

ARTICLES

Immunological Phenotyping of Fibroblast Cultures from Patients with a Mitochondrial Respiratory Chain Deficit

Siôn L. Williams, Hans R. Scholte, R. George F. Gray, James V. Leonard, Anthony H. V. Schapira, and Jan-Willem Taanman

University Department of Clinical Neurosciences (SLW, AHVS, J-WT), Royal Free and University College Medical School, University College London, and Biochemistry, Endocrinology and Metabolic Unit (JVL), Institute of Child Health, and Institute of Neurology (AHVS), University College London; Clinical Chemistry Department (RGFG), Birmingham Children's Hospital, Birmingham, United Kingdom; and Department of Biochemistry (HRS), Cardiovascular Research Institute COEUR, Erasmus University Rotterdam, Rotterdam, The Netherlands

SUMMARY: Conventional approaches to the diagnosis of mitochondrial respiratory chain diseases, using enzyme assays and histochemistry, are laborious and give limited information concerning the genetic basis of a deficiency. We have evaluated the diagnostic value of 12 monoclonal antibodies to subunits of the four respiratory chain enzyme complexes and F_1F_0 -ATP synthase. Antibodies were used in immunological studies with skin fibroblast cultures derived from patients with diverse mitochondrial diseases, including patients in which the disease was caused by a nuclear genetic defect and patients known to harbor a heteroplasmic mutation in a mitochondrial tRNA gene. Immunoblotting experiments permitted the identification of specific enzyme assembly deficits and immunocytochemical studies provided clues regarding the genetic origin of the disease. The immunological findings were in agreement with the biochemical and genetic data of the patients. Our study demonstrates that characterization of the fibroblast cultures with the monoclonal antibodies provides a convenient technique to complement biochemical assays and histochemistry in the diagnosis of mitochondrial respiratory chain disorders. (*Lab Invest* 2001, 81:1069–1077).

Mitochondrial dysfunction is associated with a large number of neuromuscular disorders (DiMauro et al, 1998). Although many metabolic processes take place in mitochondria, compromised ATP production caused by diminished activity of the mitochondrial respiratory chain is seen as the basis for the majority of these diseases. The respiratory chain is embedded in the inner mitochondrial membrane and is composed of four multisubunit enzyme complexes: NADH:ubiquinone oxidoreductase (complex I), succinate:ubiquinone oxidoreductase (complex II), ubiquinol:ferricytochrome-c oxidoreductase (complex III), and ferrocycytochrome-c: oxygen oxidoreductase (complex IV or cytochrome-c oxidase). This enzyme chain transfers electrons from NADH and $FADH_2$ to oxygen in a series of oxidation-reduction reactions and uses the free energy from these reactions to pump

protons across the inner membrane. The transmembrane proton gradient is used by F_1F_0 -ATP synthase (complex V) to generate ATP (Hatefi, 1985). All subunits of complex II are encoded by nuclear DNA, but 13 subunits of the approximately 80 subunits constituting the other complexes are encoded by mitochondrial DNA (mtDNA) (Taanman, 1999). Therefore, genetic defects that underlie respiratory chain diseases may arise from mutations of either the mitochondrial or nuclear genomes.

Somatic cells contain thousands of copies of mtDNA. Pathogenic mtDNA mutations are generally heteroplasmic, that is, wild-type and mutant mtDNA molecules coexist in the same cell. Mitochondrial encephalomyopathies, such as myoclonic epilepsy with ragged-red fibers (MERRF) and mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) are often associated with specific point mutations in mitochondrial tRNA genes. Skeletal muscle biopsies from these patients show focal histochemical defects of complex IV, reflecting the skewed distribution of mutant and wild-type mtDNA in muscle fibers. A high mutant load is thought to produce a general defect of mitochondrial protein synthe-

Received January 29, 2001.

This work was supported by The Wellcome Trust, Grant 048410.

Address reprint requests to: Dr. Jan-Willem Taanman, University Department of Clinical Neurosciences, Royal Free and University College Medical School, Rowland Hill Street, London NW3 2PF, UK. E-mail: j.taanman@rfc.ucl.ac.uk

sis. This explains why complex IV deficiency observed in patients carrying mtDNA mutations is frequently associated with deficiencies of other enzyme complexes containing mtDNA-encoded subunits (Schon, 2000).

Respiratory chain disorders are most readily identified and classified on the basis of the involvement of the enzyme complexes. This is traditionally achieved with assays of enzyme function and by histochemistry of muscle biopsies or cultured cells. However, histochemical stains are only available for a limited number of enzyme activities and these techniques are at best semiquantitative (Dubowitz, 1985), whereas biochemical assays show considerable variation (Comi et al 1998; Rahman et al 1999; Scholte et al, 1995) and require relatively large amounts of material that may be difficult to accomplish for slow-growing patient cell cultures. We have determined the diagnostic merits of 12 monoclonal antibodies to subunits of the five enzyme complexes. Antibodies were used in immunoblot and immunocytochemical experiments with primary fibroblast cultures derived from patients with diverse mitochondrial dysfunctions. The immunoblot analysis allowed the identification of specific enzyme defects and the immunocytochemical analysis provided clues concerning the genetic origin of the disease.

