Acta Neuropathol (1993) 85: 280 – 284

Acta Neuropathologica

Cytochrome *c* oxidase activity is deficient in blood vessels of patients with myoclonus epilepsy with ragged-red fibers

H. Hasegawa[1,2], T. Matsuoka[1], Y. Goto[1], and I. Nonaka[1]

[1] Division of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry (NCNP), 4-1-1 Ogawahigashi, Kodaira, Tokyo 187, Japan
[2] Department of Internal Medicine, Kitasato University School of Medicine, 2-1-1 Asamizodai, Sagamihara, Kanagawa, Japan

Received September 4, 1992/Accepted October 5, 1992

Summary. More than half of the intramuscular blood vessels in muscle biopsies from five patients with myoclonus epilepsy with ragged-fibers (MERRF) who had a point mutation in mitochondrial DNA at the $tRNA^{Lys}$ region were darkly stained with succinate dehydrogenase (SDH) stain, showing the morphologic characteristics of strongly SDH-reactive blood vessels (SSV), but they had no cytochrome *c* oxidase (CCO) activity. By electron cytochemistry, the mitochondria in the smooth muscle cells of SSV had no CCO activity. On the other hand, SSV in muscle biopsies from patients with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) had normal CCO activity as shown by light and electron microscopy. The defect in CCO activity in the arteriolar smooth muscle cells and in muscle fibers suggests that CCO deficiency is related to the pathophysiology of MERRF.

Key words: Mitochondrial encephalomyopathy – Myoclonus epilepsy with ragged-red fibers (MERRF) – Strongly succinate dehydrogenase-reactive blood vessels (SSV) – Cytochrome *c* oxidase (CCO) – Vascular involvement

The mitochondrial myopathies are a group of disorders with mitochondrial dysfunction manifesting heterogeneous clinical symptoms and biochemical defects [3, 20]. Among them, three clinically defined diseases of chronic progressive external ophthalmoplegia (CPEO), mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) and myoclonus epilepsy with ragged-red fibers (MERRF) are now proven to have different mitochondrial DNA (mtDNA) mutations: mtDNA deletion in CPEO [13, 17], a point mutation at nucleotide positions of 3243 and 3271 ($tRNA^{Leu(UUR)}$) in MELAS [8, 9], and 8344 ($tRNA^{Lys}$) in MERRF [24].

Correspondence to: H. Hasegawa (address see [1] above)

In addition to muscle weakness, convulsive episodes are an outstanding central nervous system (CNS) symptom in MELAS and MERRF, but exceptional in CPEO. In MELAS, the systemic vascular abnormalities involving CNS [19] and muscle [12, 21] have been postulated to be the basis for the stroke-like episodes. The vascular abnormalities are easily demonstrated with the succinate dehydrogenase (SDH) stain as strongly SDH-reactive blood vessels (SSV) [12] in muscle biopsies, because the small arteries in the muscle contain increased numbers of abnormal mitochondria.

CNS symptoms in MERRF are different from those in MELAS, suggesting that the pathogenetic mechanism of CNS involvement is also different. If vascular abnormality is present and plays a role in producing some symptoms of MERRF, their morphologic features must differ from those in MELAS in terms of mitochondrial enzyme defect, and distribution and size of the abnormal blood vessels. To clarify the probable pathophysiology of the vascular changes in the two diseases, we examined muscle biopsies by histochemistry and electron cytochemistry focusing on mitochondria in small arteries.

Materials and methods

Patients

Muscle biopsies were obtained from five patients who have been reported previously [16]. Patient 2 was mother of patient 1. All patients had clinical characteristics of MERRF except for patient 2 who had no clinical symptoms. All patients showed abnormal EEG and had increased serum lactate and pyruvate levels. All patients had a point mutation in mtDNA at nucleotide position 8344 in $tRNA^{Lys}$ in their muscle biopsies.

