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MINI-REVIEW

Molecular Defects in Cytochrome Oxidase in Mitochondrial Diseases

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Abstract

Defects of cytochrome *c* oxidase (COX) show remarkable clinical, biochemical, and genetic heterogeneity. Clinically, there are two main groups of disorders, one dominated by muscle involvement, the other by brain dysfunction. Biochemically, the enzyme defect may be confined to one or a few tissues (reflecting the existence of tissue-specific isozymes) or affect all tissues. Immunologically reactive enzyme protein is decreased in some forms of COX deficiency but not in others. Because COX is encoded both by nuclear and by mitochondrial genes, COX deficiencies may be due to mutations of either genome and may offer useful models to study the communication between nuclei and mitochondria. We have isolated full-length cDNA clones encoding human COX subunits IV, Vb, and VIII and a partial-length clone for subunit Va. These clones are being used as probes to analyze the DNA and RNA of patients with COX deficiency.

Key Words: Cytochrome *c* oxidase; respiratory chain; complex IV; mitochondrial myopathies; mitochondrial encephalomyopathies; maternal inheritance; isozymes; Leigh syndrome; MERRF (myoclonus epilepsy with ragged-red fibers).

Introduction

Under the labels "mitochondrial myopathies" or "mitochondrial encephalomyopathies" are included a group of clinically heterogeneous disorders characterized by morphological abnormalities of muscle mitochondria

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(DiMauro *et al.*, 1985; Morgan-Hughes, 1986). These first became apparent when electron microscopy was applied systematically to the study of muscle biopsies (Shy and Gonatas, 1964; Shy *et al.*, 1966). The introduction of a histochemical stain (modified Gomori trichrome) which reveals mitochondrial aggregates as irregular reddish patches under the sarcolemma or between myofibrils ("ragged-red fibers") further facilitated recognition of mitochondrial myopathies (Engel and Cunningham, 1963). However, morphological criteria are not adequate for the diagnosis or classification of these disorders because mitochondrial changes are not specific and may be absent in biochemically defined mitochondrial myopathies, such as carnitine palmitoyltransferase (CPT) deficiency.

Thus, although morphological abnormalities of muscle mitochondria still represent useful diagnostic clues, any rational classification of mitochondrial diseases has to rely on the identification of specific biochemical defects. In recent years, such biochemical errors have been reported in increasing number. They include: (1) defects of mitochondrial transport, such as CPT deficiency; (2) defects of substrate utilization, such as pyruvate dehydrogenase complex (PDHC) deficiency or defects of beta-oxidation; (3) defects of the Krebs cycle, such as fumarase deficiency; (4) defects of oxidation–phosphorylation coupling, exemplified by nonthyroidal hypermetabolism (Luft disease); and (5) defects of the respiratory chain.

Specific defects of individual components of the respiratory chain were first reported in the early seventies when Spiro *et al.* (1970) described lack of reducible cytochrome *b* in isolated muscle mitochondria from a father and son with a complex clinical picture including weakness and signs of brain involvement (an encephalomyopathy). In 1972, a defect of cytochrome oxidase was found in muscle, brain, and liver mitochondria in one patient with trichopoliodystrophy (Menkes disease), an X-linked recessive disorder with impaired intestinal absorption of copper (French *et al.*, 1972). However, it has been mostly in the last decade that through systematic analysis of isolated mitochondria, defects in individual complexes of the respiratory chain have been pinpointed (DiMauro *et al.*, 1985, 1987; Morgan-Hughes, 1986). In recent years, some of these defects have been further defined by studying the subunit composition of the defective complex (Darley-Usmar *et al.*, 1983; Moreadith *et al.*, 1984, 1987; Bresolin *et al.*, 1985; Takamiya *et al.*, 1986). Molecular genetic analysis is already underway and will clarify the nature of the genetic errors at the level of nuclear or mitochondrial DNA.

