Mitochondrial Respiratory Chain Dysfunction in Muscle From Patients With Amyotrophic Lateral Sclerosis

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**Background:** Amyotrophic lateral sclerosis (ALS) is a major cause of neurological disability and its pathogenesis remains elusive despite a multitude of studies. Although defects of the mitochondrial respiratory chain have been described in several ALS patients, their pathogenic significance is unclear.

**Objective:** To review systematically the muscle biopsy specimens from patients with typical sporadic ALS to search for possible mitochondrial oxidative impairment.

**Design:** Retrospective histochemical, biochemical, and molecular studies of muscle specimens.

**Setting:** Tertiary care university.

**Subjects:** Fifty patients with typical sporadic ALS (mean age, 55 years).

**Main Outcome Measure:** Number of patients showing a clear muscle mitochondrial dysfunction assessed through histochemical and biochemical muscle analysis.

**Results:** Histochemical data showed cytochrome c oxidase (COX)–negative fibers in 46% patients. Based on COX histochemical activity, patients fell into 4 groups: 27 had normal COX activity; and 8 had mild (2-4 COX-negative fibers of 100 fibers), 8 had moderate (5-10 COX-negative fibers of 100), and 7 had severe (>10 COX-negative fibers of 100) COX deficiency. Spectrophotometric measurement of respiratory chain activities showed that 3 patients with severe histochemical COX deficiency also showed combined enzyme defects. In 1 patient, COX deficiency worsened in a second biopsy taken 9 months after the first. Among the patients with severe COX deficiency, one had a new mutation in the SOD1 gene, another a mutation in the TARDBP gene, and a third patient with biochemically confirmed COX deficiency had multiple mitochondrial DNA deletions detectable by Southern blot analysis.

**Conclusions:** Our data confirm that the histochemical finding of COX-negative fibers is common in skeletal muscle from patients with sporadic ALS. We did not find a correlation between severity of the oxidative defect and age of the patients or duration of the disease. However, the only patient who underwent a second muscle biopsy did show a correlation between severity of symptoms and worsening of the respiratory chain defect. In 7 patients, the oxidative defect was severe enough to support the hypothesis that mitochondrial dysfunction must play a role in the pathogenesis of the disease.

binding protein, fused in sarcoma/translated in liposarcoma (FUS/TLS), have been described in familial ALS patients.15,16 It is likely that additional genetic factors predispose individuals to the disease17 or modify its onset and progression.18,19 Oxidative stress results from mitochondrial dysfunction and may play a role in the pathogenesis of ALS by worsening or even initiating the motor neuron injury.20-22 In fact, several reports describe mitochondrial alterations in ALS.23 Transgenic mice with muscular over-expression of uncoupling protein 1, a potent mitochondrial uncoupler, displayed age-dependent deterioration of the neuromuscular junction correlated with progressive signs of denervation and a mild late-onset motor neuron pathology.24 On the other hand, some authors are still questioning the presence of mitochondrial dysfunction.25,26

Motor neuron death might also be caused by calcium-mediated excitotoxicity or by activation of the intrinsic apoptotic pathway.27 Paradoxically, both decreased mitochondrial number and increased mitochondrial mass (with increased intramitochondrial calcium concentrations) have been reported in intramuscular nerves, spinal cord, and skeletal muscles of patients with sporadic ALS.28-30 Abnormal mitochondria were also seen by electron microscopy in muscle31 and in the anterior horn cells of the spinal cord.32 One patient with motor neuron degeneration had severe muscle cytochrome c oxidase (COX) deficiency due to a mutation in the mitochondrial DNA (mtDNA)—encoded subunit I of COX.33 Hirano et al34 have recently reviewed several articles documenting how respiratory chain defects can mimic ALS or spinal muscular atrophy. To assess the importance of mitochondria respiratory chain defects in ALS, we have studied muscle biopsies from a cohort of 50 typical patients.

METHODS

SUBJECTS

We studied biceps brachii muscle biopsy specimens from 50 patients (14 women and 36 men ranging in age from 24 to 78 years; mean age, 55 years) with sporadic ALS defined according to El Escorial35 criteria who were admitted to our clinic from 2000 to 2008. Of these specimens, 29 were biopsied within 1 year and 20 within 2 years from onset of the disease. In 1 patient, the first symptoms had appeared 4 years before the muscle biopsy.

