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Mitochondrial DNA Mutation-Elicited Oxidative Stress, Oxidative Damage, and Altered Gene Expression in Cultured Cells of Patients with MERRF Syndrome

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Abstract Myoclonic epilepsy and ragged-red fibers (MERRF) syndrome is a rare disorder characterized by myoclonus, muscle weakness, cerebellar ataxia, heart conduction block, and dementia. It has been documented that 80–90% of the patients with MERRF syndrome are caused by the A8344G mutation in the $tRNA^{Lys}$ gene of mitochondrial DNA (mtDNA). We and other investigators have reported that the mtDNA mutation results in not only inefficient generation of adenosine triphosphate but also increased production of reactive oxygen species (ROS) in cultured cells harboring A8344G mutation of mtDNA. In addition, we found an imbalance in the gene expression of antioxidant enzymes in the skin fibroblasts of MERRF patients. The mRNA, protein, and enzyme activity levels of manganese-superoxide dismutase were increased, but those of Cu,Zn-SOD, catalase, and glutathione peroxidase did not show significant changes. Recently, we showed that the excess ROS could damage voltage-dependent anion channel, prohibitin, Lon protease, and aconitase in the MERRF cells. Moreover, there was a dramatic increase in the gene expression and activity of matrix metalloproteinase 1, which may contribute to the cytoskeleton remodeling involved in the weakness and atrophy of muscle commonly seen in MERRF patients. Taken together, we suggest that mtDNA mutation-elicited oxidative stress, oxidative damage, and altered gene expression are involved in the pathogenesis and progression of MERRF syndrome.

Keyword Mitochondrial disease · MERRF · Oxidative stress · Antioxidant enzyme · Mitochondrial aconitase · Prohibitin · Lon protease · MMP-1

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Introduction

Mitochondrial diseases are mostly caused by defects in the enzymes involved in respiration and oxidative phosphorylation (OXPHOS) [1]. They may arise from mutations in nuclear DNA or mitochondrial DNA (mtDNA). It has been documented that some mitochondrial diseases are caused by specific mutations in nuclear genes, which are involved in the replication and maintenance of mtDNA and respiratory chain function of mitochondria. These diseases may be resulted from defects in the citric acid cycle, β-oxidation of fatty acids, the urea cycle, and the respiration and OXPHOS system, respectively [2]. However, a large portion of mitochondrial diseases are caused by point mutations in the transfer RNA (tRNA), ribosomal RNA (rRNA), or structural genes and by deletion, duplication, or depletion of mtDNA. Pathogenic mtDNA mutations include (a) point mutations of mtDNA: mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS), myoclonic epilepsy with ragged-red fibers (MERRF), Leigh syndrome, and Leber's hereditary optic neuropathy (LHON) and (b) large-scale mtDNA deletions: chronic progressive external ophthalmoplegia (CPEO) and Kearns-Sayre syndrome [3, 4]. These overt diseases are characterized by well-defined clinical features, but the correlation between clinical phenotype and genotype is rather poor for most of the mitochondrial diseases. It has

been established that most point mutations of mtDNA are maternally inherited and that large-scale mtDNA deletions are sporadic in patients with mitochondrial diseases [5]. Besides, most of the patients with mitochondrial diseases exhibit delayed onset and progressive clinical courses [6]. This implies that the clinical features of these diseases may involve the predisposing mtDNA mutations and age-related factors that cause a decline in the respiratory chain function, which exacerbates the inherited genetic defects [7, 8].

MERRF syndrome is a devastating neuromuscular disorder characterized by myoclonic epilepsy, general weakness, muscle wasting, cerebellar ataxia, deafness, and dementia transmitted through maternal lineages [9]. Additional manifestations such as short stature, optic atrophy, peripheral neuropathy, cardiomyopathy, myoglobinuria, and renal tubular dysfunction have also been documented [10, 11]. Besides, lactate and pyruvate are commonly elevated in serum at rest and increased excessively after moderate physical activities [12]. The A8344G mutation in the $tRNA^{Lys}$ gene of mtDNA is the most common mutation associated with MERRF syndrome, although few less frequent point mutations of mtDNA were also found in MERRF patients [13, 14]. Besides, molecular genetic studies of several MERRF pedigrees and biochemical studies of skin fibroblasts showed a positive correlation between the A8344G mutation in the $tRNA^{Lys}$ gene of mtDNA and the reduction in the activities of respiratory enzyme Complexes I and IV [7–9]. Recently, we demonstrated that the aging-associated increase of oxidative stress and oxidative damage are involved in the age-related decline of bioenergetic function of the affected tissues in patients with mitochondrial diseases [7, 8]. Moreover, we examined the gene expression profiles of the cultured cells from MERRF patients and age-matched normal subjects by complementary DNA (cDNA) microarray. The results showed that more than 350 genes were up-regulated and about 300 genes were down-regulated, respectively [15]. The up-regulated genes are associated with the stress response, inflammatory response, and alteration of cytoskeleton remodeling. Recent studies from other laboratories [16–18] have provided additional compelling evidence to support the contention that the oxidative stress and oxidative damage elicited by mtDNA mutation-induced respiratory chain defects in the affected tissues plays a crucial role in the pathogenesis and progression of mitochondrial diseases.