Results

In this study, we compared the immunological characteristics of fibroblast cultures from 10 patients with mitochondrial respiratory chain disorders and two patients with a deficiency of the mitochondrial matrix enzyme pyruvate dehydrogenase. Of the 10 patients with mitochondrial respiratory chain deficits, five were adults who presented with MELAS and focal complex IV deficiency in skeletal muscle. All of these patients carried heteroplasmic mitochondrial tRNA^{Leu(UUR)} gene mutations. The other five patients with respiratory chain disorders were pediatric cases (P1–5). The dysfunction in patients P1, P4, and P5 was caused by

mutations in *SURF1*. The *SURF1* gene is located on chromosome 9 and is involved in the biogenesis of complex IV (Tiranti et al, 1998; Zhu et al, 1998). The genetic lesions in patients P2 and P3 have not been determined at the molecular level, but mtDNA sequencing and cell fusion studies have indicated that the underlying genetic defect in these patients is of nuclear origin. Respiratory chain enzyme activities of the fibroblast cultures and skeletal muscle tissue from P1–5 are given in Table 1. Control samples showed a large variation in values. All five patients displayed a profound complex IV deficiency in fibroblasts as well as in muscle. Except for P2, complex II + III activities were normal in the patient fibroblast cultures. In contrast, complex II + III activities in muscle were below the normal range for all five patients. Complex I activity was low in muscle of all patients, but this could be due to the fact that rotenone-sensitive NADH oxidase activity was measured, which is affected by low complex IV activities (Scholte et al, 1995). Complex II activities were measured in muscle of P1, P3, and P4, and were marginally below the normal range in P3. Additional biochemical assays of muscle homogenates from P2 revealed low activities of pyruvate dehydrogenase, α -ketoglutarate dehydrogenase, and creatine kinase (respectively, 18%, 31%, and 43% of mean control values).

Immunoblot Analysis

Whole-cell protein extracts of fibroblast cultures from P1–5, a MELAS patient, and two pediatric control subjects were compared on immunoblots. The mitochondrial tRNA^{Leu(UUR)} gene mutant load in the culture from the MELAS patient was 85%. Blots were probed with monoclonal antibodies to the following nuclear-encoded mitochondrial polypeptides: the 39-kd subunit of complex I, the flavoprotein and iron-sulfur protein subunits of complex II, the core 2 subunit of complex III, subunits IV, Va, and Vb of complex IV, and subunit α of complex V. We also used monoclonal antibodies to the mitochondrially encoded subunits I,

Table 1. Spectrophotometric Measurement of Respiratory Chain Enzyme Activities^a and Protein in Homogenates

	Patients					Controls		
	P1	P2	P3	P4	P5	Mean	(Range)	(n)
Fibroblasts								
Complex II + III (μ mol ferricytochrome-c)	5.77	1.57	16.7	11.7	25.8	13.8	(3.03–27.8)	(9)
Complex IV (first order rate constant k)	<0.01	<0.01	0.28	0.15	<0.01	2.29	(0.72–5.66)	(9)
Skeletal muscle								
Complex I (μ mol NADH)	1.00	0.74	0.77	0.88	0.75	3.66	(1.60–7.30)	(45)
Complex II (μ mol p-iodotetrazolium)	0.269	ND ^b	0.188	0.412	ND ^b	0.723	(0.190–1.64)	(58)
Complex II + III (μ mol ferricytochrome-c)	1.20	0.33	1.47	1.20	0.34	5.2	(1.6–12.5)	(54)
Complex IV (first order rate constant k)	5	5	3	4	<1	95	(45–161)	(49)
Protein (mg per g wet weight)	134	166	112	88	131	174	(77–263)	(53)

^a Activities are expressed as amount of used substrate or first order rate constant $k \cdot \text{min}^{-1} \cdot (\text{g wet weight})^{-1}$.

^b Not determined.

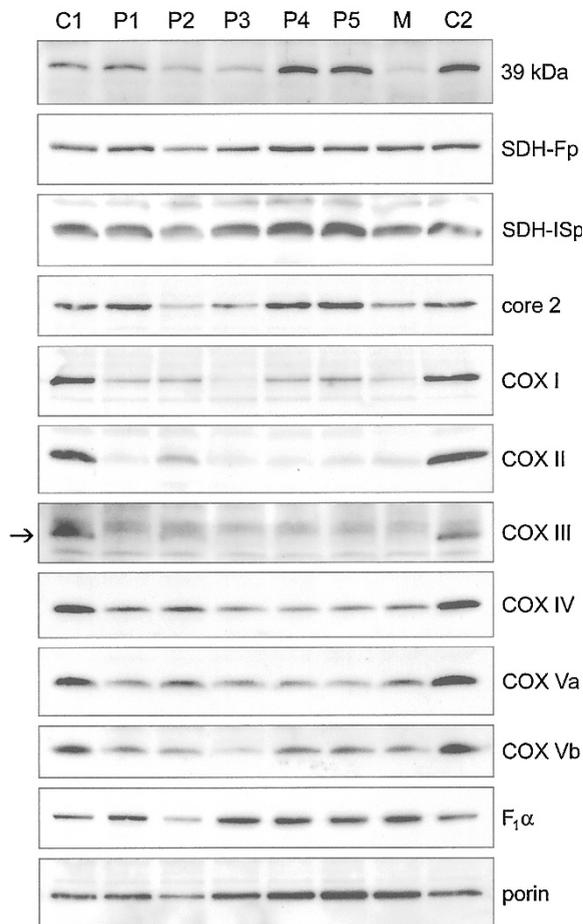


Figure 1.

Immunoblots of whole-cell protein extracts of fibroblast cultures from five pediatric patients (P1–5), a patient with mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) (M), and healthy controls (C1–2) developed with monoclonal antibodies to a 39-kd subunit of complex I (39 kDa), the flavoprotein and iron-sulfur protein of complex II (SDH-Fp and -ISp), the core 2 subunit of complex III (core 2), subunits I–Vb of complex IV (COX I, II, III, IV, Va, and Vb), subunit α of complex V ($F_{1\alpha}$), and porin. The subunit III signal of complex IV is indicated by an arrow.