Defects of complex I (NADH-Coenzyme Q reductase) and complex III (reduced Coenzyme Q-cytochrome *c* reductase) have been clearly documented and are reviewed elsewhere in this volume. Defects of complex II (succinate-Coenzyme Q reductase) have been reported in a few patients, but the biochemical defects have not been adequately characterized. In this review, we

will concentrate on defects of the fourth electron-carrier complex, cytochrome oxidase (COX), discussing clinical presentations, biochemical and genetic aspects, and preliminary results of molecular genetic analysis.

Clinical Aspects

The clinical phenotypes of COX deficiency fall into two main groups: in one myopathy is the predominant or exclusive manifestation, in the other a multisystem disorder is dominated by brain disease (Table I).

Disorders Affecting Muscle Exclusively or Predominantly

In this group, the most common clinical presentation is *severe generalized myopathy* starting soon after birth and causing respiratory insufficiency and death before one year of age. Lactic acidosis is severe and

Table I. COX Deficiency: Phenotypic Expression, Residual COX Activity, and Presence or Absence of Immunologically Reactive Material (CRM)

Phenotype	Tissue(s) affected (% COX)	CRM
A. Disorders affecting muscle exclusively or predominantly		
1. Fatal infantile myopathy		
a. Myopathy only	Muscle (< 10%)	−
b. Myopathy and nephropathy	Muscle (< 10%); kidney (40%)	−
c. Myopathy and cardiopathy	Muscle (< 10%); heart (12%)	+
d. Myopathy and hepatopathy	Muscle (< 10%); liver (< 10%)[a]	n.d.
2. Benign infantile myopathy	Muscle (< 10% to normal)	+
B. Disorders affecting predominantly the brain		
1. Subacute necrotizing encephalomyelopathy (SNE; Leigh syndrome)	Muscle (14%); brain (32%); liver (6%); kidney (35%); heart (28%); fibroblasts (22%). Liver and fibroblasts normal in some patients	+
2. Progressive sclerosing poliodystrophy (Alpers syndrome)	Muscle (10%; 42%)	n.d.
3. Myoclonus epilepsy with ragged-red fibers (MERRF)	Muscle (30%); brain (29%); heart (18%); liver (41%)	±
C. Other		
1. Progressive external ophthalmoplegia	Muscle (28% to normal)	n.d.
2. Encephalomyopathy in adults	Muscle (28–43%)	−
3. Trichopoliodystrophy (Menkes)	Muscle (54%); brain (47%); liver (23%); heart (59%)	n.d.

[a]Muscle deficiency found in one patient and liver deficiency in a first cousin; n.d. = not determined. [Modified from DiMauro *et al.* (1987), in *Molecular Genetics of Neurological and Neuromuscular Disease* (DiDonato, S., DiMauro, S., Mamoli, A., and Rowland, L. P., eds.), Raven Press, New York, p. 93].

represents an important diagnostic clue. Muscle biopsy shows abundant "ragged-red" fibers. Myopathy can be isolated or, more frequently, associated with *renal dysfunction* (DeToni–Fanconi–Debre syndrome) (DiMauro *et al.*, 1985, 1986). The association of myopathy and *cardiopathy* in the same patient (Zeviani *et al.*, 1986), and myopathy and *liver disease* in the same family but not in the same patient (Boustany *et al.*, 1983) has also been documented. Pedigree analysis in a few informative families suggested autosomal recessive transmission.

In contrast to these invariably fatal conditions, a few children with severe myopathy and lactic acidosis at birth improve spontaneously ("*benign infantile myopathy*") and are virtually normal by age 2 or 3 years. Serum lactate and muscle biopsy also return to normal (DiMauro *et al.*, 1983; Zeviani *et al.*, 1987).