Reactive Oxygen Species in the Pathophysiology of Mitochondrial Disease

It has been established that intracellular reactive oxygen species (ROS) production is directly proportional to the respiration rate of mitochondria in mammalian cells [19]. More than 90% of tissue oxygen are consumed by mitochondria in human cells and about 1–5% of the O_2 is transformed to $O_2\cdot^-$ as a result of electron leak from Complex I (NADH: ubiquinone oxidoreductase) and the protonmotive Q cycle in Complex III of the respiratory chain under normal physiological conditions [20]. Usually, $O_2\cdot^-$ anions can be converted to H_2O_2 by manganese-superoxide dismutase (Mn-SOD) in mitochondria, and are then detoxified by glutathione peroxidase (GPx) or catalase (CAT) [21]. Solid evidence has been accumulated to show that pathogenic mutations of mtDNA in the affected tissues of a patient with mitochondrial disease can impair respiration and OXPHOS when the mutant load reaches a threshold [7, 8]. The defects in the respiratory chain can lead to overproduction of ROS, which may further enhance the oxidative damage to various biomolecules in affected cells [22–24]. In a recent study, we demonstrated that the mean intracellular H_2O_2 content in the skin fibroblasts from MERRF patients was higher than that of the skin fibroblasts from age-matched normal subjects (Fig. 1a). This series of MERRF patients had different proportions of the mtDNA with A8344G mutation (M1, 78±7%; M2, 90±4%; M3, 54±8%; M4, 85±8%) and displayed ragged-red fibers (RRF) on muscle biopsies stained with modified Gomori-trichrome stain, which are characteristic of the accumulation of abnormal mitochondria. All of the four MERRF patients manifested typical clinical symptoms, including myoclonus, muscle weakness, seizures, and epilepsy.

In order to study the pathogenesis of mitochondrial diseases, scientists have developed cytoplasmic hybrids (cybrids) to examine the effects of a specific mtDNA mutation on various cell functions [23, 25–27]. These cybrids had identical nuclear background but harbored different proportions of mtDNA with a specific mutation. Such studies have established that A8344G mutation in $tRNA^{Lys}$ gene not only affects the replication and transcription of mtDNA, but also decreases protein synthesis and the efficiency of adenosine triphosphate (ATP) synthesis in mitochondria [28]. Besides, the cybrids harboring mutant mtDNA were more sensitive to extrinsic oxidative stress such as H_2O_2 and UV irradiation, which led to apoptosis as revealed by an increase in the expression of several apoptotic markers [29, 30]. It is worth mentioning that the susceptibility to UV-induced apoptosis of the cybrids harboring 4,977-bp-deleted mtDNA was related to the mutant load [31]. The oxidative stress-elicited damage and mutation to mtDNA may further compromise respiratory function and culminate in a ROS-driven vicious cycle, which is a contributory factor to the pathophysiology and progression of mitochondrial diseases [32–34].

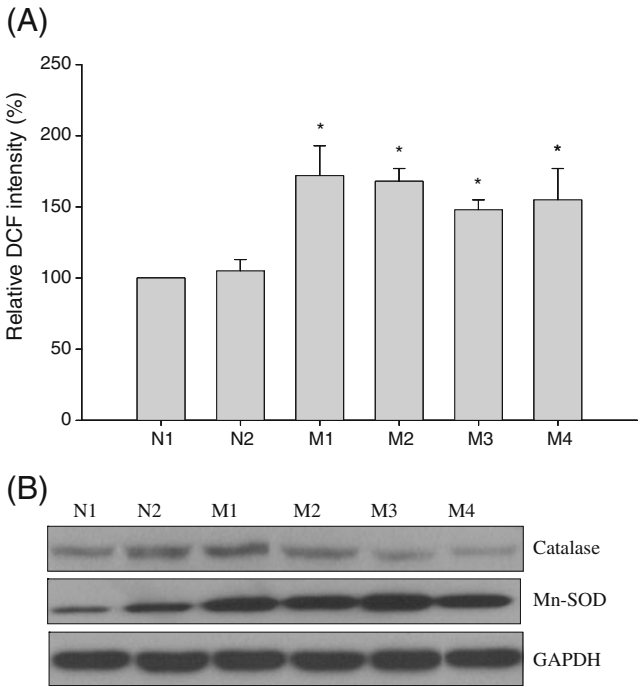

Fig. 1 The intracellular H_2O_2 content and the expression of Mn-SOD and catalase in skin fibroblasts from MERRF patients. This series of skin fibroblasts cultured from MERRF patients had different proportions of the mtDNA with A8344G mutation (M1, 78±7%; M2, 90±4%; M3, 54±8%; M4, 85±8%), and from normal subjects (N1 and N2), there was no A8344G mutation on mtDNA. **a** The level of intracellular H_2O_2 in skin fibroblasts was measured by flow cytometry using the fluorescent probe 2′, 7′-dichlorofluorescein (*DCF*). The results showed that the intracellular H_2O_2 contents of skin fibroblasts were increased in MERRF patients (M1–M4) as compared with those of the age-matched normal subjects (N1 and N2). Each data point from flow cytometry was calculated by EXPO-MFA software (Beckman-Coulter) and normalized to N1. **b** Up-regulation of Mn-SOD and down-regulation of catalase were revealed by Western blot analysis of proteins in skin fibroblasts of MERRF patients as compared with those of the age-matched normal subjects. The results are presented as mean ± SD of three separate experiments and the statistical analysis was performed by the Microsoft Excel statistical package (n=3, *p<0.05 by Student's t test)