II, and III of complex IV. A monoclonal antibody to the outer mitochondrial membrane protein porin was used to verify that equivalent amounts of mitochondrial proteins were loaded. Consistent results were achieved three times with independent extracts. Representative blots are shown in Figure 1.

Patients P1, P4, and P5, who all harbored *SURF1* mutations, showed an identical subunit expression pattern with decreased steady-state levels of all complex IV subunits, but control cell levels of subunits from complexes I, II, and III. Of the complex IV subunits, subunits II and III were most severely affected, whereas subunits IV and Va were relatively spared. In P2, steady-state levels of all subunits examined were lower than in controls. The decrease of complex II subunits was relatively mild, but the drop in the levels of the core 2 subunit of complex III was more severe than observed in any other patient. However, despite the fact that we took great care to load equal protein concentrations of whole-cell extracts, porin signals were always lower in P2 than in any of the other samples. P3 showed a subunit

expression pattern that was very similar to that of the MELAS patient. Both patients showed decreased levels of subunits from complexes I, III, and IV, but normal levels of subunits from complex II. P3, as well as the MELAS patient, had barely detectable levels of the mitochondrially encoded subunits I, II, and III of complex IV, whereas the nuclear-encoded subunits IV and Va were still present at significant levels. The α subunit of complex V was overexpressed in P1 and P3–5.

A similar immunoblot analysis was performed with whole-cell extracts of fibroblast cultures from two patients with a genetically defined deficiency of pyruvate dehydrogenase. No difference in expression patterns of respiratory chain enzyme subunits was noted between these patients and controls (data not shown).

Cytochemical and Immunocytochemical Analysis

Staining of control fibroblast cultures for complex IV activity resulted in a pale brown cytoplasmic staining of all cells (Fig. 2). The cultures from P1 and P3–5 did not show any cytoplasmic staining, whereas a faint cytoplasmic staining was observed in cultures from P2 (shown for P1–3 in Figure 2).

Fibroblast cultures from P1–5 and control cultures were immunostained with monoclonal antibodies to subunits I, IV, and VIc of complex IV, the flavoprotein of complex II, and subunit α of complex V. Experiments were repeated at least once for every cell culture/monoclonal antibody combination and gave identical results. Control cultures showed a finely branched cytoplasmic network consistent with a mitochondrial staining (Fig. 2). When cells were also labeled with the mitochondrion-selective MitoTracker dye, the antibody colocalized with the dye, confirming a mitochondrial location of the immunoreactive material (not shown).

Compared with control cell cultures, the cultures from the *SURF1* patients, P1, P4, and P5, showed a nearly complete loss of mitochondrial signal obtained with the monoclonal antibody to subunit VIc and decreased staining with the antibody to subunit IV of complex IV. With the other monoclonal antibodies, however, there was no appreciable difference in staining between control cultures and cultures from P1, P4, and P5. Representative micrographs of P1 are shown in Figure 2. P2 cells showed mitochondrial signals with an intensity similar to that of control cultures for all antibodies, but the mitochondria had a more swollen appearance (Fig. 2). P3 fibroblasts showed a considerable decrease in mitochondrial signal with the antibodies to subunits I and VIc of complex IV (Fig. 2). A less marked decrease was seen with the antibody to subunit IV (Fig. 2). Antibodies to either the flavoprotein of complex II or subunit α of complex V resulted in a signal that was indistinguishable from that of controls (Fig. 2).

We also compared fibroblast cultures from the five MELAS patients with control cultures. In contrast to the uniform immunostaining of control cell cultures and the cultures from P1–5, all MELAS cultures showed an intercellular mosaicism when stained for subunit I of complex IV, with some cells completely devoid of immunoreactive material, whereas other

