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A Biochemically Distinct Form of Cytochrome Oxidase (COX) Deficiency in the Saguenay-Lac-Saint-Jean Region of Quebec

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Summary

We report the results of biochemical and molecular investigations on a group of patients from the Saguenay-Lac-Saint-Jean region of Quebec who have an unusual form of cytochrome oxidase deficiency and Leigh disease. This group can be distinguished from the classical presentation of cytochrome oxidase deficiency with Leigh disease, by the severity of the biochemical defect in different tissues. The activity in skin fibroblasts, amniocytes, and skeletal muscle of cytochrome oxidase is 50% of normal, while in kidney and heart it is close to normal values. Brain and liver, on the other hand, have very low activities. The defect in activity appears to result from a failure of assembly of the cytochrome oxidase complex in liver, but levels of mRNA for both mitochondrially encoded and nuclear-encoded subunits in liver and skin fibroblasts were found to be the same as those in controls. The cDNA sequence of the liver-specific cytochrome oxidase subunits VIa and VIIa were determined in samples from patient liver and skin fibroblasts and showed normal coding sequence.

Introduction

Inherited forms of cytochrome oxidase (COX) deficiency in the human population are a poorly understood group of disorders (DiMauro et al. 1985). Several distinct clinical and biochemical forms of this defect have been described in the pediatric population to include fatal infantile COX deficiency (Van Biervliet et al. 1977), reversible COX deficiency (DiMauro et al. 1983), COX deficiency with Leigh disease (Willems et al. 1977), fatal infantile mitochondrial myopathy with cardiomyopathy (Sengers et al. 1984), and COX deficiency with multiple respiratory-chain defects (Robinson et al. 1992).

Some of these defects in COX activity can be distinguished on the basis of both the tissue distribution of the biochemical defect and the clinical manifestations and course of the disease. The fatal infantile form of the disease with Fanconi-Debré syndrome shows COX deficiency in kidney, muscle, and heart but not in brain, liver, and skin fibroblasts (DiMauro et al. 1985); fatal infantile COX deficiency with cardiomyopathy is deficient in heart and skeletal muscle, with partial deficiency in fibroblasts (Robinson 1989); while COX deficiency with Leigh disease shows a general depression in all tissues (DiMauro et al. 1987; Van Coster et al. 1991). In this latter disorder there is a failure of assembly of the complex, and all subunits are decreased (Glerum et al. 1988, 1989; Lombes et al. 1991; Van Coster et al. 1991). Demonstration of tissue-specific isoforms for human subunits VIa (Fabrizi et al. 1989*a*; Van Beeumen et al. 1990) and VIIa (Fabrizi et al. 1989*b*), with distribution similar to that reported for tissue-specific defects, gives a possible theoretical foundation for the presence of such deficiencies. Here we report a type of COX deficiency in Leigh disease, a type that, in its biochemical profile, is distinct from that classically re-

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ported and that occurs in the population of the Saguenay-Lac-Saint-Jean (SLSJ) region of Quebec.

Case Reports, Material, and Methods

The detailed clinical presentation of patients, the clinical course, and the autosomal recessive nature of the inheritance are fully discussed in the accompanying paper (Morin et al. 1993). In brief, we have identified 34 patients from the SLSJ region of Quebec who had chronic lactic acidosis coupled with microvesicular steatosis and accompanied, as the infants grew, by the onset of neurodegenerative disease. Most of them died of unexplained fulminant acidotic crisis at a young age (<5 years). The pathological pattern of the disease found in the brain of these patients is identical to that of subacute necrotizing encephalomyelopathy, more commonly known as Leigh disease, and the liver shows microvesicular steatosis (Reye-like syndrome).

Cultured skin fibroblasts or liver biopsies (needle) were obtained from some of these patients during the course of clinical investigation. In some cases, liver, brain, muscle, heart, and kidney samples were obtained within 2 h of death, at autopsy, and were rapidly frozen in liquid nitrogen for further analysis. In one case, in which a prenatal diagnosis of an affected pregnancy was made, tissues were obtained from the terminated fetus. Control fetal samples were obtained, with informed consent, from therapeutic abortions performed for medical reasons.

Cytochrome *c* oxidase activity was assayed in sonicated whole-cell preparations by the method of Glerum et al. (1987). Measurements of cytochrome *c* oxidase and succinate cytochrome *c* reductase were carried out in whole-tissue homogenates by the method of Moreadith et al. (1984).