Imbalanced Expression of Antioxidant Enzymes in the MERRF Skin Fibroblasts

Mitochondrial respiratory chain dysfunction can elevate the intracellular oxidative stress, which may in turn alter the expression of proteins and enzymes in the affected cells of MERRF patients. Indeed, it has been shown that the expression profile of antioxidant enzymes is altered in the affected tissues or cells of patients with mitochondrial diseases [35–38]. The protein expression and activity levels of antioxidant enzymes such as Mn-SOD were found to increase in affected tissues of patients with a specific mitochondrial disease, which was a response of cells to avoid oxidative damage of ROS released by defective mitochondria [35]. However, the antioxidant defense system may not be effective in coping with oxidative stress in the affected tissue cells of patients with mitochondrial diseases. In the past two decades, we have established the primary culture of skin fibroblasts from a number of patients with CPEO, MELAS, and MERRF syndromes, respectively, to study the alterations of antioxidant enzymes. We found that the skin fibroblasts from eight CPEO patients all had significantly higher enzyme activity and mRNA and protein expression levels of Mn-SOD, but those of catalase and GPx were not increased or even decreased in some of the patients [39]. We also detected similar changes in the skin fibroblasts of four MERRF patients (Fig. 1b). These results indicate an imbalance between the generation and disposal systems for H_2O_2 in the skin fibroblasts of those patients with CPEO or MERRF syndrome. Likewise, Kunishige et al. [40] demonstrated that the expression of Mn-SOD protein was dramatically increased in the cytochrome *c* oxidase-deficient RRFs, and that the expression levels of Cu,Zn-SOD, CAT, and GPx were only slightly increased. A decrease in the activity or expression level of the Mn-SOD relative to GPx and catalase, respectively, can lead to the accumulation of ROS such as superoxide anions. On the contrary, an increase in the activity and/or expression levels of Mn-SOD relative to GPx or catalase may result in increased production of H_2O_2. Therefore, a fine balance between different antioxidant enzymes is very important for the affected tissue cells to cope with ROS in MERRF syndrome. These findings suggest that an increase in the expression of Mn-SOD may be considered an early sign of mitochondrial disease as it often occurs at the early stage of mitochondrial encephalomyopathies such as MERRF syndrome [40].

Oxidative Damage to Mitochondrial Proteins in the MERRF Skin Fibroblasts

It was suggested that inhibition of enzymes in the Krebs cycle by H_2O_2-induced oxidative stress may contribute to neurodegenerative diseases and human aging [41]. Recently, we investigated oxidative modification to mitochondrial proteins by using proteomic techniques to further understand the pathogenesis of MERRF syndrome [42]. We observed that mitochondrial proteins were more sensitive to oxidative damage in mutant cybrids prepared from a Taiwanese MERRF patient with about 90% of mtDNA with A8344G mutation. By using the 2-D gel electrophoresis and liquid chromatography and tandem mass (LC/MS/MS) spectrometry, a total of 16 carbonylated mitochondrial proteins were identified in the MERRF cybrids after treatment with 125 μM H_2O_2 for 24 h (Table 1). Besides, by comparing with the wild-type cybrids, we found a significant increase in the carbonylation of glutamate

Table 1 Carbonylated mitochondrial proteins in human cells harboring A8433G mutation of mtDNA identified by 2-D gel electrophoresis and LC/MS/MS

Spot	Protein name	mw (kDa); p*I*	Ratio (M/W)
1	Pyruvate dehydrogenase E1 component beta subunit	39.2; 6.20	0.71
2	Chaperonin GroEL precursor	61.0; 5.70	N.D.
3	Protein disulfide-isomerase ER60 precursor (EC 5.3.4.1)	56.8; 5.98	N.D.
4	Voltage-dependent anion channel 2 (VDAC-2)	31.5; 7.49	1.98
5	Voltage-dependent anion-selective channel protein 1 (VDAC-1)	30.6; 8.63	1.80
6	Annexin A2	38.4; 7.56	1.10
7	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	36.0; 8.57	0.86
8	Fructose-bisphosphate aldolase	39.4; 8.30	1.64
9	Mitochondrial ribosomal protein L38	40.7; 6.20	1.09
10	Fumarate hydratase (FH)	54.6; 8.85	N.D.
11	Alpha enolase	47.0; 6.99	N.D.
12	Glutamate dehydrogenase (GDH)	61.4; 7.66	N.D.
13	GTP-binding regulatory protein beta-4 chain	37.4; 5.59	0.86
14	GTP-binding regulatory protein beta-2 chain	37.4; 5.60	1.15
15	Dihydrolipoyllysine-residue acetyltransferase	65.8; 5.79	N.D.
16	Prohibitin	29.8; 5.57	1.95