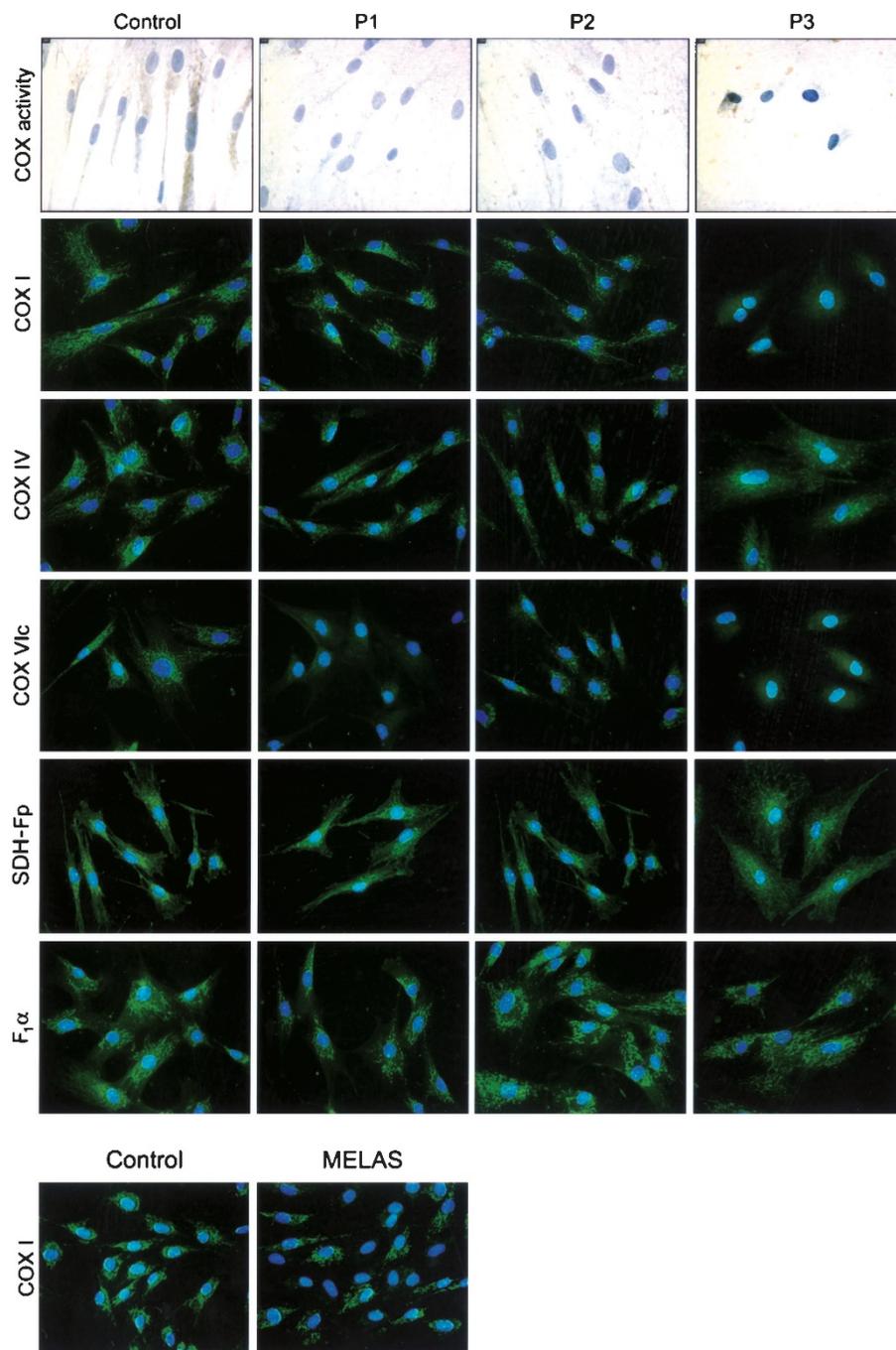


Figure 2.

The upper 24 micrographs show fibroblast cultures from a control and three pediatric patients (*P1–3*) cytochemically stained for complex IV (*COX*) activity, or immunostained for complex IV subunits I (*COX I*), IV (*COX IV*), or Vlc (*COX Vlc*), complex II flavoprotein (*SDH-Fp*) or complex V subunit α ($F_{1\alpha}$). The lower two micrographs show fibroblast cultures from a *control* and a *MELAS* patient immunostained for subunit I of complex IV. After staining for complex IV activity (brown), nuclei were counterstained purple with hematoxylin. After immunofluorescent green staining for the presence of subunits, nuclei were marked fluorescent blue with 4,6-diamidino-2-phenylindole. Original magnification: activity staining, $\times 320$; immunostaining, $\times 145$.

cells exhibited a normal staining intensity (Fig. 2). A uniform labeling pattern, similar to control cultures, was observed when cultures from the MELAS patients were immunostained with antibodies to subunit IV of complex IV or the flavoprotein of complex II, but antibodies to subunit Vlc of complex IV resulted in an intercellular mosaic staining that matched the subunit I staining pattern (not shown).

Discussion

Genetic Considerations

Respiratory chain disorders may arise from mutations of either the mitochondrial or the nuclear genomes. Early identification of the genome involved may facilitate rapid definitive diagnosis and is important for

genetic counseling because mtDNA shows a strict maternal inheritance (Giles et al, 1980). Immunocytochemical analysis of fibroblast cultures from patients P1–5 with monoclonal antibodies to the mtDNA-encoded subunit I of complex IV showed either a normal staining intensity or a uniform decreased staining intensity. This staining pattern was in stark contrast to the intercellular mosaic pattern observed in fibroblast cultures from five MELAS patients with a heteroplasmic mitochondrial tRNA^{Leu(UUR)} mutation. Intercellular mosaic staining patterns have also been observed in myoblast cultures from a patient bearing a heteroplasmic mitochondrial tRNA^{Glu} mutation (Taanman, 1997), a patient with mtDNA depletion (Taanman et al, 1997), and a patient with MERRF syndrome resulting from a heteroplasmic mitochondrial tRNA^{Lys} mutation (unpublished observation, J-WT). Moreover, a mosaic complex IV deficiency is commonly seen on histochemical analysis of skeletal muscle tissue from patients with heteroplasmic mtDNA mutations (Comi et al, 1998; Rahman et al, 1999; Taanman et al, 1996), whereas a general deficiency is found in muscle from patients with a complex IV defect caused by a nuclear mutation (Sue et al, 2000). Apparently, the intercellular mosaic staining mirrors the skewed distribution of deficient mtDNA, and the uniform staining patterns of P1–5 fibroblasts are indicative of nuclear DNA mutations.

A nuclear genetic origin of the disease of P1–5 suggested by the immunocytochemical analysis agreed with the genetic findings. Three of the five patients (P1, P4, and P5) carried mutations in *SURF1*. Mutations in this nuclear gene have previously been identified in patients with isolated complex IV deficiency (Tiranti et al, 1998; Zhu et al, 1998). Although the genetic lesion in P2 and P3 has not been determined, several observations suggest that the defect in both patients is of nuclear genetic origin: (1) sequencing of mtDNA candidate genes did not reveal any potentially pathogenic mutations; (2) cell fusion experiments between patient fibroblasts and mtDNA-less control cells resulted in complex IV-positive hybrids; (3) P2 had multiple enzyme defects, including enzymes of the citric acid cycle that do not contain subunits encoded by mtDNA; and (4) cell fusion experiments between mtDNA-less P3 fibroblasts and control platelets resulted in complex IV-negative hybrids.