Three muscle biopsies from patients with spinocerebellar degeneration, Becker type progressive muscular dystrophy and idiopathic epilepsy were chosen for control. Muscle biopsies from six MELAS patients [12] who had point mutation in mtDNA at the $tRNA^{Leu(UUR)}$ region were also examined for comparison.

Morphological examination

For histochemical analysis, muscle specimens were immediately frozen in isopentane cooled in liquid nitrogen. Serial frozen sections (10 μm thick) were stained with hematoxylin and eosin, modified Gomori trichrome (mGT), and a battery of histochemical methods. For this study, four additional serial sections were obtained: the first section was stained with mGT to identify the small thick-walled blood vessels, which were probably arteries; the second and third were stained with SDH and cytochrome *c* oxidase (CCO), respectively, to evaluate enzyme activity in the same blood vessels, and fourth was fixed for electron microscopy [4, 18]. In all specimens, the above procedure was repeated three times at intervals of 300 μm. The blood vessels with darkly stained walls and large, dense granules with the SDH stain were regarded as SSV (Fig. 1A, C and E).

For electron cytochemistry, the fourth sections were fixed in glutaraldehyde and stained with CCO [18, 23]. After washing in PBS, the sections were postfixed in OsO_4 and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate without lead nitrate.

Results

In MERRF, all muscle biopsies showed myopathic changes which consisted of variation in fiber size of both type 1 and 2 fibers, scattered ragged-red fibers, and focal CCO deficiency [16]. A total of 45 intramuscular blood vessels with thick walls (probable arteries) were identified in sections stained with mGT. Of the 45 blood vessels 29 were stained dark with SDH and contained large granules in their walls (SSV; Table 1 and Fig. 1A, C). In serial sections, none of the SSV were