The data presented in this table were obtained by comparative analysis of a cybrid clone harboring 90% A8344G mutant mtDNA from a MERRF patient and a wild-type cybrid clone. Carbonylated mitochondrial proteins were quantified by the method reported by Conrad et al. [42]. After the isolation of mitochondria, mitochondrial proteins were extracted and derivatized with 2, 4-dinitrophenyl hydrazine before conduction of 2-D gel analysis. The immobilized pH gradient (IPG) strip (18 cm, pH 4–7) was used in the IEF and an 8–15% gradient polyacrylamide gel was prepared for the 2-D electrophoresis. After analysis of the 2-D gel, the carbonylated mitochondrial proteins were identified and quantified by LC/MS/MS and primary sequence database search was performed by Mascot. The ratio is defined from the intensity of each carbonylated protein between the MERRF cybrid clone (M) and wild-type cybrid clone (W) after treatment with 125 μM H_2O_2 for 24 h. *N.D.* indicates "not detectable" for the ratio because no significant amount of carbonylated protein was detected in wild-type cybrids

mw molecular weight of the protein, *pI* isoelectric point of the protein

dehydrogenase (GDH) and fumarate hydratase (FH), which both participate in the Krebs cycle [43, 44]. It is important to point out that impairment of GDH may be involved in the pathogenesis of neurodegenerative diseases because of the disturbance of synaptic metabolism [45]. Besides, it has been reported that the degree of oxidation of GDH is positively correlated with the clinical stage of Alzheimer's disease (AD) patients [46]. MERRF syndrome is a neuromuscular disease accompanied by myoclonus, epilepsy, and neurological disorders among others. Further study is warranted to determine whether GDH is involved in the pathogenesis of MERRF syndrome. On the other hand, FH has been proven to be involved in the devastating infantile encephalopathies, similar to those caused by a recessive mtDNA mutation or defects of the respiratory chain [44]. Besides, FH was found to be a target of ROS in experimental traumatic brain injury [47], and thus, the inhibition of FH activity may possibly underlie the breakdown of mitochondrial energy production and cause eventual cell death in this model. Pyruvate dehydrogenase complex, which is composed of three subunits (E1, E2, and E3 subunits) and is the point to control mitochondrial respiration, was shown to be more extensively modified in the MERRF cybrids. Other studies also showed that pyruvate dehydrogenase (PDH) is quite susceptible to oxidative damage [48]. Chronic lactic acidosis and neuron development defects are often manifested in patients with PDH deficiency [49]. In addition, patients with Leigh syndrome, one of the mitochondrial diseases caused by the A8993G mutation of mtDNA, are often associated with a decrease in the PDH activity [50]. Further, we also observed that voltage-dependent anion channel (VDAC) and prohibitin (PHB) were susceptible to oxidative damage in the MERRF cybrids compared with wild-type cybrids after treatment with 125 μM H_2O_2 for 24 h. VDAC is a major component of the permeability transition pore complex on the outer mitochondrial membrane, which regulates the transport of ions and metabolites in and out of the mitochondria. The role of VDAC in mitochondrial diseases is not known, but a recent study showed that oxidative modification of VDAC is a cardinal feature of AD [51]. We speculate that the accumulated oxidative damage to VDAC may cause a loss of bidirectional fluxes of ions and metabolites across mitochondrial membranes, which in turn leads to the bioenergetic breakdown in the MERRF cybrids. On the other hand, the key function of

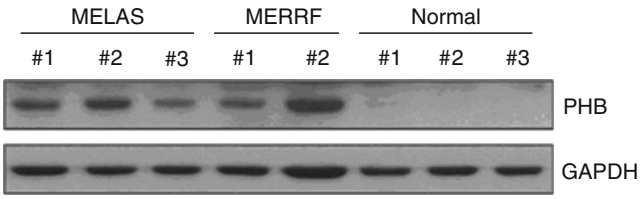

Fig. 2 Increase in the expression of prohibitin (*PHB*) in skin fibroblasts from patients with MERRF or MELAS syndrome. Western blot analysis revealed that the PHB protein expression was significantly increased in skin fibroblasts harboring the MERRF-specific A8344G mutation and MELAS-specific A3243G mutation of the mtDNA, respectively. GAPDH was used as internal control, and the data are presented as mean ± SD of the results from three separate experiments

PHB is to act as a chaperone to stabilize proteins and prevent misfolding of mitochondrial proteins [52, 53]. It was demonstrated that oxidative damage to the PHB protein might aggravate the mitochondrial dysfunction [54]. Besides, we observed an increase in the expression of PHB in the skin fibroblasts from MERRF and MELAS patients, respectively (Fig. 2). This finding suggests that increased PHB expression could be a good marker for mitochondrial dysfunction, especially in patients who carry a pathogenic mutation of mtDNA.