Biochemical Considerations

In support of the results of the biochemical assays, the immunological studies revealed a complex IV deficiency in the cultures from P1–5 and a complex II + III deficiency in the culture from P2. However, although the biochemical assays did not reveal a complex II + III deficiency in P3, the immunological characterization indicated a clear complex III defect in P3 fibroblasts. Thus the immunological phenotyping provided more detailed information on the enzyme deficiencies than the biochemical assays. In skeletal muscle tissue from P1–5, most respiratory chain enzyme activities were

affected. Unfortunately, no tissue was available for immunological analysis.

Subunit Expression Patterns

In accordance with earlier reports (Poyau et al, 2000; Von Kleist Retzow et al, 1999), immunoblot analysis of fibroblasts from the patients carrying *SURF1* mutations (P1, P4 and P5) revealed a partial deficiency of complex IV subunits. Recently, it was shown that assembly of complex IV in these patients is stalled and assembly intermediates containing subunit I accumulate (Coenen et al, 1999; Tiranti et al, 1999). Studies in yeast have revealed proteolytic pathways controlling the clearing of unassembled and improperly folded polypeptides in the various subcompartments of mitochondria (Van Dyck and Langer, 1999). The components of this system are evolutionary conserved and are likely to be responsible for the degradation of complex IV subunits in cells with a *SURF1* defect. In contrast to the markedly decreased levels of subunit I on immunoblots, immunocytochemistry suggested normal subunit I levels in cells from patients with *SURF1* mutations. A possible explanation for these divergent findings is that immunocytochemistry may only detect substantial changes in subunit I levels, whereas immunoblotting allows the detection of more subtle changes. Alternatively, these divergent findings may suggest that in cells with a *SURF1* defect, subunit I is embedded in the membrane in its more or less native state or is bound to a chaperone protein, protecting it from proteolytic attack. However, disruption of the mitochondrial membranes during protein extraction with the mild detergent laurylmaltoside (which keeps enzyme complexes intact) exposes the bare or partly assembled subunit I to endogenous proteases and, despite the presence of a protease inhibitor cocktail in the extraction buffer, subunit I is partly degraded.

Patient P3 showed a protein expression pattern on immunoblots that was strikingly similar to the MELAS patient carrying a heteroplasmic mitochondrial tRNA mutation. Both P3 and the MELAS patient exhibited decreased subunit levels of the bigenomic complexes I, III, and IV, whereas complex II subunits were unaffected. In particular, the mtDNA-encoded subunits I, II, and III of complex IV were present at very low levels. Northern blot analysis revealed normal transcript levels for subunits I and II (unpublished observations, SLW). Therefore, the similarity of the molecular phenotype of P3 fibroblasts to that of the MELAS fibroblasts suggests an impairment of mitochondrial translation in P3, possibly arising from a defect in a mitochondrial translation factor, ribosomal protein, or aminoacyl-tRNA synthase, all of which are of nuclear origin. To our knowledge, no diseases have been attributed to mutations in such genes; however, they constitute a large pool of nuclear candidate genes for disorders involving bigenomic respiratory chain complexes.

Patient P2 showed a relatively mild decline of all respiratory chain subunits levels, with the exception of

the core 2 subunit of complex III and subunit α of complex V, which were severely decreased. The immunoblots of whole-cell protein extracts also revealed lower levels of the outer mitochondrial membrane protein porin, and the immunocytochemical experiments indicated an abnormal mitochondrial morphology. In addition to decreased activities of respiratory chain enzymes, we found decreased activities of other mitochondrial enzymes. Thus, the deficiency of respiratory chain subunits may in part be explained by a general failure of mitochondrial biogenesis, for example, caused by a defect in mitochondrial protein import or processing.

Concluding Remarks

Biochemical assays have been used to diagnose respiratory chain diseases for decades. Although biochemical assays are a reliable and quantitative index for diagnosis, a number of drawbacks are associated with the technique. The assays usually show a large variation of enzyme activities in control samples, which may complicate the interpretation of patient values. The assays are relatively difficult to perform and are, therefore, predominantly conducted at specialized centers. Furthermore, biochemical assays require quite large quantities of material and provide only limited information regarding the genetic basis of a deficiency. Histochemical staining may offer an alternative for assessing the activities of some respiratory chain enzymes when sample size is small and has the additional advantage that individual cells can be studied. However, although satisfactory histochemical results are normally obtained with tissue and cultured tumor cells, primary cultures tend to stain poorly. The library of monoclonal antibodies used in this study permits a rapid initial evaluation of fibroblast cultures from patients suspected of having a respiratory chain dysfunction. The antibodies may provide more detailed information concerning the enzymatic defect and genetic origin of the disease than traditional approaches to diagnosis. The entire immunological analysis requires less than one confluent 10-cm cell culture plate ($\sim 0.5 \times 10^6$ cells) and can, therefore, be performed soon after the primary culture has been established. Alternatively, the monoclonal antibodies can be used for immunohistochemical staining of tissues (Rahman et al, 2000; Taanman et al, 1996). Thus, immunological analysis offers a convenient approach that can be used in conjunction with biochemical assays and histochemistry to enhance the accuracy of diagnosis and to help discern the underlying pathology of these complicated metabolic diseases.

Material and Methods

Case Reports and Genetic Characterization

Patient P1 was born to healthy unrelated parents. She presented with hypertrichosis, lactic acidemia, hypotonia, and ataxia. Nystagmus was noted from 2.5 years. A muscle biopsy, taken 3 months later, showed

normal morphology. A computed tomography (CT) scan was normal. She had delayed bone age, was never able to walk independently, suffered from excessive sweating with polyuria, and there was a progressive loss of motor and mental achievements. The patient died at the age of 3.3 years from respiratory insufficiency. Autopsy was not performed. A younger sister died of a similar illness. DNA sequencing revealed that P1, as well as her sister, were homozygous for a 312insATdel10 rearrangement (numbering according to Tiranti et al, 1998) in exon 4 of *SURF1*, whereas both parents were confirmed as heterozygous carriers. This is the most common mutant *SURF1* allele. The mutation is predicted to result in a truncated *SURF1* protein product and causes complex IV deficiency (Tiranti et al, 1998; Zhu et al, 1998).