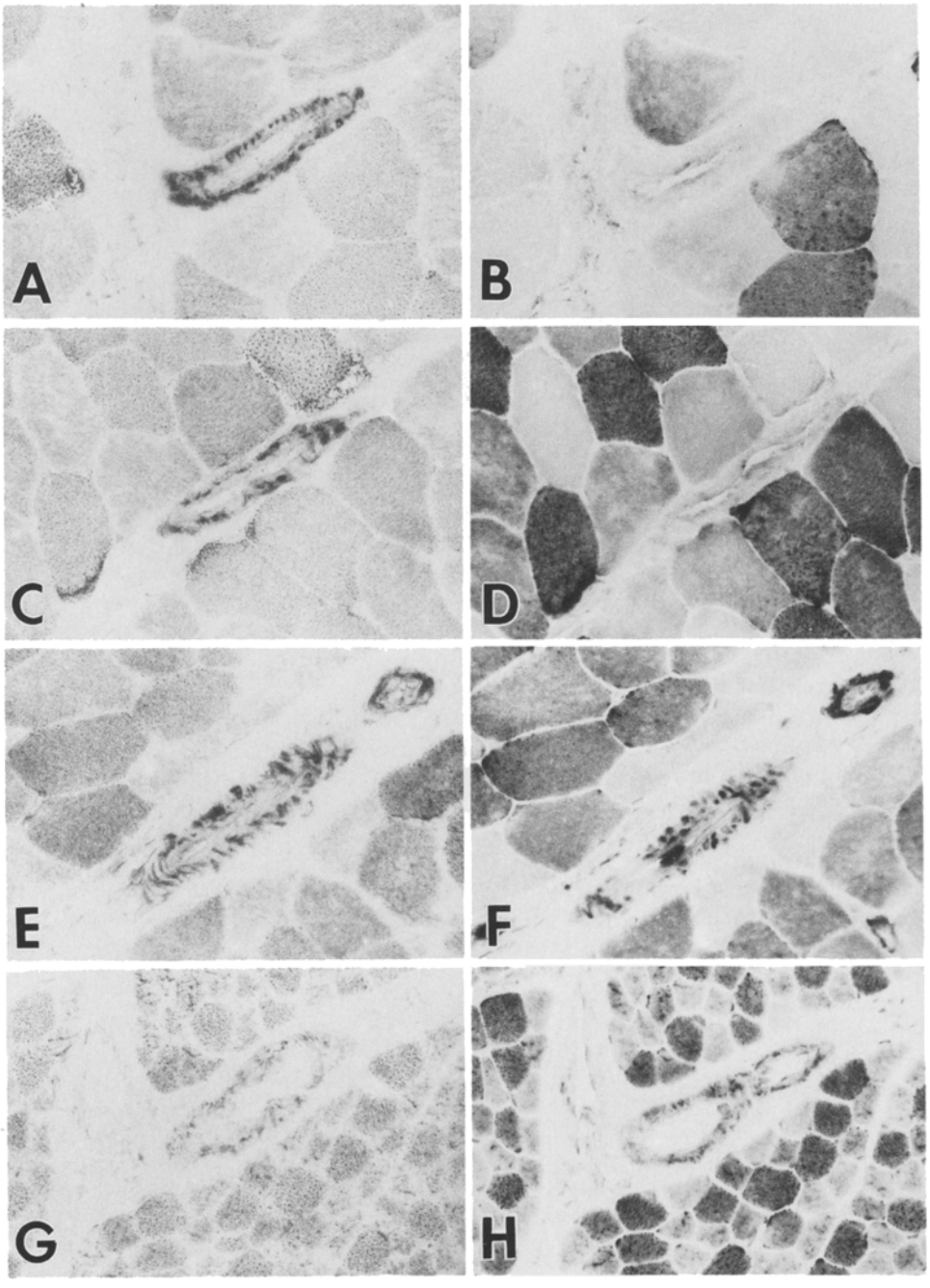

Fig. 1A–H. Intramuscular arteries in patients with myoclonus epilepsy with ragged-red fibers (MERRF) (**A–D**) are stained darkly with succinate dehydrogenase (SDH) with large dense granules in their walls (**A, C**) showing strongly SDH-reactive blood vessels (SSV), which are similar to those seen in patients with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) (**E**). On serial sections, the SSV in MERRF have negative cytochrome *c* oxidase (CCO) activity (**B, D**), whereas in MELAS they are strongly positive (**F**). Patient 3 (**A, B**), patient 4 (**C, D**), 30-year-old male with MELAS (**E, F**), 9-year-old male with idiopathic epilepsy (**G, H**). **A, C, E, G** SDH stain, **B, D, F, H** CCO stain; **A–H** × 240