For controls, we used biceps brachii muscle biopsy specimens from 8 normal, healthy controls, 10 biopsies from patients with peripheral neuropathy, and 10 biopsies from patients with nonmitochondrial metabolic diseases (5 carnitine palmitoyltransferase II deficiencies, 2 myo-adenylate deaminase deficiencies, and 3 glycogenoses). Control muscle samples came from individuals of similar ages as the ALS patients. All specimens were obtained from the DNA, Muscle and Nerve Tissue Bank of the Neurological Unit, Fondazione Ospedale Maggiore, University of Milan, Milan, Italy, with informed consent according to a protocol approved by the institutional review board of Foundation IRCCS Ospedale Maggiore Policlinico, Mangiagalli and Regina Elena, Milan, in compliance with Italian and European Union laws.

MUSCLE BIOPSY

Muscle biopsy specimens from the left biceps brachii were frozen in isopentane-cooled liquid nitrogen, and cryostat cross-sections were processed according to standard histological techniques.36 Histochemistry for COX, succinic dehydrogenase (SDH), and combined COX/SDH stains were performed as previously described.37

BIOCHEMISTRY

Mitochondrial respiratory chain enzyme and citrate synthase activities were measured spectrophotometrically in all patients with histochemical evidence of COX deficiency by described assays.38 The specific activity of each complex was normalized to that of citrate synthase.

MOLECULAR GENETICS

In the 7 patients with severe COX deficiency, we sequenced both the SOD1 gene (OMIM 147450) and mtDNA. We also performed Southern blot analysis of muscle mtDNA.39 Molecular examination for TARDBP (OMIM 605078) was also performed.40 Quantitation of mtDNA was performed using Southern blot analysis as described.

Genomic DNA was extracted from peripheral blood of all patients and used as a template for polymerase chain reaction (PCR) amplification of each of the 5 exons of SOD1. Mitochondrial DNA was PCR-amplified using the VariantSEQr Resequencing System (Applied Biosystem, Foster City, California). All PCR products were purified and directly sequenced using the BigDye Terminator protocol on an automated 3100 ABI Prism Genetic Analyzer (Applied Biosystem). The following nuclear genes were analyzed in patient 7: PEO1, POLG1, POLG2, ANT1, and OPA1.

RESULTS

CLINICAL AND MUSCLE BIOPSY FINDINGS

In all patients, histopathologic examination showed a chronic neurogenic pattern, with small groups of atrophic fibers and fiber-type grouping. Histochemically, COX-negative fibers were observed in 23 patients (46%). The oxidative defect was mild in 8 patients (2-4 COX-negative fibers per 100 fibers), moderate in 8 (5-10 COX-negative fibers per 100), and severe in 7 (>10 COX-negative fibers per 100) (Figure 1). In patient 6, a second muscle biopsy performed 9 months after the first one documented further decreasing of the COX activity (almost all muscle fibers lacked COX activity) (Figure 2). The clinical, morphological and biochemical features of the 7 patients with severe oxidative defects are presented in the Table.

All patients were men; age at onset ranged from 31 to 75 years; and two-thirds of patients were younger than 50 years. At onset, all patients had predominantly lower motor neuron involvement and variable disease severity.

BIOCHEMISTRY

Respiratory chain enzyme activities were normal in all patients but patients 3, 6, and 7. The enzyme activities were normal in all patients with scattered (<10) COX-negative muscle fibers. In the more severe group, as defined by the presence of more than 10 histochemically deficient COX fibers, the degree of respiratory chain deficiency paralleled the histochemical defect. For example, patients 1, 2, 4, and 5, who had borderline histochemical COX defects, had either normal respiratory chain activities (patients 1,
4, and 5) or showed moderate complex III deficiency (patient 2). On the other hand, patients 3, 6, and 7 showed severe combined deficiencies involving all respiratory chain complexes, but more markedly COX activity (Table). Respiratory complex activities were normal in muscle biopsy from patients with peripheral neuropathy.