Patient P2 fed poorly at birth and rapidly developed a metabolic acidosis with profound lactic acidemia. He failed to gain skills and died aged 7 months. A muscle biopsy revealed a marked type IIb fiber atrophy with accentuated T-tubules but no other abnormalities. A male sibling was found to have severe intrauterine growth retardation, was born with a gross lactic acidemia, hypotonia, hypospadias, and died at 27 hours of age. Sequencing of all 22 mitochondrial tRNA genes and the three mitochondrial genes encoding subunits of complex IV did not identify any known mutations in P2 mtDNA; however, two homoplasmic base changes were found that have not been reported previously: a 5894 A→G transition in the untranslated region between the genes for tRNA^{Tyr} and subunit I of complex IV and a 6852 G→A transition in the gene for subunit I of complex IV. The region of the first base change is unlikely to be critical for mtDNA expression because a poly-C insertion in the same region (5895–5899) has been documented in healthy individuals (Chen et al, 1995). The second base change causes a Gly³¹⁷→Ser residue change in subunit I of complex IV. This residue is conserved from yeast to human. Nevertheless, the base change is unlikely to contribute to the disease because amino acid substitution at the homologous position of subunit I in yeast had no effect on the activity of the yeast enzyme (Meunier, 2001). The presence of pathogenic mutations of mtDNA was excluded further by cell fusion experiments between P2 fibroblasts and control cell line ρ^0 A549.B2, which is a mtDNA-less derivative of the human carcinoma cell line A549 (Taanman et al, 1997). In contrast to P2 fibroblasts, all P2 hybrid cells showed complex IV activity. This result indicates that A549 nuclear DNA complements P2 mtDNA and that pathogenic mutations are not present in P2 mtDNA.

Patient P3 was born to healthy unrelated parents after a normal pregnancy. A heart murmur was noted at 6 weeks and a right divergent squint from 1.25 years. He was first admitted to a hospital at 1.5 years with a viral infection. On arrival, the patient was shocked, poorly perfused and drowsy. Blood lactate concentrations were raised, the CT brain scan was normal, the electromyogram (EMG) showed no evidence of peripheral neuropathy, but an echocardiogram revealed hypertrophic cardiomyopathy and peri-

cardial effusion. A renal tubular defect was also noted. The patient died at the age of 2.4 years. Postmortem examination established a diagnosis of Leigh syndrome. Two older siblings had died, one a stillbirth and the other a sudden death after a mild upper respiratory tract infection at the age of 1.5 years. A third sibling was 9 years old and well at the time of admission of P3. Sequencing of all 22 mitochondrial tRNA genes and the three mitochondrial genes encoding subunits of complex IV did not identify any known mutations in P3 mtDNA. The presence of pathogenic mutations of mtDNA was excluded further by a cell fusion experiment between P3 fibroblasts and control cell line ρ^0 A549.B2. As found for P2, all resulting P3 hybrid cells showed complex IV activity, suggesting that pathogenic mutations are not present in P3 mtDNA. This conclusion was supported by a second cell fusion experiment in which mtDNA-depleted, transformed P3 fibroblasts were fused with control platelets. In the latter experiment, none of the P3 hybrids showed complex IV activity, indicating that control platelet mtDNA is unable to complement P3 nuclear DNA. Thus, the defect in the patient is likely to be the result of a nuclear genetic lesion.

Patient P4 was the first child of healthy unrelated parents. He presented with vomiting at 8 months, by 1.2 years was failing to thrive and subsequently developed progressive cerebellar ataxia, nystagmus, hypotonia and developmental regression. Muscle biopsy showed normal complex IV histochemistry but increased lipid. Plasma lactate was elevated. An EMG showed mixed peripheral neuropathy, a CT brain scan was normal, but an MRI of the brain showed lesions consistent with Leigh syndrome. There was a progressive deterioration leading to death. Autopsy confirmed Leigh syndrome. A subsequent sibling died with a similar clinical presentation. Like P1, P4 was homozygous for the common 312insATdel10 rearrangement in exon 4 of *SURF1*.

The clinical details of patient P5 have been reported elsewhere (Das et al, 1994). Postmortem examination demonstrated Leigh syndrome. DNA sequencing revealed that P5 was compound heterozygous for the common 312insATdel10 rearrangement in *SURF1* exon 4 and a 370 G→A mutation in *SURF1* exon 5. The latter mutation results in a Gly¹²⁴→Arg substitution in the *SURF1* protein product. This missense mutation and a different missense mutation affecting the same codon, leading to a Gly¹²⁴→Glu replacement, were recently described in two other patients with complex IV-deficient Leigh syndrome (Coenen et al, 1999; Poyau et al, 2000).

Five adult patients with MELAS syndrome and a partial complex IV deficiency in muscle were shown to carry a heteroplasmic 3243 A→G point mutation in their mitochondrial tRNA^{Leu(UUR)} gene. Two pediatric patients presented with congenital lactic acidosis suggestive of a mitochondrial disorder. Detailed biochemical investigations indicated isolated pyruvate dehydrogenase deficiency in both patients, which was corroborated by genetic analysis. Pediatric (healthy)

control tissues were acquired from children aged <3 years having orthopedic surgery.

Cell Cultures and Growth Conditions

Fibroblast cultures were established from skin biopsies after informed (parental) consent. Cells were grown in Dulbecco's modified Eagle's medium with 25 mM glucose and 4 mM L-alanyl-L-glutamine, to which 10% fetal bovine serum, 0.2 mM uridine, 1 mM sodium pyruvate, 50 U/mL of penicillin, and 50 μ g/mL of streptomycin was added. Cultures were maintained at 37° C in a humidified atmosphere of 8% CO₂ in air and were checked regularly for mycoplasma infection.

