pattern changed to Lennox–Gastaut syndrome at that time (Fig. 1A). Nerve conduction velocity in both motor and sensory nerve showed low amplitude with delayed velocity indicating severe neuropathy. At this point, we thought that severe neuropathy seen in the patient may caused by the severe adverse effects of DCA, since he received the DCA supplementation for more than 17 months period. Because of the severe neuropathy, we decided to terminate the DCA at his age of 18 months-old, and after received written informed consent, we started the oral supplementation of sodium pyruvate at 0.5 g/kg/day TID. Three months later, he started to roll over and showed the facial expression of happiness and sadness. He could start to chatter and swallow the liquid food. Six months after starting pyruvate supplementation, he had almost no epileptic seizure and was demonstrated the significant improvement by EEG (Fig. 1B). The entire clinical course is summarized in Fig. 2 and Table 2.

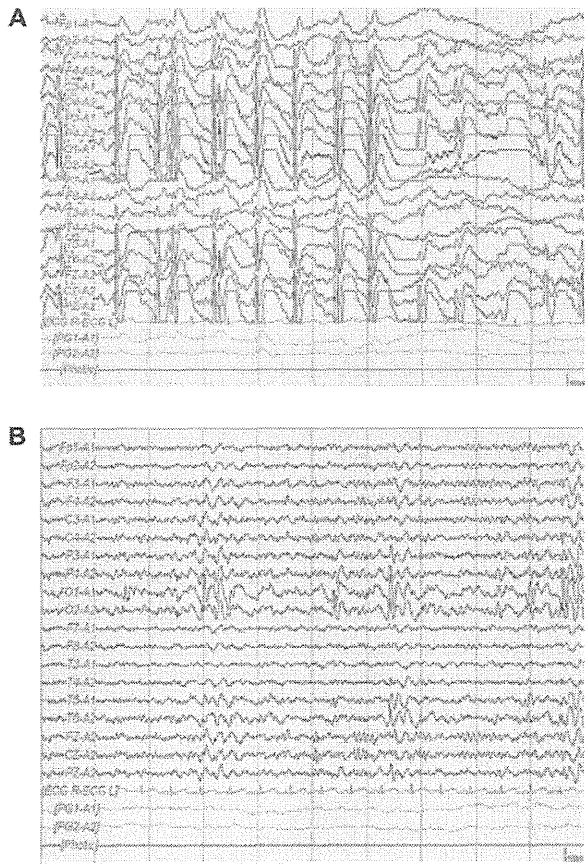

Fig. 1. (A) EEG taken at 18 months old. A grossly abnormal inter-ictal EEG showed continuous, high-amplitude, sharp-slow-waves or spike-slow-waves indicating a multifocal and generalizing sharp-slow-wave-discharges at 1.5–2.5 Hz. Patient showed intractable epilepsy with 15–20 times a day of grandmal, and/or myoclonic type seizure. (B) EEG taken at 36 months old. An abnormal inter-ictal EEG pattern showed with continuous, sharp-slow-waves or spike-slow-waves. However it showed low-amplitude and less multi-focality. Patient showed no grandmal or myoclonic type seizure by daily base frequency.

The lactate and pyruvate concentrations in cerebral spinal fluid were 8.23 mM, and 1.26 mM under the period of DCA therapy, and 4.61 mM and 0.68 mM under the period of pyruvate therapy (Fig. 2).

2.2. Lactate, pyruvate, L/P ratio and alanine determination

In order to investigate the energy state of patient in each time period of therapy, we measured the plasma level of lactate, pyruvate and aminogram including alanine, 8 times in the periods of 31 days with free of DCA and pyruvate, 12 times in 17 months during DCA therapy, and 10 times in 58 months during pyruvate therapy. Analysis of amino acids was performed on protein-free extracts of fresh plasma using described methods.

2.3. Enzyme assays

The PDHC activity in cultured skin fibroblasts was assayed using two different concentrations of TPP (0.4 and 1104 mM) after the activation of PDHC using DCA as previously described [10].

2.4. Genetic analysis

Mutation analysis of the E1α gene, a major cause of PDHC deficiency, was performed using genomic DNA from cultured skin fibroblasts. For the genetic analysis of the 11 exons of the E1α gene, the individual exons were amplified using primer pairs and conditions as described previously [11].

2.5. Statistical analysis

Statistical analysis of the biochemical data including lactate, pyruvate, L/P ratio, and alanine was performed using two-tailed Mann–Whitney *U*-test or Student's *t*-test. A value of $P < 0.05$ was considered as statistically significant.