**MOLECULAR GENETICS**

Besides having severe muscle COX deficiency, patient 2 also harbored a missense mutation (c.65A>G, p.Q22R) in SOD1,41 and patient 4 had an A382T mutation in TARDBP.13 To rule out the presence of rearrangements in mtDNA, we performed a Southern blot analysis and a specific PCR assay on muscle-derived DNA in all patients. Patient 7 showed multiple mtDNA deletions detectable by Southern blot analysis. In this same patient, there were no mutations in PEO1, POLG, POLG2, ANT1, or OPA1. Sequence analysis of the entire mtDNA was performed in all patients belonging to the group with severe histochemical COX deficiency. The nucleotide variations that were found had been reported in the probands’ ethnic groups as polymorphisms and are not likely to explain the observed COX deficiency. The Table summarizes the genetic findings in the 7 patients with severe COX deficiency.

**COMMENT**

Amyotrophic lateral sclerosis is a major cause of neurological disability and its pathogenesis remains elusive despite a multitude of studies. Because the diagnosis is based on clinical and neurophysiologic criteria, few patients undergo muscle biopsy.

Although defects of the mitochondrial respiratory chain have been described in several ALS patients, their pathogenic significance is unclear. Comi and colleagues33 described a patient with early onset and rapidly progressive motor neuron disease who harbored a heteroplasmic microdeletion of the mtDNA-encoded subunit I of COX. Rubio-Gozalbo et al13 reported on a child with spinal muscular atrophy, cardiomyopathy, and reduced COX ac-

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**Figure 1.** Representative light microscopy images. A, Normal cytochrome c oxidase (COX) activity. B, Moderate COX deficiency (5-10 COX-deficient fibers per 100 fibers). C, Severe COX deficiency (>10 COX-deficient fibers per 100) (original magnification ×25).

**Figure 2.** Light microscopic image of sequential muscle biopsies from patient 6. Both the first biopsy (A) and the second (D) show a neurogenic pattern with small and angular muscle fibers. However, the first biopsy (B and C) shows many cytochrome c oxidase–deficient large fibers, while the second biopsy obtained 9 months later shows generalized cytochrome c oxidase deficiency (E and F) (original magnification ×25).
tivity in muscle and fibroblasts; the absence of mutations in SMN1 and the presence of cardiomyopathy suggest that this child might have had mutations in the COX assembly gene SCO2. Finsterer described a mother and 2 daughters with symptoms compatible with ALS. All 3 patients showed COX-negative muscle fibers, ultrastructurally abnormal mitochondria, and no mutations in SOD1, but harbored 3 mtDNA mutations, one in the transfer RNAle gene, a second in the cytochrome b gene, and the third in the adenosine triphosphatase 6 gene. Fetoni et al described a man with monomelic amyotrophy, diabetes mellitus, and COX-negative ragged red fibers in the muscle biopsy. Other family members had maternally inherited hearing loss. A mutation in the transfer RNADlecU gene of mtDNA was found in the patient and in a maternal niece. Borthwick et al described a patient with clinical features suggestive of ALS, diabetes mellitus, and cardiac arrhythmia who died of cardiac arrest during the study. Autopsy results showed a normal cortex and corticospinal tracts but numerous COX-deficient motor neurons. Sequencing of mtDNA showed a heteroplasmic mitochondrial transfer RNAle mutation different from that reported by Finsterer. Recently, Hirano and colleagues reported on a 65-year-old man with typical ALS in whom muscle biopsy specimens showed 10% ragged red fibers, which were unexpectedly abundant even at his age and suggested mitochondrial dysfunction.

Given the provocative but still anecdotal evidence of mitochondrial involvement in muscle of patients with ALS, we decided to review systematically the remarkable collection of muscle biopsy specimens from patients with typical ALS in our possession. Our histochemical data in 50 patients showed variably severe but unequivocal