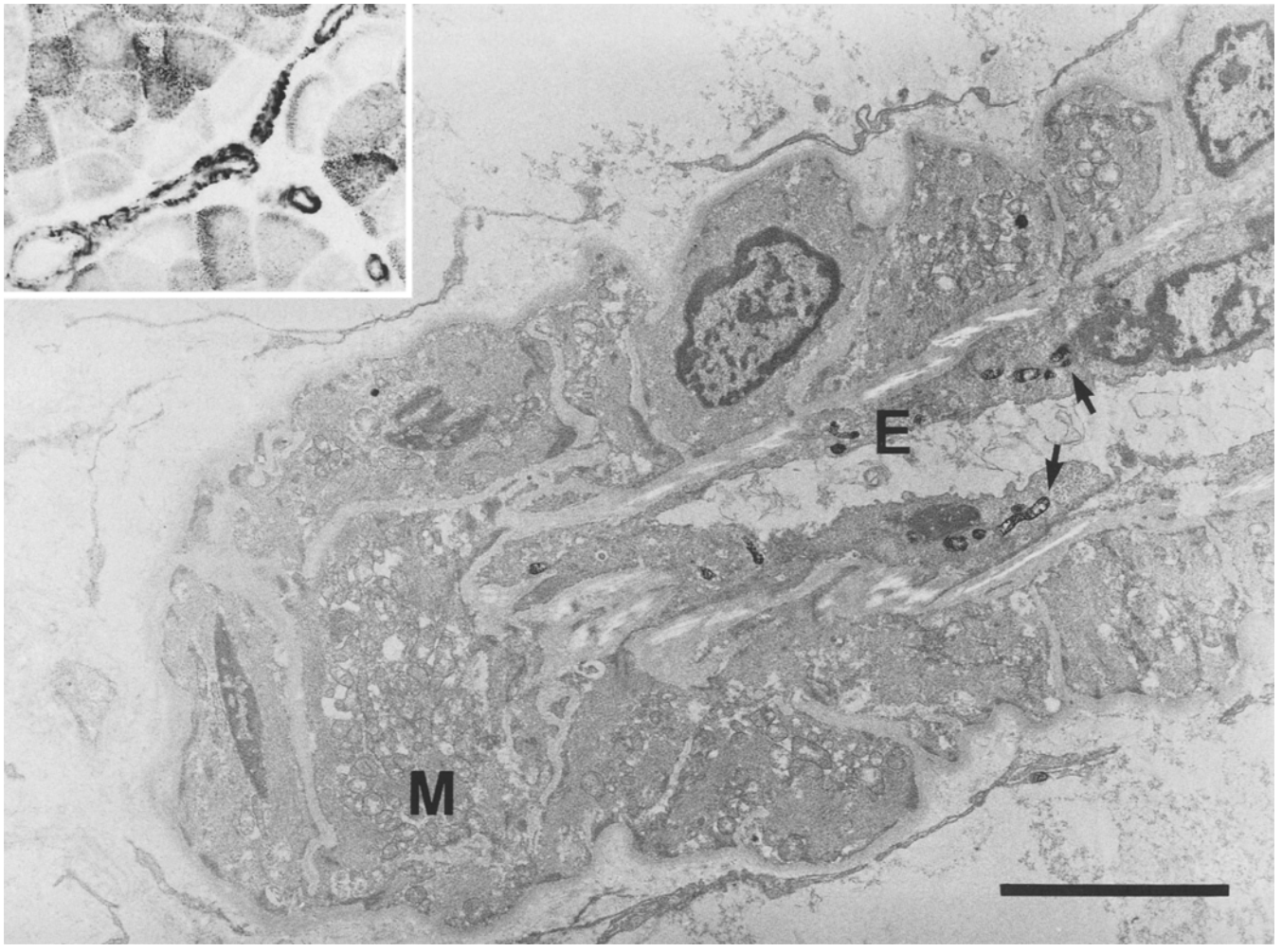

Fig. 2. A strongly SDH-reactive blood vessel (SSV) by light microscopy (*inset*) is confirmed to contain markedly proliferated mitochondria in smooth muscle cell (*M*) by electron microscopy of serial frozen sections. These proliferated mitochondria show no CCO activity. Mitochondria in endothelial cells (*E*) appear normal with normal CCO activity (*arrow*). Patient 3. CCO and uranyl acetate without lead nitrate stain; *inset* SDH stain. *Bar* = 5 μm; *inset* × 190

darkly stained with CCO (Fig. 1B, D) except for three faintly stained vessels.

By electron microscopy, all ten SSV examined were confirmed to be arteries. The smooth muscle cells had markedly increased number of mitochondria. The proliferated abnormal mitochondria were not stained with CCO, indicating CCO deficiency (Fig. 2). In an SSV in patient 3, the smooth muscle cells with CCO-positive mitochondria and those with CCO-negative mitochondria coexisted in a mosaic distribution. There was no intracellular mosaicism of CCO-positive and -negative mitochondria in the same smooth muscle cell even in such an artery. Mitochondria in the endothelial cells appeared normal with CCO activity (Table 1).