Disorders Affecting Predominantly the Brain

In the second group of disorders, dominated by brain involvement, the most common syndrome is *subacute necrotizing encephalomyelopathy* (*SNE, Leigh syndrome*), a devastating encephalopathy of infancy or childhood characterized by psychomotor retardation, brainstem dysfunction, and respiratory abnormalities. The characteristic neuropathological alterations are focal, symmetrical necrotic lesions affecting mostly the brainstem. Microscopically, these "spongiform" lesions show demyelination, vascular proliferation, and astrocytosis. Muscle histochemistry is normal, but electron microscopy may show increased number of mitochondria. Most patients with Leigh syndrome and COX deficiency have been sporadic cases (DiMauro *et al.*, 1987b), but at least one set of affected siblings was reported (Miyabayashi *et al.*, 1983), and parents are normal, suggesting autosomal recessive inheritance. COX deficiency was also shown in muscle biopsies from two unrelated patients with *Alpers disease* (*progressive sclerosing poliodystrophy*), a disorder clinically and pathologically similar to Leigh syndrome, but with more severe involvement of the cerebral cortex (Prick *et al.*, 1983).

COX deficiency has recently been reported in two families with *myoclonus epilepsy and ragged-red fibers* (*MERRF, Fukuhara syndrome*) (Mendell *et al.*, 1987; Berkovic *et al.*, 1987), an encephalomyopathy characterized by myoclonus, ataxia, hearing loss, weakness, and generalized seizures (Fukuhara, 1983). As implied by the acronym, muscle biopsies show abundant ragged-red fibers. Analysis of several pedigrees has suggested non-mendelian, maternal inheritance (Rosing *et al.*, 1985). Consistent with maternal inheritance, the clinical expression varies markedly in different generations and in different individuals of the same generation.

Other Disorders

Partial defects of COX, sometimes only evidenced by histochemically nonreactive fibers in muscle biopsies, have been described in different forms of *progressive external ophthalmoplegia (PEO)* (Johnson *et al.*, 1983; Muller-Hocker *et al.*, 1983; Byrne *et al.*, 1985). Partial defects of COX have also been found in three adult patients with symptoms and signs of muscle and brain dysfunction (*encephalomyopathy*) (Servidei *et al.*, 1987; Pezeshkpour *et al.*, 1987).

It is uncertain whether COX deficiency is the primary biochemical defect in these disorders or merely the consequence of an underlying and as yet undefined process.

COX deficiency is certainly a secondary phenomenon in *trichopoliodystrophy* (*Menkes disease*), an X-linked recessive disorder characterized by seizures, developmental regression, hair abnormalities, tortuous arteries, fragile bones, hypopigmentation, and temperature instability. These diverse manifestations have been attributed to deficiencies of copper-dependent enzymes, including COX, secondary to defective intestinal transport of copper. The different levels of COX activity found in different tissues probably reflect varying concentrations of copper.

Biochemical Considerations

The clinical heterogeneity illustrated above is not surprising when one considers the complexity of the enzyme. COX, the last component of the respiratory chain, catalyzes the transfer of reducing equivalents from cytochrome *c* to molecular oxygen, utilizing the energy liberated in the reaction to sustain a transmembrane proton-pumping activity (Hatefi, 1985). The holenzyme contains as redox centers two copper atoms and two unique heme A iron porphyrins bound to a multisubunit protein frame embedded in the mitochondrial inner membrane.

In mammals, the apoprotein is composed of 13 subunit polypeptides. The three larger subunits (I to III) are associated with the prosthetic groups and perform both catalytic and proton pumping activities (Capaldi *et al.*, 1983). They are encoded by mitochondrial DNA (mtDNA) and are synthesized in mitochondria; both their coding sequences and primary structures have been established in humans (Anderson *et al.*, 1981). The 10 smaller subunits [IV, Va, Vb, VIa, VIb, VIc, VIIa, VIIb, VIIc, VIII; nomenclature of Kadenbach *et al.* (1983)] are encoded by nuclear DNA and synthesized on cytoplasmic ribosomes, presumably as precursors carrying N-terminal basic presequences that allow them to be transported into the mitochondria (Van

Loon *et al.*, 1986; Rosenberg *et al.*, 1986). The functions of the nuclear-encoded subunits have not been fully elucidated, but a regulatory role has been proposed by Kadenbach and coworkers, who have also shown that some of these subunits occur as tissue-specific isoforms (Kadenbach and Merle, 1981; Kadenbach *et al.*, 1983; Kuhn-Nentwig and Kadenbach, 1985). These COX isozymes could optimize the enzymatic activity to the metabolic requirements of different tissues.