Immunological Analysis

Mouse monoclonal antibodies to the flavoprotein (clone 2E3-GC12-FB2-AE2) and iron-sulfur protein (clone 21A11-AE7) of complex II, the core 2 protein of complex III (clone 13G12-AF12-BB11), subunits I (clone 1D6-E1-A8), II (clone 12C4-F12), III (clone DA5), IV (clone 10G8-C12-D12), Va (clone 6E9-B12-D12), Vb (clone 16H12-H9), and Vlc (clone 3G5-F7-G3) of complex IV, and subunit α of complex V (clone 7H10-BD4) have been described elsewhere (Marusich et al, 1997; Rahman et al, 1999; Taanman and Capaldi, 1993; Taanman et al, 1996). In addition, a mouse monoclonal antibody directed against the 39-kd subunit of complex I (clone: 20C11-B11-B11) was used. All of these antibodies are available through Molecular Probes (Eugene, Oregon). The anti-porin 31HL mouse monoclonal antibody was obtained from Calbiochem (CN Biosciences UK, Nottingham, United Kingdom).

Immunoblot analysis of whole-cell extracts was performed essentially as described (Taanman et al, 1997). For immunocytochemical analysis, cells seeded on glass coverslips were washed in PBS, fixed with 4% paraformaldehyde in PBS for 20 minutes, and washed again. Coverslips to be immunostained with the anti-flavoprotein antibody were incubated in 10 mM sodium citrate buffer (pH 6.0) at 90° C for 20 minutes before treatment with methanol for 15 minutes at -20° C and a PBS wash. The sodium citrate treatment was omitted for other coverslips. All subsequent incubations were carried out at 37° C in a humidified atmosphere. Protein binding sites were saturated with 10% normal goat serum in PBS for 30 minutes, followed by a 45-minute incubation with primary antibodies in PBS at optimized concentrations. Coverslips were washed with PBS, incubated with 2 μ g Alexa 488 goat antimouse IgG conjugate (Molecular Probes) per 100 μ L of PBS for 45 minutes, washed again, and mounted to glass slides in Citifluor-glycerol-PBS solution (Agar Scientific, Stansted, United Kingdom) supplemented with 1 μ g/mL of 4,6-diamidino-2-phenylindole (DAPI). In some experiments, coverslips were treated with MitoTracker Red CM-H₂XRos (Molecular Probes) preceding paraformaldehyde fixation (Taanman et al, 1997). Fluorescence was inspected with a Zeiss Axiophot photomi-

croscope. Photographs were taken on Kodak Ektachrome 400× film.

Cytochemical Staining and Respiratory Chain Enzyme Assays

The cytochemical staining procedure for complex IV activity was adapted from a technique described elsewhere (Seligman et al, 1968). Cells, grown on coverslips, were rinsed with PBS supplemented with 0.5 mM MgCl₂ and 0.9 mM CaCl₂, briefly air-dried, and incubated for 1 hour at 37° C in 50 mM sodium phosphate buffer (pH 7.4) containing 1 mg/mL of horse cytochrome-c, 0.5 mg/mL of 3,3'-diaminobenzidine, and 2 μg/mL of catalase. The staining was intensified by treating the coverslips with DAB Enhancer Reagent Solution (Zymed Laboratories, San Francisco, California) for 10 minutes at room temperature. Cell nuclei were counterstained with Meyer's hematoxylin solution.

Spectrophotometric assays were performed on freeze-thawed homogenates of skeletal muscle and primary skin fibroblast as described (Scholte et al, 1987, 1995). Homogenates were prepared in 0.25 M sucrose, 10 mM N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES) KOH (pH 7.4), and 1 mM ethylenediamine tetraacetic acid (EDTA) (pH 7.4).

Acknowledgements

We thank Drs. H. Przyrembel and S. Schweitzer-Krantz for patient samples.

References

Chen Y-S, Torroni A, Excoffier L, Santachiara-Benerecetti AS, and Wallace DC (1995). Analysis of mtDNA variation in African populations reveals the most ancient of all human continent-specific haplogroups. *Am J Hum Genet* 57:133–149.

Coenen MJH, Van den Heuvel LP, Nijtmans LGJ, Morava E, Marquardt I, Girschick HJ, Trijbels FJM, Grivell LA, and Smeitink JAM (1999). SURFEIT-1 gene analysis and two-dimensional blue native gel electrophoresis in cytochrome c oxidase deficiency. *Biochim Biophys Res Commun* 265:339–344.

Comi GP, Bordoni A, Salani S, Franceschina L, Sciacco M, Prella M, Fortunato F, Zeviani M, Napoli L, Bresolin L, Bresolin N, Moggio M, Ausenda CD, Taanman J-W, and Scarlato G (1998). Cytochrome c oxidase subunit I microdeletion in a patient with motor neuron disease. *Ann Neurol* 43:110–116.

Das AM, Schweitzer-Krantz S, Byrd DJ, and Brodehl J (1994). Absence of cytochrome c oxidase activity in a boy with dysfunction of renal tubules, brain and muscle. *Eur J Pediatr* 153:267–270.

DiMauro S, Bonilla E, Davidson M, Hirano M, and Schon EA (1998). Mitochondria in neuromuscular disorders. *Biochim Biophys Acta* 1366:199–210.

Dubowitz V (1985). *Muscle biopsy: A practical approach*, 2nd ed. London: Baillière Tindall, 19–40.

Giles RE, Blanc H, Cann HM, and Wallace DC (1980). Maternal inheritance of human mitochondrial DNA. *Proc Natl Acad Sci USA* 77:6715–6719.