Human skin fibroblasts were grown from explants of forearm skin biopsy tissue (taken with informed parental consent). Culture medium was Eagle's X—Minimal essential medium supplemented with 10% FCS and 5 mM glucose. Lactate:pyruvate ratios were determined by incubation of confluent cultures of skin fibroblasts in a medium containing 1 mM glucose, followed by determination of lactate and pyruvate according to a method described elsewhere (Robinson et al. 1986).

Immunoblotting was carried out using 100 μg of fibroblast or liver mitochondrial protein. Samples were separated on 6 M urea, 0.1% SDS polyacrylamide gel (modified from the procedure of Kadenbach et al. 1983) and were electroblotted onto nitrocellulose by the method of Towbin et al. (1979), with the addition of 0.1% SDS to the electrode buffer. The electroblotted nitrocellulose sheets were then blocked with 2% gelatin-TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5) for 1 h, followed by overnight incubation in primary antibody. Incubation in secondary antibody was for 2 h and used affinity-purified goat anti-rabbit IgG-alkaline phosphatase conjugate (Bio-Rad). Bovine subunit-specific antibodies used were to subunits II and VIa of COX (provided by R. Capaldi, Eugene, OR); an antibody raised in our laboratory against the beef-heart holoenzyme COX was also used.

Total RNA was prepared from either liver or cultured skin fibroblasts, by the method of Chirgwin et al. (1979), and northern blotting was carried out by using the procedure described by Fourney et al. (1988): 20 μg of total RNA was separated by electrophoresis through a 1.5% (mass/v) agarose, 0.66 M formaldehyde gel. cDNA clones obtained by PCR amplification using the published sequence for subunits VIa (liver) and VIIa (liver) were radiolabeled with [^{32}P] CTP (Amersham) by random-primed DNA synthesis and were used as a probe.

Results

The clinical course of this disease, with its chronically elevated blood lactate levels (3.5–7 mM) and the neurodegenerative course culminating in a pathological diagnosis of Leigh disease and microvesicular steatosis, strongly suggested that this was an inborn error of energy metabolism (Morin et al. 1993). Since we know that Leigh disease can be caused by defects in the pyruvate dehydrogenase complex and by defects in complex I, IV, or V of the respiratory chain (Robinson et al. 1986, 1987, 1990; Robinson 1989; Tatuch et al. 1992), tests for the presence of these defects were carried out. Fibroblast cell lines from a group of SLSJ patients had normal activity of the pyruvate dehydrogenase complex and had only slightly raised or normal lactate:pyruvate ratios. This was a perplexing result, because respiratory-chain defects usually give high lactate:pyruvate ratios in fibroblasts. The cells did not carry the mtDNA mutation at 8993 that causes Leigh disease, thus ruling out the only known defect of complex V as a causative factor (Tatuch et al. 1992).

The only abnormality detected was a consistently low activity of COX in skin fibroblasts, compared with that in control cell lines. Comparison of the residual COX activity in fibroblast culture as a function of the measured cellular lactate:pyruvate ratio relative both to other patients with COX deficiency and to the normal population suggested that this SLSJ group was a cohort

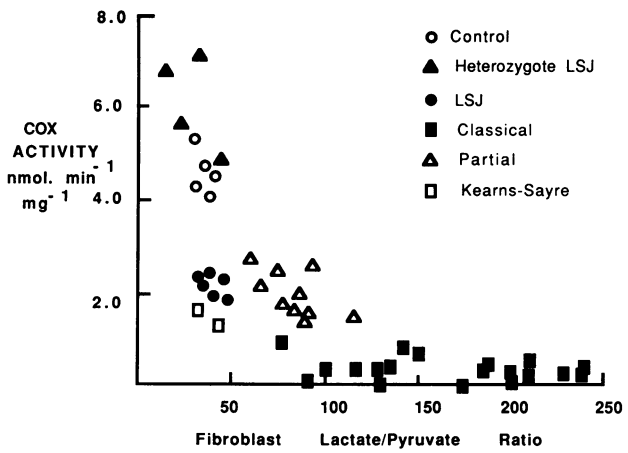

Figure 1 Residual activity of COX in cultured skin fibroblasts, as a function of the observed lactate:pyruvate ratio. The lactate:pyruvate ratio was measured for the following groups: normal controls, classical COX deficiency with Leigh disease, partial COX deficiency with Leigh disease (non-SLSJ), patients with Kearn-Sayre syndrome, patients with partial COX deficiency and Leigh disease who were from SLSJ, and obligate heterozygotes for the SLSJ deficiency. The whole-cell residual COX activity is plotted as a function of the fibroblast lactate:pyruvate ratio (the value shown is the mean of at least three determinations for each value).

distinct from other groups (fig. 1). This activity in the patients was decreased by 50%, with the results being highly significant and reproducible and seen in every patient cell line (table 1). We measured COX activity in the tissues of a patient that were obtained at autopsy and frozen in liquid nitrogen. We found low activities in liver and brain, about 50% activity for kidney and skeletal muscle, and almost normal activity in heart muscle (fig. 2). Examination of liver tissue obtained either by biopsy or at autopsy showed deficient activity in 13 cases (table 1). In contrast, succinate cytochrome *c* reductase activity was essentially normal in all cases except two autopsy cases, where poor preservation had decreased it to 50%. In both of these cases, COX activity in the liver was zero. Muscle COX activity was much less decreased than that of the liver enzyme in the five cases in which it was measured.