Biochemical studies of patients with different forms of COX deficiency support the notion of tissue-specific isozymes. In agreement with clinical and pathological observations, in cases with *fatal infantile myopathy* COX deficiency was confined to skeletal muscle, sparing heart, liver, and brain (Van Biervliet *et al.*, 1977; DiMauro *et al.*, 1980; Minchom *et al.*, 1983; Bresolin *et al.*, 1985). Accordingly, reduced-minus-oxidized cytochrome spectra showed that the cytochrome aa_3 peak was lacking in mitochondria isolated from muscle (Van Biervliet *et al.*, 1977; DiMauro *et al.*, 1980; Minchom *et al.*, 1983) but present in heart mitochondria (Bresolin *et al.*, 1985). A partial defect of COX in kidney was demonstrated both by enzyme assay and by spectral analysis in two patients with *myopathy and renal dysfunction*. Of the three cases with *myopathy and cardiopathy*, the heart was studied in two: COX activity was, surprisingly, normal in one (Rimoldi *et al.*, 1982) but markedly decreased in the other (Zeviani *et al.*, 1987).

Differential tissue involvement was also demonstrated by muscle histochemistry in patients with fatal infantile myopathy: the enzyme stain was virtually absent in extrafusal fibers but present in intrafusal fibers of muscle spindles and in smooth muscle cells of blood vessels (Zeviani *et al.*, 1985).

In the two encephalomyopathies, *Leigh syndrome* and *MERRF*, COX deficiency appears to be generalized, although partial (DiMauro *et al.*, 1987; Mendell *et al.*, 1987). The amount of residual enzyme activity in Leigh syndrome varied in different tissues in a study of five patients: it was about 35% of the normal mean in brain, 15% in muscle, and less than 10% in liver. One of the five patients, however, had normal COX activity in liver and fibroblasts, suggesting genetic heterogeneity even within this group of patients (DiMauro *et al.*, 1987).

The spontaneous clinical improvement in children with *benign infantile myopathy* correlates with a return of COX activity in muscle, which can be demonstrated histochemically and biochemically. In one patient, COX activity was 6, 33, and 174% of normal in muscle biopsies taken at two, seven, and 36 months (DiMauro *et al.*, 1983; Zeviani *et al.*, 1987). In both cases, histochemistry showed that only scattered fibers stained for COX in the first biopsies, but the number of COX-positive fibers increased with time (DiMauro *et al.*, 1983; Zeviani *et al.*, 1987).

The biochemical heterogeneity of COX deficiency extended to the presence or absence of immunologically cross-reacting material (CRM). Patients with fatal infantile myopathy showed markedly decreased CRM in muscle extracts or isolated mitochondria by enzyme-linked immunosorbent assay (ELISA) or by immunocytochemistry of frozen sections (Bresolin *et al.*, 1985). In contrast, children with benign infantile myopathy due to reversible COX deficiency have normal amounts of CRM in muscle, even in biopsies taken early in the course of the disease and showing very little COX activity (Zeviani *et al.*, 1987). The presence of CRM in muscle biopsies of infants with mitochondrial myopathies is a potentially useful tool in distinguishing malignant (irreversible) from benign (reversible) COX deficiency. In both Leigh syndrome (DiMauro *et al.*, 1987) and MERRF (Mendell *et al.*, 1987), CRM in all organs was normal or moderately decreased.