Recently, we also detected dramatic oxidative damage to the proteins containing iron–sulfur (Fe–S) clusters in the mitochondria of cells that had been cultured from MERRF patients. Enzymes containing Fe–S clusters can serve as excellent donors and acceptors of electrons in a variety of biological reactions such as mitochondrial respiratory enzyme Complexes I–III, ferredoxins, and hydrogenases [55]. A number of enzymes or proteins containing Fe–S clusters have been shown to be quite labile under oxidative stress [56]. It is conceivable that oxidative damage to proteins or enzymes containing Fe–S clusters is associated with or responsible for mitochondrial dysfunction in the target tissues of patients with mitochondrial diseases. Aconitase is one of the Fe–S cluster-containing enzymes that participate in the Krebs cycle in the matrix of mitochondria. It has been known that aconitase is a preferred target of ROS and can be inactivated by ROS that cause disassembly of the Fe–S clusters [57]. Besides, it was reported that the Fe^{2+} ions released from the damaged aconitase can cause further oxidative damage through the Fe^{2+}-catalyzed Fenton reaction [58]. Most importantly, many studies revealed that decrease of mitochondrial aconitase activity was associated with neurodegenerative diseases such as Parkinson's disease, Huntington's disease, and Friedreich's ataxia [59–61]. In one of the previous studies, we assayed the activities of mitochondrial (m-) and cytosolic (c-) aconitases by an in-gel analysis method [62] in the cultured cells of patients with MERRF syndrome. The results revealed that the m-aconitase and c-aconitase activities in the MERRF cells were largely decreased as compared with age-matched normal subjects (Fig. 3). These results suggest that the increase in oxidative stress elicited by respiratory chain dysfunction due to A8344G mutation in the $tRNA^{Lys}$ gene can damage proteins and enzymes containing Fe–S clusters such as m- and c-aconitases.

Further, we noted two cytosolic enzymes, α-enolase and fructose-bisphosphate aldolase, which were shown to carry more oxidative modifications in the MERRF cybrids after 125 μM H_2O_2 treatment for 24 h as compared to wild-type cybrids. α-Enolase, a subunit of enolase that is responsible for the inter-conversion of 2-phosphoglycerate and phosphoenolpyruvate in glycolysis, may be located near the mitochondria for substrate accessibility. Thus, enolase could become the immediate targets of free radicals produced by defective mitochondria. Recently, α-enolase was reported to be more oxidatively damaged in AD and in the brain tissues of old mice, which suggest that the enolase activity may be reduced in the affected tissues [63] and that the decrease of α-enolase activity could result in abnormal cell growth and reduced metabolism in the pathogenesis of AD [64]. Therefore, the increase of oxidatively modified α-enolase observed in the MERRF cybrids may imply the alteration of glucose metabolism in the pathology of the MERRF syndrome. Instead of enolase, it was reported that fructose-bisphosphate aldolase, located in the cytoplasm and associated with mitochondria, would catalyze the breakdown of fructose-1,6-bisphosphate during glycolysis to glyceraldehyde 3-phosphate and dihydroxyacetone phosphate [65]. The significance of the increase of the oxidation modification of aldolase in the MERRF cybrids has never been reported, but it may be related to metabolic demands

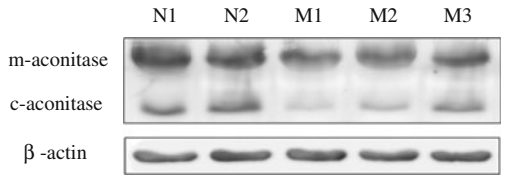

Fig. 3 The mitochondrial (*m*-) and cytosolic (*c*-) aconitase activities in skin fibroblasts from MERRF patients and normal subjects. In-gel analytic method [62] was used to assay for the activity of aconitase in skin fibroblasts. An aliquot of 100 μg freshly prepared proteins was subjected to the gel for electrophoresis. After completion of the electrophoresis, the aconitase activities were assayed by incubating the gels in the dark at 37°C in a reaction buffer (100 mM Tris–HCl, pH 8.0) containing 1 mM $NADP^+$, 2.5 mM cis-aconitic acid, 5 mM $MgCl_2$, 1.2 mM MTT, 0.3 mM phenazine methosulfate, and 5 U/ml isocitrate dehydrogenase. Aconitase activity will be assayed and performed by the ImageJ 1.40g software (NIH, USA). A representative set of data of the in-gel analysis is shown to indicate the decrease of m- and c-aconitase activities in skin fibroblasts of MERRF patients (M1–M3) as compared with those of age-matched normal subjects (N1 and N2). The β-actin expression is shown as a loading control, and the data are presented as mean ± SD of the results from three separate experiments

for MERRF patients. Taken together, we have identified mitochondrial proteins with oxidative damage in the MERRF cybrids, and these oxidized proteins could be involved in the breakdown of energy metabolism and impairment of the protein quality control system. These observations may provide us new insight to understand the pathophysiology of the MERRF syndrome.