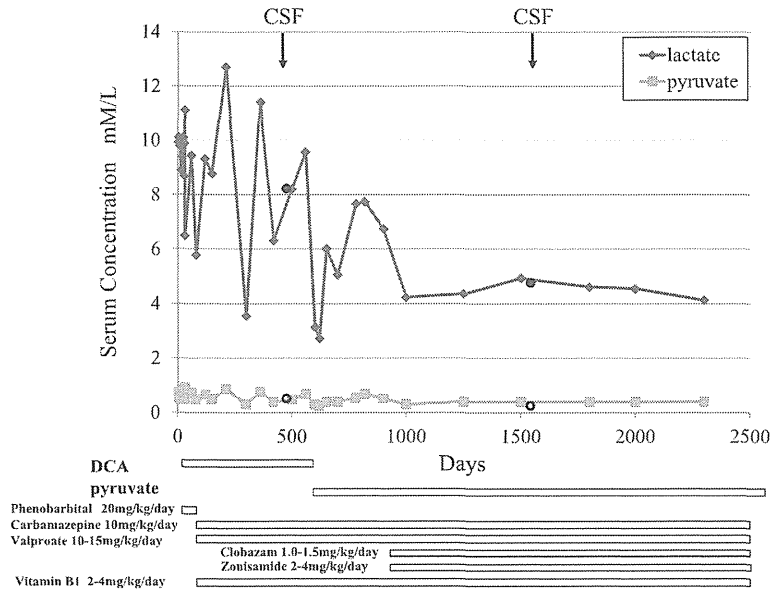

Fig. 2. Entire clinical course.

3. Results

Since patient showed lactic acidosis with normal lactate/pyruvate ratio, we measure the PDHC activity in cultured skin fibroblasts cells. The PDHC activity was 0.94 in the presence of DCA and 0.4 mM TPP (normal: 4.07 ± 0.68 nmol/min/mg protein). Mutation analysis of PDH E1α subunits revealed a point mutation of an A–C at nucleotide 599 in exon 6, resulting in the substitution of aspartate for threonine at position 200 (N200T). Though this mutation has not been reported before, we considered it as the responsible gene defect in this patient because; (1) no other mutations were found in entire PDH E1α gene, (2) conserved amino acid in different species, (3) mother has the mutation in hemizygous condition, and (4) no same mutation found in 50 normal females.

The laboratory data before, and after the treatment by DCA, and after pyruvate treatment are shown in Table 1 and Fig. 2. The concentration of lactate and pyruvate in blood before the treatment was 51–58 times higher than normal range, with normal lactate/pyruvate ratio (Table 1). The concentration of alanine was also increased 2.1–3.5 times higher than normal range. After the treatment by DCA, though the concentration of lactate and pyruvate showed no significance, the concentration of alanine was significantly decreased. The patient showed intractable seizures, and decreased the activity of daily living. After the treatment by pyruvate, the concentration of lactate and pyruvate were significantly decreased in comparison with those without therapy, and with DCA treatment, with significantly decreased level of alanine (Table 1 and Fig. 2). The concentrations of lactate and pyruvate in the CSF were also significantly decreased with significantly decreased plasma level of alanine (Fig. 2).

4. Discussion

LS, the most dominant sub-type of mitochondrial disorders in children, are clinically more severe and patients usually die before the first decade of the life. In another words, LS showed the most severe cytopathy among subtypes of mitochondrial disorders. Therapeutic target of mitochondrial angiopathy is now on-going of L-arginine as an investigator-mediated clinical trial on MELAS [12]. However there are no clinical trial of therapeutic approach for mitochondrial cytopathy especially LS. Since the severe adverse events of DCA reported in 2006 [13], the new therapeutic drugs to prevent or improve the mitochondrial cytopathy or lactic acidosis have to be developed as a substitute for DCA.

In the present study, we reported a patient with LS caused by a novel PDH E1α mutation who responded to pyruvate administration for 3 years period. Pyruvate therapy significantly decreased the lactate, pyruvate and alanine levels, showed no adverse effects such as severe neuropathy seen in this patient under the DCA therapy, and had better clinical response on development and epilepsy. It was reported that pyruvate percolates through the blood brain barrier via monocarboxylate transporters and provides an excellent energy state for neurons and astroglia [14]. As shown in our patient (Fig. 2), pyruvates decreased lactate and alanine levels not only in blood but in CSF, and improved the electroencephalogram in our patient, suggested that pyruvate