None of the blood vessels in control muscles were strongly positive for SDH (Fig. 1G) or CCO (Fig. 1H) since normal arteries have only a few numbers of mitochondria in the smooth muscle cells [21]. All mitochondria in the endothelial and smooth muscle cells had normal morphology with normal CCO activity by electron microscopy (Fig. 3; Table 1).

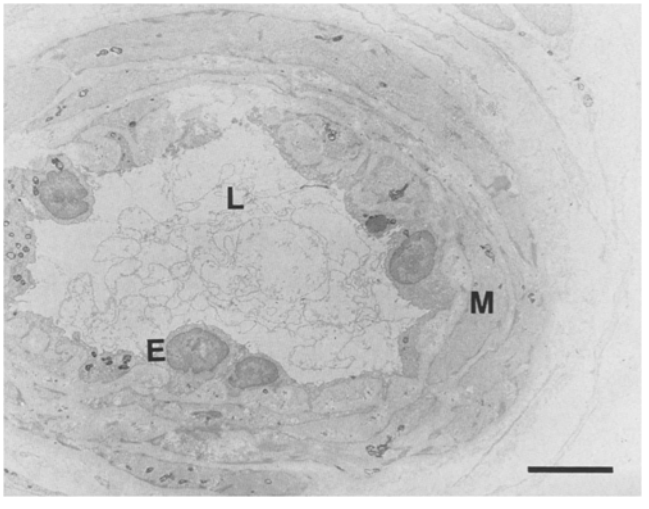

Fig. 3. An intramuscular artery (*control*). Endothelial and smooth muscle cells contained a few scattered mitochondria with positive CCO activity. Lumen (*L*), endothelial cell (*E*) and smooth muscle cell (*M*). CCO and uranyl acetate without lead nitrate stain. *Bar* = 5 μm

Table 1. Intramuscular vessels

Patients	Age/sex	Light microscopy			Electron cytochemistry	
		Number of vessels	SSV	CCO^+ SSV	Number of SSV with CCO^+ mitochondria in smooth muscle cells	Number of SSV with CCO^+ mitochondria in endothelial cells
1	37/F	10	6	0	0/1[a]	1/1
2	61/F	7	4	0 (1)[b]	0/2	2/2
3	16/F	10	9	0	1[c]/5	5/5
4	15/F	9	5	0	0/2	2/2
5	35/M	9	5	0 (2)	NE	NE
Total		45	29	0	1/10	10/10
Controls ($n = 3$)	18	0	0		All 10 small arteries examined had CCO^+ mitochondria in their smooth muscle and endothelial cells.	
MELAS ($n = 6$)		32	25	25	22 + 3[c]/25	25/25

[a] Number of vessels examined
[b] Number of SSV with faintly positive CCO activity
[c] Intercellular mosaicism of smooth muscle cells with CCO^+ and CCO^- mitochondria in a single artery
SSV, Strongly SDH-reactive blood vessels; CCO^+: Cytochrome *c* oxidase-positive activity; NE: not examined

In MELAS, 25 of the 32 blood vessels identified were SSV. The morphology of SSV was not different from that seen in MERRF, but all were stained darkly not only with SDH but also with CCO (Fig. 1E, F). These findings corresponded to the proliferated mitochondria with normal CCO activity seen by electron microscopy. In three arteries there were some smooth muscle cells with mitochondria without CCO activity, but even in these arteries, the mitochondria in the remaining smooth muscle cells had normal CCO activity (Table 1).

Discussion

Vascular abnormality in CNS [19] and in muscle [12, 21] has been reported in MELAS, but not in MERRF. Since such abnormal blood vessels contain increased number of mitochondria in smooth muscle cells [12, 19, 21], they are strongly stained with SDH, and so have been designated SSV, strongly SDH-reactive blood vessels [12]. SSV are found in most muscle biopsies from patients with MELAS [10, 12].