Alteration in the Expression of Mitochondrial Proteins in MERRF Syndrome

In order to better understand the pathophysiology of MERRF syndrome, we further investigated the alteration of mitochondrial protein expression in the MERRF cybrids harboring >90% mtDNA with A8344G mutation by 2-D gel electrophoresis. The fist-dimensional isoelectric focusing (IEF) strip was chosen with a small range of p*I* system [4–7] in our study. We found that about 1,200 spots in the MERRF cybrids were up- or down-regulated as compared to wild-type cybrids after analysis of the 2-D gels. The spots with a change in the intensity above 50% were selected and identified by LC/MS/MS spectrometry (Table 2). Further analysis of the results showed that the mitochondrial proteins with a decreased intensity in the MERRF cybrids were mostly related to the respiratory chain, especially the subunits of Complex I. This protein expression profile change in the MERRF cybrids was caused by the A8344G mutation in the $tRNA^{Lys}$ gene of mtDNA, which results in defects in the translation in mitochondria. However, the ATP synthase d subunit of Complex V was up-regulated as a compensatory response in the MERRF cybrids, which was also observed in the cultured cells of patients with Parkinson's disease [66]. Besides, metabolic enzymes, mitochondrial aldehyde dehydrogenase, and delta-3, delta-2-enoyl-CoA isomerase, were more than twofold up-regulated in the MERRF cybrids as compared to wild-type cybrids, although there are still unknown roles in the pathology of MERRF syndrome. Most importantly, mitochondrial Lon protease was significantly up-regulated in the MERRF cybrids. Lon protease is a mitochondrial ATP-dependent protease, which has been shown to degrade oxidized aconitase [67]. Inactivation of Lon protease was reported to associate with several neurodegenerative diseases [68]. Recently, it was reported that Lon protease is a stress response enzyme [69], which was particularly vulnerable to inactivation under the conditions of elevated oxidative stress [60]. In a recent study, we determined the rate of degradation of flourescein isothiocyanate (FITC)-casein, an artificial fluorescence substrate, by Lon protease in mitochondria [70]. The results showed that the activity of Lon protease in the MERRF cybrids was significantly lower than that of the wild-type cybrids (Fig. 4),

Table 2 Assignment of proteins in the 2-D gel spots with quantitative changes from human cells harboring A8433G mutation of mtDNA

Spot	mw/p*I*	Ratio (M/W)	Protein name	Location
1	26.2/4.40	0.37	Myosin regulatory light chain 2	Cyt
2	33.2/5.20	0.33	Chloride intracellular channel protein 1	Nuc
3	76.5/5.35	0.29	NADH-ubiquinone oxidoreductase, 75 kDa subunit	Mito
4	52.8/5.52	0.08	Cytokeratin 8	Cyt
5	47.5/5.63	0.31	Ubiquinol–cytochrome *c* reductase complex core protein I	Mito
6	37.2/5.88	0.41	L-lactate dehydrogenase B chain	Cyto
7	27.4/6.05	0.48	NADH-ubiquinone oxidoreductase, 30 kDa subunit	Mito
8	19.6/5.33	3.96	Endoplasmic reticulum protein ERp19	ER
9	24.5/5.25	1.56	ATP synthase d subunit	Mito
10	160.0/5.24	3.23	Oxygen-regulated protein precursor, 150 kDa	ER
11	30.4/5.93	2.98	Endoplasmic reticulum lumenal protein 28	ER
12	55.3/5.88	3.70	Mitochondrial aldehyde dehydrogenase 2	Mito
13	93.8/5.93	2.00	Lon protease homolog, mitochondrial precursor	Mito
14	87.8/5.95	2.99	Aldehyde dehydrogenase 1 family, member L2	Cyto
15	30.9/6.30	2.46	Delta-3, delta-2-enoyl-CoA isomerase	Mito

The data presented in this table were obtained by comparative analysis of mitochondrial proteins expressed in a cybrid clone harboring 90% A8344G mutant mtDNA from a MERRF patient and those expressed in a wild-type cybrid clone. For the 1-D electrophoresis, the IEF strip with a mid-range p*I* (4–7) was chosen and an 8–15% gradient polyacrylamide gel was prepared for the 2-D electrophoresis. After analysis of the 2-D gel, about 1,200 spots were detected by silver staining. The spots with a change in the intensity above 50% between mutant and wild-type cybrids were selected and identified by LC/MS/MS. The ratio indicates the relative intensity of the protein spot in mitochondria of the MERRF cybrid clone (M) as compared with that of the wild-type cybrid clone (W)

mw molecular weight of the protein, *pI* isoelectric point of the protein, *Cyt* cytoplasm, *Mito* mitochondria, *ER* endoplasmic reticulum

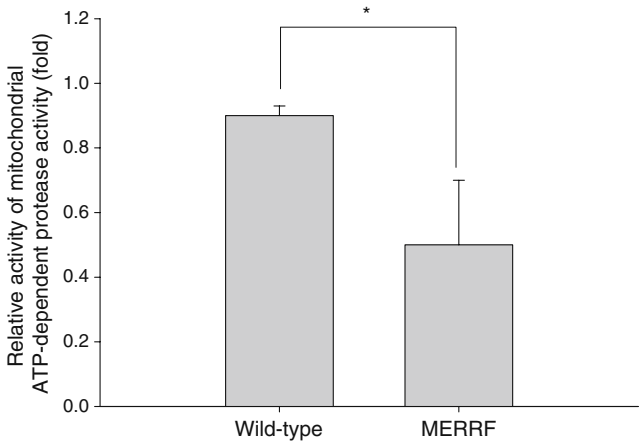

Fig. 4 Decrease of mitochondrial Lon protease activity in the MERRF cybrids. Mitochondrial ATP-dependent Lon protease activity was assayed by using FITC-casein, an artificial fluorescent substrate, according to the method described by Bulteau et al. [70]. Proteolysis of FITC-casein by Lon protease resulted in the production of fragmented peptides, which were determined on a fluorescence spectrophotometer. The results showed that the mitochondrial ATP-dependent Lon protease activity was significantly decreased in the MERRF cybrids as compared with that of the wild-type cybrid. The data are presented as mean ± SD of the results from three separate experiments, and the statistical analysis was performed by using the Microsoft Excel statistical package (n=3, *p<0.05 by Student's t test)

while the expression of Lon protease was twofold up-regulated in the MERRF cybrids as compared with the wild-type cybrids by the 2-D proteomics approach. We believe that up-regulation of Lon protease and PHB in the MERRF cells may be a compensation for the decrease of their enzymatic activities caused by ROS damage. The up-regulation of Lon protease and PHB contributes to the quality control of mitochondrial proteins, which may be useful in the elucidation of the pathology of MERRF syndrome (Fig. 5).