When immunoblotting experiments were carried out with liver mitochondria isolated from autopsy specimens, it was found that all subunits visualized by the holoenzyme antibody (subunits II, IV, and VIb) were decreased compared with levels in controls, in about the same proportion as the decrease in activity of the complex (fig. 3). Immunoblotting for subunit VIa (liver) showed only a faint band for human liver, which was decreased in the SLSJ patients (fig. 4). If this antibody cross-reacted with the heart form of VIa, it suggests that even the small amount of heart-specific VIa found in the liver COX is decreased below that in controls. Thus the residual activity in the liver of patients cannot be due to the proportion of COX serviced by the heart

Table 1

COX Activity in Liver, Cultured Fibroblasts, and Amniocytes

	COX Activity[a] (μmol/g wet wt/min)	
	Patient	Controls
Liver:		
NH	.33	2.38
PP	.00	2.68
AMB	.23	2.38
SM	.27	2.38
NG	.10	.68
AT	.14	.68
MT	.081	1.40
CC	.00	...
JSG	.034	...
JF	.00	...
AM	.101	...
JS	.00	.63
MGP	.00	.92
	Mean ± SEM COX Activity (no. of determinations)[a] (nmol/mg protein/min)	
	Patient	Controls
Fibroblasts:		
AGD	2.228 ± .277 (9)	4.874 ± .488 (12)
JF	2.37 ± .82 (7)	5.34 ± .92 (7)
AM	1.82 ± .25 (6)	3.60 ± 1.33 (4)
JS	2.49 ± .14 (2)	5.28 ± 1.42 (2)
NH	1.99 ± .18 (6)	4.24 ± .69 (4)
SMB	1.84 ± .21 (4)	4.80 ± .67 (4)
Amniocytes:		
CGD	3.254 ± .236 (5)[b]	7.280 ± .834 (9)
CLG	7.57 ± .91 (7)	8.20 ± 1.04 (8)
DC	4.14 ± .45 (5)[b]	8.92 ± 1.01 (7)
MB	4.09 ± .60 (6)[b]	8.08 ± .11 (10)
NP	5.45 ± .56 (5)	4.83 ± .86 (10)
GB	5.05 ± .06 (4)	6.98 ± .70 (8)
CD	4.70 ± .67 (4)	5.44 ± .34 (4)

[a] Measured in liver, fibroblast, and amniocyte homogenates as described in the Case Reports, Material, and Methods section. This was done for (1) patient and control liver samples, (2) patient and control skin fibroblasts, and (3) aminocytes from pregnancies at risk for COX deficiency (SLSJ group) and from control amniocytes. Control values were measurements performed on either control liver, fibroblast, or amniocyte homogenates and done on the same day under the same conditions.

[b] Case was predicted to be affected.

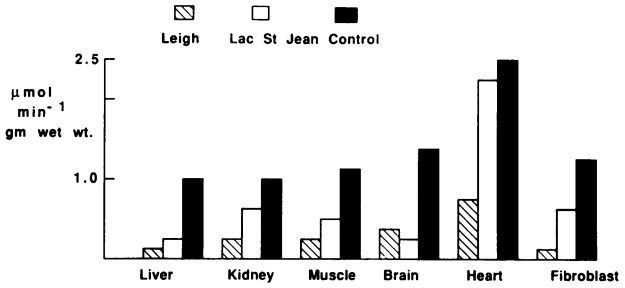

Figure 2 Activity of COX in tissues of a COX-deficient patient from the SLSJ region. The activities in liver, kidney, muscle, brain, heart, and cultured skin fibroblasts are compared with the COX activities in a patient with classical COX deficiency with Leigh disease and in three normal controls.

form of VIa. Since the homogenization of patient and control liver unavoidably includes the blood vessels of the liver, there is also a possibility that the cross-reactivity is provided by the small amount of muscle COX present in the vessel.