In MERRF, the intramuscular arteries showed similar vascular changes to those seen in SSV in MELAS. These blood vessels, identified as SSV in MELAS, had high CCO activity, but in MERRF they had no enzyme activity. Electron cytochemistry confirmed that these enlarged mitochondria which were present in increased numbers in smooth muscle cells in MERRF had no CCO activity. Since CCO deficiency in mitochondria in smooth muscle cells in MELAS was exceptional, CCO deficiency may not be the primary enzyme defect in arteries in MELAS.

Patient 2, mother of patient 1 with an identical mutation in mtDNA, had no apparent clinical abnormality except for an abnormal EEG and elevated serum lactate and pyruvate levels. In the muscle biopsy of the patient, however, not only ragged-red fibers but also SSV were found. The presence of SSV in such an unaffected patient suggests that abnormal vascular pathology starts from an early stage of the disease and play a certain role in pathogenesis of this disease.

In contrast to the autopsy findings of multiple focal cortical necrosis and basal ganglionic calcification in MELAS [11, 14, 19], the pathology in MERRF is system degeneration, involving the dentate nuclei, superior cerebellar peduncles, inferior olivary complex, globus pallidus, red nuclei, and cerebellar cortex, where neuronal loss is prominent but where there is little morphologic alteration of the neuronal mitochondria [6, 7, 22]. Although focal ischemic lesions [1, 15] and 'stroke-like episodes' [2, 5] have been described, no blood vessel abnormalities in CNS were found in autopsied patients. From the present study, however, it is likely that vascular involvement in CNS does exist and explains, at least in part, the neuronal loss and the multiple ischemic lesions in MERRF.

Focal CCO deficiency in muscle biopsy is a constant histochemical change in MERRF patients [16]. In addition, as we have shown, this enzyme activity was also absent in the arteriolar smooth cells. Therefore, CCO deficiency seems to relate to the pathomechanism to induce MERRF symptoms, although it remains unknown how a point mutation in $tRNA^{Lys}$ leads to the enzyme defect.

Acknowledgements. The authors wish to express their appreciation to Prof. H. Kowa, Department of Internal Medicine, Kitasato University School of Medicine, Kanagawa and Prof. S. M. Sumi, Department of Pathology, University of Washington, Seattle, USA for helpful suggestions and advice on this work.