Availability of antibodies against COX has allowed studying the subunit composition of mutant enzymes by SDS-PAGE of mitochondrial extracts followed by immunoblotting, or by immunoprecipitation of mitochondrial extracts followed by SDS-PAGE of the immunoprecipitate. No alteration of the subunit pattern has been found in brain mitochondria from two patients with Leigh syndrome (DiMauro *et al.*, 1987) or in muscle mitochondria from a child with fatal myopathy (Bresolin *et al.*, 1985).

Genetic Considerations

The large number of subunits, some encoded by nuclear DNA, others by mtDNA, and the complexity of post-translational events required for the import and correct assembly of nuclear-encoded subunits explain how inherited COX deficiencies may be due to many different genetic errors. At least three mechanisms can be considered: (1) alterations of transcription or translation of mtDNA-encoded polypeptides (I to III); (2) alterations of transcription or translation of nuclear-encoded polypeptides (IV to VIII); (3) alterations of the post-translational processing of the cytoplasmically synthesized precursors of subunits IV to VIII. Interaction with mitochondrial receptors, translocation and proteolytic processing of these precursors are indispensable for correct assembly in the inner mitochondrial membrane (Van Loon *et al.*, 1986; Rosenberg *et al.*, 1986). Diseases due to mechanisms 2 and 3 ought to be transmitted by mendelian inheritance, while diseases due to mutations of mtDNA should be transmitted by non-mendelian, maternal inheritance.

In addition, because all mitochondria of one individual have identical DNA molecules, tissue-specific COX deficiencies should be due to mutations of nuclear-encoded subunits. On the other hand, generalized COX deficiencies

could result either from mutations of nuclear-encoded subunits that are not tissue-specific or from mutations of mtDNA-encoded subunits. Fatal infantile myopathy, Leigh syndrome, and MERRF might exemplify, respectively, each of these three genetic mechanisms, but the affected subunits have not yet been identified in any of these conditions.

The basis for the reversibility of COX deficiency in the benign form of infantile myopathy is not known, but two hypothetical explanations can be considered. (1) If the genetic error affects mtDNA, there could be a gradual selection of fibers containing predominantly wild-type mitochondria over those that contain a majority of mutant mitochondria and are presumably not viable. In favor of this hypothesis is the histochemical observation that fibers appear to be affected in an all-or-none fashion: it is the number of normal fibers that increases with time, not the intensity of the reaction in each fiber (DiMauro *et al.*, 1983; Zeviani *et al.*, 1987). Against this hypothesis is the lack of any evidence of maternal transmission in the families of the three patients. Also, the exclusive involvement of muscle seems incompatible with the general assumption that mtDNA is identical in all cells of one individual. (2) If the mutation involves nuclear DNA, it could affect a subunit of COX that is not only tissue-specific but also developmentally regulated. Expression of immunologically different isoforms during development was demonstrated for several COX subunits by comparing fetal and adult rat tissues (Kuhn-Nentwig and Kadenbach, 1985). Mutations of a fetal or neonatal isozyme would correct spontaneously when the mature isozyme begins to be expressed.

Molecular Genetic Studies

To define at the molecular level the different genetic defects responsible for the clinical and biochemical heterogeneity of COX deficiency, we are isolating cDNA clones for the ten human COX nuclear-encoded subunits. To date we have obtained full-length clones for subunits IV, Vb, and VIII, and a partial-length clone encoding subunit Va.

Subunit IV

Using a monoclonal antibody as a probe (Nakagawa and Miranda, 1987), a full-length cDNA (678 bp long) for subunit IV was isolated from a library of human liver cDNA inserted into lambda gt11 (Zeviani *et al.*, 1987b). Its sequence is highly homologous to both a liver-derived bovine COX IV cDNA (Lomax *et al.*, 1984) and to a bovine COX IV processed pseudogene (Bachman *et al.*, 1987). The presumed presequence is 22 amino acids long: it contains 4 basic and no acidic residues.