Northern blots of RNA from cultured fibroblasts (fig. 5) and from liver (not shown) showed normal mRNA for the liver-specific subunits of COX VIa and VIIa and for the mtDNA-encoded COX II subunit. Sequencing of cDNA reverse transcripts obtained from liver and skin fibroblasts of affected patients yielded normal coding sequence for both subunit VIa and subunit VIIa.

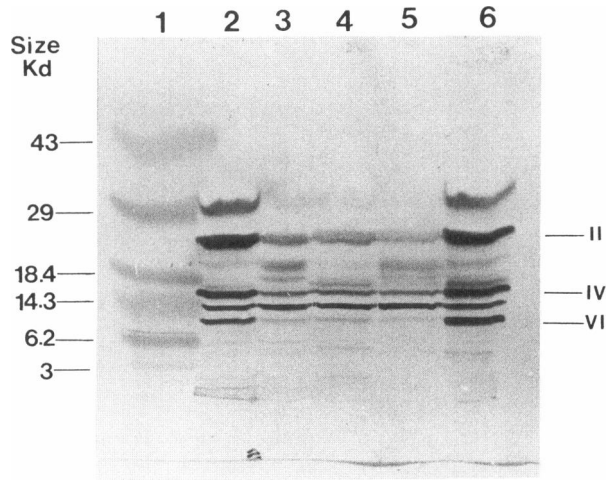

Figure 3 Immunoblot of liver mitochondrial proteins, with the antibody to beef-heart COX. Mitochondria were prepared by differential centrifugation from liver obtained at autopsy and rapidly frozen in liquid nitrogen. The resulting mitochondrial protein (100 μg) was electrophoresed through a urea/SDS/polyacrylamide gel as described, electroblotted onto nitrocellulose, and probed with an antibody to beef-heart COX raised in rabbits. Lane 1, Protein standards. Lane 2, Control human liver mitochondria. Lane 3, Patient CC. Lane 4, Patient JSG. Lane 5, Patient SM. Lane 6, Second control human liver mitochondria. Bands visualized consistently with this antibody in human liver are COX II, COX IV, and a COX VI subunit tentatively identified as VIb.

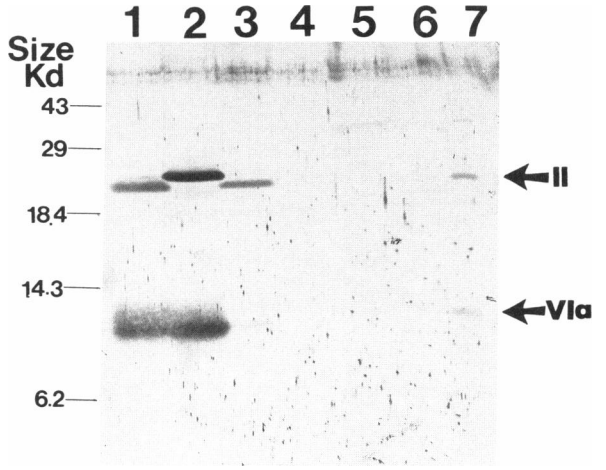

Figure 4 Immunoblot of liver mitochondrial proteins, with antibodies to COX II and COX VIa (liver). The blot was done as described in the legend to fig. 3, except that monospecific antibodies to COX II and COX VIa were used to probe. Lane 1, Human muscle mitochondria. Lane 2, Beef-heart mitochondria. Lane 3, Human liver mitochondria (control 1). Lane 4, Patient liver mitochondria, CC. Lane 5, Patient liver mitochondria, JSG. Lane 6, Patient liver mitochondria, SM. Lane 7, Human liver mitochondria (control 2).

Prenatal Diagnosis

Prenatal diagnosis by measurement of enzyme activity of COX in amniocytes has been attempted by us,

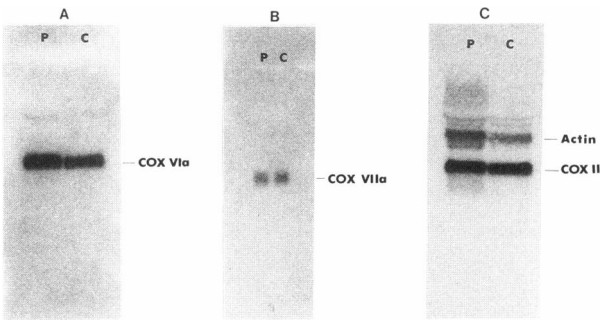

Figure 5 Northern blots of total RNA from cultured skin fibroblasts. Total RNA (20 μg) isolated from patient (lanes P) and matched controls (lanes C) fibroblast cell lines was separated by electrophoresis on agarose/formaldehyde gels and was blotted onto a Hybond N® membrane. Blots were then hybridized to ^{32}P-labeled cDNA probes corresponding to (*A*) COX VIa (human liver form), (*B*) COX VIIa (human liver form), and (*C*) COX II and actin. All mRNA levels were accessed for equal loading, by comparison with actin mRNA levels.