References

1. Berkovic SF, Carpenter S, Evans A, Karpati G, Shoubridge EA, Andermann F, Meyer E, Tyler JL, Diksic M, Arnold D, Wolfe LS, Andermann E, Hakim AM (1989) Myoclonus epilepsy and ragged red fibers (MERRF). 1. A clinical, pathological, biochemical, magnetic resonance spectrographic and positron emission tomographic study. Brain 112:1231–1260
2. Byrne E, Trounce I, Dennett X, Gilligan B, Morley JB, Marzuki S (1988) Progression from MERRF to MELAS phenotype in a patient with combined respiratory complexes I and IV deficiencies. J Neurol Sci 88:327–337
3. DiMauro S, Bonilla E, Zeviani M, Nakagawa M, DeVivo DC (1985) Mitochondrial myopathies. Ann Neurol 17:521–538
4. Eisenberg BR, Kuda AM (1977) Retrieval of cryostat sections for comparison of histochemistry and quantitative electron microscopy in a muscle fiber. J Histochem Cytochem 25:1169–1177
5. Fukuhara N (1985) Stroke-like episodes in MERRF. Ann Neurol 18:368
6. Fukuhara N (1991) MERRF: a clinicopathological study, relationships between myoclonus epilepsies and mitochondrial myopathies. Rev Neurol (Paris) 147:476–479
7. Fukuhara N, Tokiguchi S, Shirakawa K, Tsubaki T (1980) Myoclonus epilepsy associated with ragged-red fibers (mitochondrial abnormalities): disease entity or a syndrome? Light- and electron-microscopic studies of two cases and review of literature. J Neurol Sci 47:117–133
8. Goto Y, Nonaka I, Horai S (1990) A mutation in the $tRNA^{Leu(UUR)}$ gene associated with the MELAS subgroup of mitochondrial encephalomyopathies. Nature 348:651–653
9. Goto Y, Nonaka I, Horai S (1991) A new mtDNA mutation associated with mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS). Biochim Biophys Acta 1097:238–240
10. Goto Y, Horai S, Matsuoka T, Koga Y, Nihei K, Kobayashi M, Nonaka I (1992) Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS): a correlative study of the clinical features and mitochondrial DNA mutation. Neurology 42:545–550
11. Hamazaki S, Okada S, Kusaka H, Fujii T, Okuno T, Kashu I, Midorikawa O (1989) Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. Report of an autopsy. Acta Pathol Jpn 39:599–606
12. Hasegawa H, Matsuoka T, Goto Y, Nonaka I (1991) Strongly succinate dehydrogenase-reactive blood vessels in muscles from patients with mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes. Ann Neurol 29:601–605
13. Holt IJ, Harding AE, Morgan-Hughes JA (1988) Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. Nature 331:717–719
14. Kuriyama M, Umezaki H, Fukuda Y, Osame M, Koike K, Tateishi J, Igata A (1984) Mitochondrial encephalomyopathy with lactate-pyruvate elevation and brain infarctions. Neurology 34:72–77
15. Lombes A, Mendell JR, Nakase H, Barohn RJ, Bonilla E, Zeviani M, Yates AJ, Omerza J, Gales TL, Nakahara K, Rizzuto R, Engel WK, DiMauro S (1989) Myoclonic epilepsy and ragged-red fibers with cytochrome oxidase deficiency: neuropathology, biochemistry, and molecular genetics. Ann Neurol 26:20–33
16. Matsuoka T, Goto Y, Yoneda M, Nonaka I (1991) Muscle histopathology in myoclonus epilepsy with ragged-red fibers (MERRF). J Neurol Sci 106:193–198
17. Moraes CT, DiMauro S, Zeviani M, Lombes A, Shanske S, Miranda AF, Nakase H, Bonilla E, Werneck LC, Servidei S, Nonaka I, Koga Y, Spiro AJ, Brownell AKW, Schmidt B, Schotland DL, Zupanc M, DeVivo DC, Schon EA, Rowland LP (1989) Mitochondrial DNA deletions in progressive external ophthalmoplegia and Kearns-Sayre syndrome. N Engl J Med 320:1293–1299
18. Nonaka I, Koga Y, Ohtaki E, Yamamoto M (1989) Tissue specificity in cytochrome *c* oxidase deficient myopathy. J Neurol Sci 92:193–203
19. Ohama E, Ohara S, Ikuta F, Tanaka K, Nishizawa M, Miyatake T (1987) Mitochondrial angiopathy in cerebral blood vessels of mitochondrial encephalomyopathy. Acta Neuropathol (Berl) 74:226–233
20. Petty RKH, Harding AE, Morgan-Hughes JA (1986) The clinical features of mitochondrial myopathy. Brain 109:915–938
21. Sakuta R, Nonaka I (1989) Vascular involvement in mitochondrial myopathy. Ann Neurol 25:594–601
22. Sasaki H, Kuzuhara S, Kanazawa I, Nakanishi T, Ogata T (1983) Myoclonus, cerebellar disorder, neuropathy, mitochondrial myopathy, and ACTH deficiency. Neurology 33:1288–1293
23. Seligman AM, Karnovsky MJ, Wasserkrug HL, Hanker JS (1968) Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmiophilic reagent, diaminobenzidine (DAB). J Cell Biol 38:1–14
24. Shoffner JM, Lott MT, Lezza AMS, Seibel P, Ballinger SW, Wallace DC (1990) Myoclonic epilepsy and ragged-red fiber disease (MERRF) is associated with a mitochondrial DNA $tRNA^{Lys}$ mutation. Cell 61:931–937