### Table. Characteristics of Seven Patients With Amyotrophic Lateral Sclerosis and Severe Oxidative Defects

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal Value, Mean (SD)</th>
<th>Patient No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex/age at biopsy, y</td>
<td></td>
<td>M/62</td>
</tr>
<tr>
<td>Neurological signs at biopsy</td>
<td>Walking difficulties for 1 y; weakness and hypotrophic in distal limbs; had high CK serum levels</td>
<td>Fasciculations in the whole body for 2 y; lingual hypotrophy; episodic dysphagia; high CK serum levels</td>
</tr>
<tr>
<td>Histological morphology</td>
<td>Neurogenic aspect with atrophic and angular fibers; large fiber type 1 grouping</td>
<td>Neurogenic aspect with angular fibers and moderate muscle fiber variability</td>
</tr>
<tr>
<td>Respiratory chain activity, nM/min/mg of protein</td>
<td>CI 15.7 (3.2) 18.4 16.2 5.0b 10.8 17.1 3.9b 4.2b 7.3b</td>
<td>CII 9.9 (2.3) 10.1 10.6 2.8b 8.9 14.5 4.2b 3.2b 3.5b</td>
</tr>
<tr>
<td>mtDNA Southern blot results SOD1</td>
<td>Negative</td>
<td>p.022Rb</td>
</tr>
<tr>
<td>TARDBP</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>mtDNA sequence</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Age at onset, y</td>
<td>61</td>
<td>38</td>
</tr>
</tbody>
</table>

Abbreviations: C, complex of mitochondrial respiratory chain; CK, creatine kinase; CS, citrate synthase; mtDNA, mitochondrial DNA.

a Sequence analysis of the coding regions of the following genes was negative: ANT1, PED1, POLG1, POLG2, and OPAL1.
b Activities reduced by at least 30% of the normal mean values.
c Mean (SD) values of the respiratory chain complex activities are normalized to CS.
COX deficiency in 46% of patients, an unexpectedly high proportion. Patients can be divided into 4 groups according to COX histochemical activity: 27 had normal COX activity; and 8 had mild, 8 had moderate, and 7 had severe COX deficiency. When respiratory chain enzyme activities were measured spectrophotometrically in whole muscle extracts, only 3 patients showed combined enzyme defects (Table, patients 3, 6, and 7). In patient 6, COX activity worsened in a second biopsy taken 9 months after the first. The fact that respiratory enzyme analyses in 4 of 7 patients with severe histochemical COX deficiency were normal is not surprising because biochemistry does not accurately reflect the extent of histochemical changes, which are nonetheless significant.

Notably, among the patients with severe COX deficiency, 1 (patient 2) had a mutation in SOD1 (c.65A>G, p.Q22R), another (patient 4) had a mutation in TARDBP, whereas another patient (patient 7) with biochemically confirmed COX deficiency had multiple mtDNA deletions detectable by Southern blot analysis, though sequencing all the known nuclear genes associated with multiple mtDNA deletions failed to show any pathogenic mutation. The patient's advanced age may explain this finding, at least in part; however, a substantial proportion of mtDNA multiple deletion syndromes is not assigned to any known gene.

Therefore, the possibility of a mutation in a yet unidentified gene cannot be ruled out. Vielhaber et al described multiple deletions of mtDNA in 1 patient with sporadic ALS and attributed the mtDNA alteration to the decreased activity of membrane-associated mitochondrial Mn-superoxide dismutase activity, but did not study nuclear genes associated with multiple mtDNA deletions.

Our data confirm that varyingly severe histochemical COX deficiency is a common finding in skeletal muscle from ALS patients and does not correlate with age. In fact, it is noteworthy that most patients with severe COX deficiency by histochemistry (group 4) were in their 30s, 1 was aged 40 years, another was in his 60s, and only 1 was aged 75 years. Paradoxically, the patient with the most severe histochemical COX deficiency was one of the youngest in our cohort (32 years of age at disease onset).

We failed to detect a general correlation between severity of the oxidative defect and duration of the disease. Most patients underwent muscle biopsy within 1 year from the onset of symptoms. However, in the only patient who underwent a second muscle biopsy, we did find a correlation between worsening of the respiratory chain defect and severity of symptoms. Echaniz-Laguna et al twice performed biopsies in 7 patients with sporadic ALS and showed worsening of complex IV deficiency in the later biopsies.

In 7 patients, the oxidative defect was severe enough to support the hypothesis that, at least in some cases, mitochondrial dysfunction plays a role in the pathogenesis of the disease. Although aging can cause mitochondrial changes and onset is typically late in ALS, it is important to note that most of our ALS patients were still young and that the changes we observed were much more pronounced than those described in healthy elderly individuals and were not present inagematched controls.

The partial oxidative defect in patients with pathogenic mutations in SOD1 (patient 2) and TARDBP (patient 4) further suggests that the COX deficiency may be secondary to identifiable genetic defects. This concept is reinforced by the molecular findings in the single patient with muscle multiple mtDNA deletions, though we could not identify the nuclear gene responsible for this defect of intergenicomic signaling.

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REFERENCES


