Much interest has recently been shown in apoptosis-mediated roles in the pathophysiology of mitochondrial diseases, because mitochondrial defects are implicated in a wide variety of degenerative diseases. We investigated whether apoptotic events occurred in skeletal muscles of patients with mitochondrial diseases, including chronic progressive external ophthalmoplegia (CPEO), Kearns–Sayre syndrome (KSS), and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS). In a immunohistochemical study, stainings for 8-hydroxy-deoxyguanosine (8-OH-dG), 4-hydroxy-nonenal (4-HNE), Mn-SOD, Bcl-2, cytochrome c, DNase I and Bcl-xL showed a pronounced granular distribution in the cytochrome c oxidase (COX)-negative ragged-red fibers (RRFs). On the other hand, the signals for Bax, p53, Fas and caspase 3 were not obviously increased in RRFs. In situ labeling of DNA breaks demonstrated preferential signals not only in myonuclei but also in subsarcolemmal regions of RRFs, indicating that mitochondrial as well as myonuclear DNA is fragmented in RRFs. An immunoblotting study demonstrated that cytochrome c was increased in the cytosol of diseased muscles and that DNase I was increased in mitochondria, compared to that of normal muscles. No difference was observed between protein bands at 20 kDa corresponding to caspase 3 in diseased and normal muscles. These findings demonstrate that these mitochondrial diseases harbor unique apoptosis-related changes that differ from caspase 3-dependent apoptosis. It is thought that these changes are induced by superoxide overproduction and cytochrome c release resulting from an inherent mitochondrial defect and that the events are associated with DNase I activation.

Mitochondrial diseases were originally characterized by morphologic abnormalities in muscle mitochondria and have been classified into several groups based on clinical, biochemical and genetic characteristics [5]. Chronic progressive external ophthalmoplegia (CPEO) and Kearns–Sayre syndrome (KSS) are generally characterized by large-scale deletions of mitochondrial DNA (mtDNA); mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) are characterized by point mutations of mtDNA [5, 21]. These diseases have a delayed onset and a progressive course. This implies that the phenotype expression depends on two factors, a predisposing mutation that arises as a fresh mutation either during oogenesis or early embryonic development, and an age-related factor that causes a decline in mitochondrial function, which exacerbates the inherited factor [32]. Recently, mitochondrial defects have been implicated not only in the classical mitochondrial disease but also in a wide variety of degenerative diseases, aging and cancer. Furthermore, it has come to much notice that apoptosis underlies the pathophysiological mechanisms of these disorders.

Apoptosis, a programmed cell death characterized by cell shrinkage, membrane blebbing, nuclear breakdown and DNA fragmentation, is a multi-step process and an important mechanism of maintenance of homeostasis during development [8, 30]. On the other hand, malfunctions of apoptosis have been involved in many forms of human diseases such as cancer, neurodegenerative disease and ischemic stroke [31]. Mitochondria are thought to play a central role in initiation of the process of apoptosis. Release of cytochrome c from mitochondria inactivates the electron transfer chain and triggers activation of a proteolytic cascade including caspases 3, 6 and 7 [10, 26]. The Bcl-2 family is known to regulate cytochrome c release; i.e. Bcl-2 and Bcl-xL block the release but Bax enhances...
it. Although mitochondrial function alters during apoptosis, mitochondrial morphology reportedly remains intact throughout the process [26].

Given these findings, mitochondrial diseases, which harbor both malformation and malfunction of mitochondria, may not undergo ordinary apoptosis. However, little is known about the apoptotic processes involved in these disorders. In the present study, we investigated the apoptosis-related changes including cytochrome c release, DNA breaks, caspase activation and oxidative damage in skeletal muscles of patients with CPEO, KSS and MELAS. In addition, the expression of apoptosis-related proteins such as p53, Fas and Bcl-2-associated proteins were immunohistochemically analyzed. We found a unique proteolytic process that depended on DNAse I and differs from that in ordinary apoptosis associated with caspases.

### Materials and methods

#### Patients and muscle specimens

The subjects included four patients with sporadic CPEO, one patient with KSS and one patient with MELAS, as summarized in Table 1. The diagnosis of KSS, CPEO or MELAS was made on the basis of muscle biopsy specimen findings, including ragged-red fibers (RRFs) observed by light microscopy, abnormal mitochondria observed by electron microscopy, and large-scale mtDNA deletions or a point mutation, in addition to clinical observations [5]. To analyze mtDNA deletions, mtDNAs were purified from biopsied muscle, digested with a restriction enzyme, and processed for Southern blotting with a probe corresponding to mtDNA (nt 8483–13459) were detected in all patients with CPEO or KSS. In addition, the expression of apoptosis-related proteins such as p53, Fas and Bcl-2-associated proteins were immunohistochemically analyzed. We found a unique proteolytic process that depended on DNAse I and differs from that in ordinary apoptosis associated with caspases.