with seven pregnancies from mothers of previously (affected) diagnosed children. Prenatal diagnostic tests were performed as preliminary experimental procedures only, and patients were fully informed of this by their genetic counselor. They were made fully aware that prenatal diagnosis on the basis of 50% activity was a very difficult and untried procedure. On four of these occasions the activity in amniocytes was the same as that in controls. In three of the pregnancies, the COX level in the amniocytes was 50% of the control level, and the pregnancies were terminated electively (table 1). In two of these cases we were able to obtain fetal tissue; one was obviously affected with much lower COX activity than was present in controls, and the other had normal activities both in tissues and in skin fibroblasts. The four cases predicted to be normal were normal.

Discussion

We have demonstrated that, in the population in the SLSJ region of Quebec, a unique form of COX deficiency exists. This form of the deficiency has some features of classical COX deficiency with Leigh disease (neurodegeneration, with death occurring usually at <5 years of age) (Morin et al. 1993). The difference, at the biochemical level, between this form and the classical form involves a lesser involvement of kidney, muscle, heart, and skin fibroblasts. Until more autopsy samples from both groups can be measured, it is difficult to say whether there is any statistical difference in the involvement of brain and liver. It suffices to say, at present, that brain is similarly affected in both groups, which most certainly accounts for the similarity in the clinical course of the disease. The microvesicular steatosis seen in the SLSJ cohort may reflect a somewhat lower COX activity in liver.

Measurement of the lactate:pyruvate ratio in skin fibroblasts shows that this ratio is almost normal in the SLSJ patients and suggests that in the mildly affected tissues the residual COX activity is sufficient for the normal operation of the respiratory-chain, without compromise of the redox state of the intramitochondrial and cytosolic pyridine nucleotide couples. A similar situation occurs in obligate heterozygotes, whereby normal cytochrome *c* oxidase and lactate:pyruvate ratios are observed. This is almost certainly not the case in liver and brain, where there is very little activity of COX. We showed that in these tissues the amount of immunodetectable subunits was similarly decreased for all subunits tested. The conclusion that the complex is not assembled to any great degree in liver and brain, in this form of the disease, is justified. A similar conclusion was reached with respect to the classical form of COX deficiency with Leigh disease (Glerum et al. 1988; Van Coster et al. 1991). Partial forms of COX deficiency with Leigh disease have been described by us that have either partial or complete assembly of the complex in fibroblasts (Glerum et al. 1987, 1988, 1989). In nearly all of these cases the lactate:pyruvate ratio in fibroblasts is elevated, whereas in the SLSJ cases it is normal (fig. 1). We suggest that the 50% complex that does assemble in fibroblasts constitutes 50% expression of a normal functional enzyme.

The pattern of deficiency for these patients would very well fit the profile for the expression of one of the liver-specific subunits, VIa or VIIa. Despite several attempts, we have not been able to make an effective antibody against the human liver subunits VIa and VIIa, and thus we cannot yet investigate their individual synthesis. Immunoblotting with anti-bovine (anti-ASA) VIa antiserum suggests that the residual activity in liver is not due to COX assembled with the heart-specific form in the absence of liver-specific form. In addition, it seems that the coding sequence of both VIa and VIIa for the liver forms deduced from amplified cDNA are absolutely normal in these patients. Because the northern blots for VIa and VIIa liver forms also appear to be no different in patient and control livers and fibroblasts, it therefore seems unlikely that these tissue-specific subunits are responsible for this defect. However, until the details of these two genes and their expression are worked out, there is a considerable gap in our knowledge about this aspect of tissue-specific expression. Such observations suggest the presence of a tissue-specific component or factor that is necessary for the assembly of the cytochrome *c* oxidase complex. Such assembly factors/components have been shown to be absolutely necessary for cytochrome *c* oxidase assembly in yeast (Norbrega et al. 1990; Tzagoloff et al. 1990). Codominant expression of this component in tissues such as fibroblasts may explain the 50% activity and expression of a heart-specific isoform and the apparently normal activity in heart.

Finally, it would seem that the accurate measurement of COX activity in amniocytes in affected pregnancies may be a possible way to carry out prenatal diagnosis for this defect, although one false positive has occurred in our series to this point. Further investigation to uncover the molecular basis of this defect will undoubtedly lead to more reliable methods for prenatal diagnosis.

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