On the other hand, it is well-established that proper folding, post-translational modifications, and oligomerization of the secretory proteins are controlled in the endoplasmic reticulum (ER) by endoplasmic reticulum luminal proteins (ERp) [71]. We found significant increase of ER proteins in the MERRF cybrids, indicating the crisis of protein quality control in mutant cells. ERp19 and Erp28, the members of the protein disulfide isomerase family that may be involved in protein folding and secretion in the ER [72], were increased by more than 2.5-fold in the MERRF cybrids as compared to the wild-type cybrid. In addition, we also found that the ER-associated 150-kDa oxygen-regulated protein (ORP-150), a member of heat shock protein family that functions as a

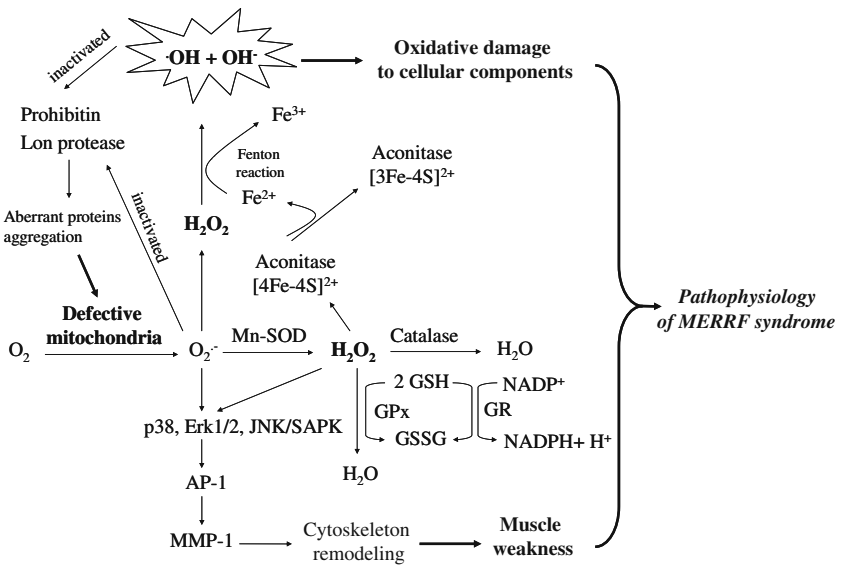

Fig. 5 Illustration of the mtDNA mutation-elicited oxidative stress and oxidative damage in the pathogenesis of MERRF syndrome. The defects in the mitochondrial respiratory chain in mutant cells from MERRF patients can cause overproduction of ROS by the electron leak from the respiratory chain. We found an up-regulation of the protein expression and enzymatic activity of Mn-SOD, but not those of Cu,Zn-SOD, catalase, glutathione peroxidase (*GPx*), and glutathione reductase (*GR*), respectively. This scenario results in oxidative stress due to the accumulation of H_2O_2 and oxidative damage in the MERRF cells. On the other hand, aconitase is a protein containing Fe–S clusters and ROS-induced damage to aconitase can lead to the release of Fe^{2+} ion. The released Fe^{2+} ion might enhance the generation of hydroxyl radicals through Fenton reaction. Prohibitin (*PHB*) and Lon protease are susceptible to oxidative damage brought about by ROS in mitochondria. Inactivated PHB and Lon protease can cause the accumulation of misfolded or damaged proteins, which further amplify the mitochondrial defects. Moreover, ROS increased the gene expression and activity levels of matrix metalloproteinases 1 (*MMP-1*), which could cause degradation of the cytoskeleton of the skin fibroblasts of MERRF patients. Improper cytoskeleton remodeling is linked to the weakness, wasting, and atrophy of skeletal muscle in patients with MERRF syndrome. The above-mentioned molecular events together suggest that respiratory chain dysfunction-triggered oxidative stress and oxidative damage play an important role in the pathogenesis and progression of MERRF syndrome

molecular chaperone in the ER, was increased by more than threefolds in the MERRF cybrids as compared with the wild-type cybrids [73]. These implied that the up-regulation of these protein quality control enzymes may play an essential role in the prevention of accumulation of the oxidized or misfolded proteins in mitochondrial diseases such as MERRF syndrome. Finally, we also found several non-mitochondrial or non-ER proteins including myosin regulatory light chain 2, cytokeratin 8, and chloride ion channel protein 1 that were also found to be altered in the MERRF cybrids. In summary, these findings suggest that the 2-D proteomics approach is useful and powerful for the detection of novel proteins that may play a role in the pathogenesis and progression of MERRF syndrome.