### Immunohistochemistry

Unfixed 6-µm-thick frozen sections were fixed with ice-cold acetone for 10 min and incubated with a buffer containing 3% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 20 min at room temperature. Monoclonal antibodies against 8-deoxy-2′-deoxyguanosine (8-OH-dG), 4-hydroxy nonenal (4-HNE), Mn-superoxide dismutase (Mn-SOD) (all purchased from NOF, Tokyo, Japan), Cu-Zn-SOD (Sigma Chemical), Bcl-2 (Upstate, N.Y., USA), Bcl-xL (Transduction, Ky., USA), Fas (Transduction, Ky., USA), p53 (Progen, Heidelberg, Germany) and cytochrome c (Pharmingen, Calif., USA) were used as primary antibodies. Polyclonal rabbit antibodies against Bax (Upstate, N.Y., USA) and deoxyribonucleic acid (DNA) (DNase I) (Chemicon, Calif., USA) and polyclonal goat antibodies against caspases 1, 3, 6 and 8 (Santa Cruz, Calif., U.S.A.) were also used. Following incubation with the primary antibody diluted from 1:10 to 1:200 in BSA/PBS buffer for 60 min, the sections were washed three times with PBS for 10 min, incubated with biotinylated anti-mouse, anti-rabbit and anti-goat IgG (Vector, Calif., USA) diluted 1:400 for 60 min, and then washed three times. The biotin-labeled specimens were then incubated with streptavidin–alkaline phosphatase and detected by nitroblue tetrazolium and 5-brome-4-chloro-3-indolyl phosphate, as previously described [19].

### Quantitative analysis of immuno-positive fibers

As described in the results, immunostained signals were preferentially localized in some muscle cells, which were defined as immuno-positive fibers. The immuno-positive fibers were easily distinguished from immuno-negative fibers. Immuno-positive fibers were analyzed in six transverse sections from three frozen muscle blocks obtained from one patient. RRFs, defined as myofibers with ragged or rimmed red-staining granular deposits with modified Gomori trichrome stain [21], were also analyzed in serial sections. The ratios of immuno-positive fibers in RRFs and those of RRFs in immuno-positive fibers were statistically analyzed by two-factor ANOVA and a post-hoc test. We used StatView for Windows (version 5.0) for statistical analysis.

### In situ labeling of DNA breaks

Unfixed 6-µm-thick frozen sections were fixed with 4% paraformaldehyde in PBS for 5 min, rinsed twice in PBS, and dehydrated in a graded ethanol series. The sections were digested by proteinase K (20 µg/ml) for 15 min at room temperature and then washed twice in PBS. In situ labeling of DNA breaks was performed by terminal deoxynucleotidyl transferase with biotin-conjugated nucleotides in the presence of CoCl₂ using Genzyme TACS in situ Apoptosis Detection kits (Genzyme, Mass., USA). The biotin-labeled specimens were visualized using immunohistochemical methods and then the sections were counterstained with methyl-green [19].

### Table 1  Summary of patients examined in the present study (CPEO chronic progressive external ophthalmoplegia, KSS Kearns-Sayer syndrome, MELAS mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes, RRF ragged-red fiber)

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Phenotype</th>
<th>Biopsied skeletal muscle</th>
<th>Mitochondrial DNA % RRF</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>F</td>
<td>CPEO</td>
<td>Mitochondrial DNA</td>
<td>0.2</td>
</tr>
<tr>
<td>2</td>
<td>36</td>
<td>F</td>
<td>CPEO</td>
<td>Mitochondrial DNA</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>28</td>
<td>M</td>
<td>CPEO</td>
<td>Mitochondrial DNA</td>
<td>8.8</td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>F</td>
<td>CPEO</td>
<td>Mitochondrial DNA</td>
<td>0.07</td>
</tr>
<tr>
<td>5</td>
<td>56</td>
<td>M</td>
<td>KSS</td>
<td>Mitochondrial DNA</td>
<td>9.2</td>
</tr>
<tr>
<td>6</td>
<td>21</td>
<td>M</td>
<td>MELAS</td>
<td>Mitochondrial DNA</td>
<td>8.6</td>
</tr>
</tbody>
</table>

#### Histochemistry

Unfixed 6-µm-thick sections were stained with modified Gomori trichrome, cytochrome c oxidase (COX), succinic dehydrogenase, hematoxylin–eosin and NADH-TR stains according to routine procedures. To identify the relationship between RRFs and immunostained signals, three sets of serial sections were prepared from three respective frozen muscle blocks and subjected to histochemistry and immunohistochemistry. Histochemical and immunohistochemical stainings were performed twice in each serial section.