INCREASED DENSITY OF MITOCHONDRIA ISOLATED FROM THE SKELETAL MUSCLE OF A PATIENT WITH MITOCHONDRIAL MYOPATHY

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ABSTRACT
Mitochondria from the muscle of a patient with mitochondrial myopathy contained paracrystalline inclusion bodies. To characterize the mitochondrial fraction containing the crystalline materials, the muscle homogenate was subjected to a Ficoll density gradient centrifugation. The mitochondrial fraction was identified by immunoblotting with specific antisera against the F₁-ATPase β-subunit and lipoamide acetyltransferase of the pyruvate dehydrogenase complex. The mitochondria from the patient had a density higher than in mitochondria from controls. This method is useful for isolating abnormal mitochondria from muscle specimens of about 0.1 g.

Paracrystalline inclusion bodies have often been observed in mitochondria from muscles of patients with mitochondrial myopathy (14, 15). These crystalloids in diseased human muscle have been described (1, 16), but few information is available on their significance in the pathogenesis of the mitochondrial myopathies. To characterize these inclusion bodies, we first attempted to isolate abnormal mitochondria by density gradient centrifugation. Mitochondria from muscle of a patient with mitochondrial myopathy had a density higher than that of mitochondria from control muscle.

MATERIALS AND METHODS
Case
The patient was a 54-year-old Japanese female with progressive ophthalmoplegia and generalized muscle weakness during the last ten years. In 1984 at the age of 53, she had an episode of apnea requiring hospitalization. When she was admitted to the Hospital of Jichi Medical School, laboratory data on electrolytes, and creatine phosphokinase and aldolase activities were normal. The concentration of lactate and pyruvate was elevated in blood and cerebrospinal fluid. A brain CT scan showed a low density in the periventricular region and diffuse cortical atrophy. Retinal and cardiac involvement was absent. She died of respiratory failure at the age of 54 in 1985. One of her brothers and one of her two sons have been diagnosed as the same disease. In addition, her grandmother, mother, three brothers, one sister and four cousins had symptoms of the same disease. Thus the family history suggested autosomal dominant inheritance or maternal inheritance.

Muscle
With the family's consent, a specimen was
obtained from the quadriceps femoris of the patient after she died. Control specimens were obtained from the quadriceps femoris of patients who were free from myopathy.

**Isolation of the Mitochondrial Fraction**

Muscle specimen (about 1 g) was carefully minced with a razor and homogenized in 1 ml of Chappell-Perry solution (2) in a Waring blender and Potter-type Teflon homogenizer. The homogenate was centrifuged at 150 g for 5 min to remove cell debris and nuclei, and the supernatant was applied to a linear density gradient of 2.5–35% Ficoll (8) in Chappell-Perry solution. The gradient was centrifuged for 3 h at 32,000 g in a Hitachi 35 Ti swing-out rotor and then collected in 20 fractions.

**Immunoblotting**

A sample of 100 µl was heat-denatured in 1% SDS and 1% 2-mercaptoethanol for 5 min and subjected to SDS-polyacrylamide gel (10%) electrophoresis (12). The proteins were electrophoretically transferred to a nitrocellulose membrane as described by Towbin et al. (18). The nitrocellulose membrane was incubated with the antiserum (1,000 dilution) in 20 mM Tris-HCl (pH 8.0), 100 mM NaCl and 0.5% defatted milk powder overnight at room temperature with gentle shaking. Then the membrane was washed four times with defatted milk solution (9) for 20 min each time and incubated with 0.1 µCi/ml of iodinated Pro-

<table>
<thead>
<tr>
<th>Table 1 Enzyme Activities in Skeletal Muscle Mitochondria</th>
<th>Patient</th>
<th>Control (n=5) mean±SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complex I</td>
<td>0.21</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td>Complex II</td>
<td>0.36</td>
<td>0.30±0.13</td>
</tr>
<tr>
<td>Complex III</td>
<td>0.15</td>
<td>0.20±0.13</td>
</tr>
<tr>
<td>Complex IV</td>
<td>0.24</td>
<td>0.14±0.03</td>
</tr>
</tbody>
</table>

Values are expressed as µmol of substrate oxidized or reduced min/mg protein.