Human COX IV has a relatively simple hybridization pattern in genomic Southern analysis, suggesting that the COX gene family is small, located at only two or three chromosomal loci. There is no evidence for tissue-specific isoforms, because Northern analysis of muscle, liver, and HeLa cell RNA shows an identically sized transcript of about 700 nt in each cell type. The human cDNA clone is highly homologous to its bovine counterpart in the coding regions for both the mature polypeptide and the presequence (Zeviani *et al.*, 1987).

Subunit Va

A partial-length cDNA (440 bp long) encoding COX subunit Va was isolated from a library of human fetal muscle cDNA inserted into lambda gt11, using a synthetic oligonucleotide probe based on the known polypeptide sequence of bovine heart subunit Va (Tanaka *et al.*, 1977). Using this clone as a nick-translated probe, we have rescreened this library and obtained a longer, possibly full-length clone that is currently being sequenced.

Subunit Vb

We have isolated a full-length cDNA (490 bp long) encoding COX subunit Vb from a library of human muscle cDNA inserted into lambda gt11, using a polyclonal antibody to COX Vb as a probe. The sequence of the mature polypeptide is highly homologous to that of beef heart COX Vb. The presumed human presequence is 31 amino acids long, and contains 5 basic, but no acidic, residues. As with subunit IV, there is no evidence for tissue-specific isoforms of COX subunit Vb: Northern analysis shows a transcript of identical size (about 550 nt) in muscle and liver RNA. In addition, COX Vb cDNA clones derived from human fetal muscle and endothelial cell cDNA libraries had the same sequence as the adult muscle-derived clone.

Subunit VIII

Using a partial-length cDNA clone for subunit VIII derived from a rat hepatoma cDNA library (Suske *et al.*, 1987) as a nick-translated probe, we have isolated a full-length clone (475 bp long) encoding COX subunit VIII from libraries of human fetal muscle and liver cDNA inserted into lambda gt11. The presumed presequence is 25 amino acids long and contains 5 basic, but no acidic, residues. In contrast to subunits IV, Va, and Vb, the deduced amino acid sequence of this clone has only 40% homology with the published amino acid sequence of beef heart (Meineke *et al.*, 1984), and 60% homology with that of rat liver (Suske *et al.*, 1987). These findings suggest that subunit

VIII may be encoded by different isoform genes. However, this hypothesis is not supported by Northern analysis, because the size of the hybridizing signal in five cell types (muscle, heart, brain, liver, and HeLa cell) was approximately the same.

Molecular Genetic Analysis of COX Deficiencies

We are using the cDNA clones described above as probes to analyze the DNA and RNA from tissues of patients with COX deficiency.

Northern analysis of RNA isolated from brain of one patient with Leigh syndrome using probes for four nuclear-encoded subunits (IV, Va, Vb, and VIII) and the three mtDNA-encoded subunits (kindly provided by Dr. Giuseppe Attardi) showed a disproportion between the steady-state transcriptional level of nuclear as compared to mitochondrial genes, with a marked decrease of the mitochondrial : nuclear ratio of transcription. We are going to extend these studies to tissues other than brain and to other patients with Leigh syndrome. The results in Leigh syndrome, a generalized COX deficiency, will be compared to those obtained from Northern analysis of RNA isolated from muscle and from nonaffected tissues in patients with fatal infantile myopathy.

In the patient with MERRF, Western blots of mitochondrial proteins separated by SDS-PAGE suggested a selective defect of subunit II, which would be consistent with a mutation of mtDNA and with maternal inheritance. However, Northern analysis of mitochondrial RNA using cDNA clones COX I, II, and III as probes has failed to show any alteration in RNA amount or size. Further studies are needed to reconcile these apparently contradictory results.

When unraveled at the molecular level, the different hereditary COX deficiencies will provide naturally occurring human mutations that can be used to study intracellular communication between nuclear and mitochondrial genome.

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