Disruption of Cytoskeleton by Up-regulated MMP-1 in MERRF Syndrome

In a previous study by cDNA microarray, we discovered that a number of genes were over-expressed in skin fibroblasts from patients with MERRF syndrome [10]. These include not only the antioxidant enzymes (notably Mn-SOD) but also several matrix metalloproteinases (MMPs), which include MMP-1, MMP-3, and MMP-9. The up-regulation of MMP-1 was apparently induced by the increased production of ROS in affected cells because the same change could be observed by treatment of the cells with H_2O_2, and the phenomenon could be abolished by pre-treatment of cells with *N*-acetyl cysteine (NAC). It is worth noted that the features of MERRF syndrome include myoclonus, clonic–tonic seizure, and weakness and atrophy of skeletal muscle [3]. The atrophy of the ECM in vitro and in vivo has been correlated with the increased expression of MMPs [74]. Besides, several studies have shown that alterations of MMPs are involved in the progression of neurodegenerative diseases, including the degradation of β-amyloid peptides in AD, and the apoptosis of dopaminergic neurons in PPD [75]. Moreover, MMP-1 was found to be highly induced in human fibroblasts upon treatment with H_2O_2 [76, 77], and ROS caused a decrease in fibrillar collagen synthesis, which was associated with an increase in the activities of MMPs in the myocardial cells [78]. Most importantly, the elevated ROS production and Mn-SOD activity can alter a number of distinct signaling cascades that can specifically activate the expression of MMPs, executing extracellular matrix degradation that is associated with various degenerative diseases [79, 80]. Taken together, we suggest that the morphological changes in the mitochondria of the affected cells of patients with MERRF syndrome are a result of intracellular H_2O_2-induced up-regulation of the MMP-1. This scenario suggests that oxidative stress and its subsequent response to induce MMPs play an important role in the degradation and deterioration of the extracellular matrix and structural proteins of skeletal muscle in patients afflicted with MERRF syndrome.

Concluding Remarks

Defects in mitochondrial respiratory enzyme Complexes I and IV accompanied with RRF are the most prominent biochemical defects in the muscle of MERRF patients [81]. Mitochondrial dysfunction not only decreases the production of ATP but also increases the ROS generation through the electron leak from the respiratory chain in mitochondria [7, 8]. Consequently, enhanced oxidative stress and oxidative damage have been often observed in the affected tissues of MERRF patients [26]. Normally, the expression and activity levels of antioxidant enzymes are induced to change to prevent cells from ROS-induced oxidative damage. Moreover, we first observed an imbalance in the expression of antioxidant enzymes in cultured cells of patients with CPEO and MERRF syndrome, respectively. Although the activity of Mn-SOD was found to be dramatically increased to transform superoxide anions to H_2O_2 in the skin fibroblasts from the MERRF patients, the activities of catalase and GPx were not increased in a coordinated manner to metabolize H_2O_2 to H_2O. The inefficient regulation and imbalanced expression of the antioxidant enzymes lead to the accumulation of H_2O_2 in the affected cells of patients with CPEO, MERRF, and MELAS syndrome, respectively [82]. We may suggest that the increase in protein expression of Mn-SOD can be considered as an early sign of the onset of mitochondrial disorders such as MERRF syndrome [40].

Long-term exposure to ROS of the affected cells in MERRF patients may initiate and expedite a vicious cycle to result in further increase of ROS production and enhanced oxidative damage to DNA, RNA, lipids, and proteins in mitochondria [83]. Damage to aconitase may promote the intracellular ROS production because of the fact that the released Fe^{2+} ion can act as a catalyst of Fenton reaction (Fig. 5). On the other hand, we found that the mitochondrial Lon protease was more sensitive to oxidative damage and thereby inactivated in cultured cells of patients with MERRF syndrome (Fig. 4). Loss of Lon protease activity might aggravate the biochemical defects caused by the A8344G mutation of mtDNA due to defective degradation and accumulation of damaged proteins in the mitochondria [84, 85]. Besides, we found a significant increase in the protein expression of prohibitin in skin fibroblasts of MERRF and MELAS patients, respectively (Fig. 2). This finding suggests that increased expression of prohibitin can serve as a good marker for the defects in

tRNA, rRNA, and other factors that are essential for the protein synthesis in mitochondria. Moreover, several glycolytic enzymes were found to be quite susceptible to oxidative damage in the MERRF cybrids, indicating that the breakdown of energy metabolism may be involved in the pathophysiology of MERRF syndrome. Finally, we demonstrated that H_2O_2-induced MMP-1 expression is increased in skin fibroblasts from MERRF patients and the effect can be abolished by treatment with the antioxidant NAC [10]. MMP-1 is involved in the cytoskeleton remodeling, and the regulation of MMP-1 requires the interplay between the oxidative stress-responsive signaling pathways of p38, Erk1/2, and JNK/SAPK followed by the activation of the transcription factor AP-1 to up-regulate the expression of the MMP-1 gene [77, 86]. Taken together, the aforementioned molecular events that occur in the affected cells may be involved in the pathological manifestation of weakness, wasting, and atrophy of skeletal muscle that contribute to the clinical progression of MERRF syndrome (Fig. 5). Further study of the global change in the expression and modification of proteins in response to oxidative stress induced by A8344G mutation of mtDNA is warranted. This line of research will shed new insights to help us to understand the molecular mechanisms of pathogenesis and to facilitate the development of better treatment for mitochondrial diseases.

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