Hatefi Y (1985). The mitochondrial electron transport and oxidative phosphorylation system. *Annu Rev Biochem* 54:1015–1069.

Marusich MF, Robinson BH, Taanman J-W, Soo JK, Schillace R, Smith JL, and Capaldi RA (1997). Expression of mtDNA and nDNA encoded respiratory chain proteins in chemically and genetically derived Rho0 human fibroblasts: A comparison of subunit proteins in normal fibroblasts treated with ethidium bromide and fibroblasts from a patient with mtDNA depletion syndrome. *Biochim Biophys Acta* 1362:145–159.

Meunier B (2001). Site-directed mutations in the mitochondrially encoded subunits I and III of yeast cytochrome oxidase. *Biochem J* 354:407–412.

Poyau A, Buchet K, Bouzidi MF, Zobot M-T, Echenne B, Yao J, Shoubridge EA, and Godinot C (2000). Missense mutations in *SURF1* associated with deficient cytochrome c oxidase assembly in Leigh syndrome patients. *Hum Genet* 106:194–205.

Rahman S, Lake BD, Taanman J-W, Hanna MG, Cooper JM, Schapira AHV, and Leonard JV (2000). Cytochrome oxidase immunohistochemistry: Clues for genetic mechanisms. *Brain* 123:591–600.

Rahman S, Taanman J-W, Cooper JM, Nelson I, Hargreaves I, Meunier B, Hanna MG, Garcia JJ, Capaldi RA, Lake BD, Leonard JV, and Schapira AHV (1999). A missense mutation of cytochrome oxidase subunit II causes defective assembly and myopathy. *Am J Hum Genet* 65:1030–1039.

Scholte HR, Busch HFM, Bakker HD, Bogaard JM, Luyt-Houwen IEM, and Kuyt LP (1995). Riboflavin-responsive complex I deficiency. *Biochim Biophys Acta* 1271:75–83.

Scholte HR, Busch HFM, Luyt-Houwen IEM, Vaandrager-Verduin MHM, Przyrembel H, and Arts WFM (1987). Defects in oxidative phosphorylation: Biochemical investigations in skeletal muscle and expression of the lesion in other cells. *J Inher Metab Dis* 10(Suppl 1):81–97.

Schon EA (2000). Mitochondrial genetics and disease. *Trends Biochem Sci* 11:555–560.

Seligman AM, Karnovsky MJ, Wasserkrug HL, and Hanker JS (1968). Nondroplet ultrastructural demonstration of cytochrome oxidase activity with a polymerizing osmophilic reagent, diaminobenzidine (DAB). *J Cell Biol* 38:1–14.

Sue CM, Karadimas C, Checcarelli N, Tanji K, Papadopoulou LC, Pallotti F, Gou FL, Shanske S, Hirano M, De Vivo DC, Coster RV, Kaplan P, Bonilla E, and DiMauro S (2000). Differential features of patients with mutations in two COX assembly genes, SURF-1 and SCO2. *Ann Neurol* 47:589–595.

Taanman J-W (1997). Human cytochrome c oxidase: Structure, function, deficiency. *J Bioenerg Biomembr* 29:151–163.

Taanman J-W (1999). The mitochondrial genome: Structure, transcription, translation and replication. *Biochim Biophys Acta* 1410:103–123.

Taanman J-W, Bodnar AG, Cooper JM, Morris AAM, Clayton PT, Leonard JV, and Schapira AHV (1997). Molecular mechanisms in mitochondrial DNA depletion syndrome. *Hum Mol Genet* 6:935–942.

Taanman J-W, Burton MD, Marusich MF, Kennaway NG, and Capaldi RA (1996). Subunit specific monoclonal antibodies show different steady-state levels of various cytochrome-c oxidase subunits in chronic progressive external ophthalmoplegia. *Biochim Biophys Acta* 1315:199–207.

Taanman J-W and Capaldi RA (1993). Subunit VIa of yeast cytochrome c oxidase is not necessary for assembly of the enzyme complex but modulates the enzyme activity. *J Biol Chem* 268:18754–18761.

Tiranti V, Galimberti C, Nijtmans L, Boenta S, Perini MP, and Zeviani M (1999). Characterization of *SURF-1* expression and Surf-1p function in normal and disease conditions. *Hum Mol Genet* 8:2533–2540.

Tiranti V, Hoertnagel K, Carozzo R, Galimberti C, Munaro M, Granatiero M, Zelante L, Gasparini P, Marzella R, Rocchi M, Bayona-Bafaluy P, Enriquez J-A, Uziel G, Bertini E, Dionisi-Vici C, Franco B, Meitinger T, and Zeviani M (1998). Mutations of *SURF-1* in Leigh disease associated with cytochrome c oxidase deficiency. *Am J Hum Genet* 63:1609–16021.

Van Dyck L and Langer T (1999). ATP-dependent proteases controlling mitochondrial function in the yeast *Saccharomyces cerevisiae*. *Cell Mol Life Sci* 56:825–842.

Von Kleist Retzow JC, Vial E, Chantrel-Groussard K, Rötig A, Munnich A, Rustin P, and Taanman J-W (1999). Biochemical, genetic and immunoblot analyses of 17 patients with an isolated cytochrome c oxidase deficiency. *Biochim Biophys Acta* 1455:35–44.

Zhu Z, Yao J, Johns T, Fu K, De Bie I, Macmillan C, Cuthbert AP, Newbold RF, Wang J, Chevrette M, Brown GK, Brown RM, and Shoubridge EA (1998). SURF1, encoding a factor involved in the biogenesis of cytochrome c oxidase, is mutated in Leigh syndrome. *Nat Genet* 20:337–343.