Fig. 1 Cross section of biopsied muscle stained with modified Gomori trichrome (3). Arrows show the cell containing the 'ragged-red fiber'. ×400
tein A (Amersham) for 2 h at room temperature. After exhaustive washing with the same washing buffer, the bands were visualized by autoradiography.

**Electron Microscopy**

The fractions of mitochondria obtained by centrifugation were pooled, diluted 3-fold with Chappell-Perry solution, and precipitated by centrifugation at 32,000 g for 3 h. The pellet was pre-fixed with 2% glutaraldehyde for 1 h and then fixed with OsO₄ and embedded. Thin sections were cut and stained with lead citrate and uranyl acetate for electron microscopy.

**Antiseras**

Antisera against pig lipoamide acetyltransferase (LAT) of the pyruvate dehydrogenase complex (11), and against yeast F₁ ATPase β-subunit (10) were gifts from Dr K. Koike (Nagasaki University, Japan) and Dr G. Schatz (Biocenter, Basel, Switzerland), respectively.

**Others**

Enzyme activities were measured by the method of Hatefi (4-7). Light and electron microscopic examinations were carried out in a routine way. Silver staining of proteins in electrophoresis was performed by the method of Oakley et al. (13). Ficoll and ¹²⁵I-iodinated Protein A were obtained from Pharmacia and Amersham, respectively.

**RESULTS AND DISCUSSION**

Mitochondrial enzyme activity was essentially similar between the patient and controls (Table 1). Light microscopic examination of the mitochondrial preparations from the patient stained with modified Gomori trichrome stain (3) showed the presence of ragged-red fibers (Fig. 1). Electron microscopy showed that mitochondria contained a large number

Fig. 2  Electron micrograph of biopsied muscle. Mitochondria with paracrystalline inclusion bodies are shown (arrows). Arrowheads indicate the myofibril. Bar, 1 μm
Fig. 3 Immunoblotting of fractions from muscle homogenates separated on a Ficoll-density gradient. The fractions are numbered from the bottom of the gradient. Arrows indicate the positions of $F_1\beta$-subunit or pyruvate dehydrogenase LAT.

Fig. 4 Electron micrograph of the mitochondrial fraction from patient with mitochondrial myopathy. Mitochondria contain the obscure inner structure (arrow).
of paracrystalline inclusion bodies (Fig. 2).
The muscle homogenates from the patient and controls were subjected to Ficoll density gradient centrifugation. Immunoblotting showed that the mitochondrial proteins were distributed in fractions of 12–15% Ficoll in case of patient, while in the fraction of 12% Ficoll in controls (Fig. 3). The F₁ β-subunit and LAT were found in the same fractions as mitochondria. Since the F₁ β-subunit is located on the inner-membrane and LAT is located in the matrix, these fractions were thought to contain whole mitochondria. The results show that mitochondria from the patient were higher in density than those from the control. This finding was reproducibly seen in four independent experiments. Since the same fractions from the patient and the controls gave the same protein profiles on electrophoresis with silver nitrate staining (data not shown), the fractions with the same fraction number contained the same densities of Ficoll. This finding confirmed that the density of the mitochondrial fraction from the patient was higher than that from the controls.

Mitochondria were sedimented by centrifugation, and examined by electron microscopy. Irregular vacuole-like structures (17) rather than inclusion bodies were found in mitochondria with abnormally high density (Fig. 4). It is possible that the pelleting step damaged the mitochondria resulting in the loss of paracrystalline inclusion bodies.

The method described here will be useful for isolating abnormal mitochondria from a small amount of muscle. Since the sensitivity of the immunoblotting is enough to analyze 1/10 of the materials described here, the amount of starting materials may be reduced to 0.1 g.

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REFERENCES