Biochemical and Molecular Studies of Mitochondrial Function in Diabetes Insipidus, Diabetes Mellitus, Optic Atrophy, and Deafness

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OBJECTIVE — To determine if diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (DIDMOAD) combined with a cerebellar syndrome is associated with a systemic disorder of respiratory chain function as found in similar genetic syndromes.

CASE — A muscle biopsy was taken from a patient with DIDMOAD, and a mitochondrial fraction was prepared. Respiratory chain function was assessed by analysis of intermediary metabolites, histochemical analysis of muscle biopsy, measurement of the activity of individual respiratory chain complexes, oxidative flux through the respiratory chain, and cytochrome concentration and compared with a population with normal respiratory chain function. Mitochondrial DNA from skeletal muscle, brain, and pancreas was examined for major rearrangements and specific point mutations. Brain tissue was examined neuropathologically for abnormalities, particularly those previously described in association with DIDMOAD.

RESULTS — No abnormality was found in mitochondrial oxidation, individual complex activity, or cytochrome concentration. Histochemical analysis and electron microscopy showed no abnormality known to be associated with mitochondrial dysfunction. A single-base substitution at position 12308 of the mitochondrial genome was found, but no major rearrangement of mitochondrial DNA was demonstrated. Neuropathological examination revealed severe demyelination and gliosis in the optic nerves and loss of Purkinje cells associated with gliosis in the white matter in the cerebellum.

CONCLUSIONS — We have found no evidence that DIDMOAD is associated with a systemic abnormality of respiratory chain function. The mitochondrial DNA single-base substitution noted is likely to be a polymorphism rather than a pathogenic point mutation. We have confirmed that DIDMOAD may be associated with a neurodegenerative disorder, but the cause of this remains undetermined.

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DIDMOAD, diabetes insipidus, diabetes mellitus, optic atrophy, and deafness; IDDM, insulin-dependent diabetes mellitus; PCR, polymerase chain reaction.

The underlying metabolic abnormality in patients with diabetes insipidus, diabetes mellitus, optic atrophy, and deafness (DIDMOAD) is unknown (1). Patients with this condition may also develop an ataxic syndrome (2), an unusual combination of features that is also found in a group of neurodegenerative disorders as a result of abnormalities of mitochondrial respiratory chain function, the mitochondrial encephalomyopathies (3,4). These are a heterogeneous group of diseases that may present at any age. In childhood, they are commonly associated with lactic acidosis and progressive psychomotor retardation; in adults, they may present with a myopathy (often selectively affecting the extraocular muscles) or other neurological symptoms. The last 10 years has seen a great increase in knowledge of respiratory chain diseases, not only in their biochemical and molecular features but also in their presentation. Apparently, mitochondrial disorders may present in an extraordinary variety of ways and are no longer diseases seen almost exclusively by pediatricians and neurologists. Individuals with endocrine dysfunction (5), sideroblastic anemia (6), gastrointestinal symptoms (7), or cardiomyopathy (8) have all been reported, although ptosis, progressive ophthalmoplegia, and myopathy are currently thought to be the most common manifestations of mitochondrial disease in adults.

The mitochondrial respiratory chain consists of ~70 different proteins arranged as 5 multisubunit complexes (numbered I through V) situated in the inner mitochondrial membrane. It performs two vital functions: the reoxidation of reduced cofactors formed by the metabolism of metabolic fuels, e.g., carbohydrate and fatty acids, and the phosphorylation of ADP to ATP (9). The proteins of the respiratory chain are derived from DNA encoded on two separate genomes, nuclear DNA, found in the chromosomes, and mitochondrial DNA (mtDNA). mtDNA is a small, circular genome found

in mitochondria; it is almost exclusively inherited from the mother and is present in multiple copies within each mitochondrion (10). mtDNA encodes 13 of the 70 or more proteins of the respiratory chain. Disorders of the respiratory chain, therefore, may be sporadic or show either Mendelian inheritance (when the nuclear DNA is abnormal) or a maternal pattern of inheritance. Occasionally, Mendelian patterns of inheritance are associated with mtDNA abnormalities, which is thought to reflect an abnormality of a nuclear factor controlling mtDNA synthesis or maintenance (11).

Function of the respiratory chain may be studied in a number of ways: indirectly, by measurement of intermediary metabolites such as lactate and pyruvate, or more specifically, by skeletal muscle biopsy with histochemical analysis and biochemical studies of a mitochondrial fraction prepared from the biopsy. Histochemical preparations are used to demonstrate mitochondrial accumulation and the activity of two enzymes of the respiratory chain, succinate dehydrogenase and cytochrome *c* oxidase (complexes II and IV; 12). Electron microscopy gives further information about mitochondrial content and structure. Preparation of a mitochondrial fraction allows direct measurement of the activity of all the individual respiratory chain complexes, the concentration of cytochromes aa_3, *b*, and *c*, which are associated with the respiratory chain, and measurement of oxidative flux through the chain (13).

Mitochondrial disorders may be associated with diabetes mellitus (14,15), and recent reports have suggested that DIDMOAD may be associated with morphological mitochondrial abnormalities and an abnormality of mtDNA, which encodes components of the mitochondrial respiratory chain (16). In view of these reports and the remarkable similarity in phenotype between some patients with DIDMOAD and those with certain types of respiratory chain disease, we have examined respiratory chain function and mtDNA in a patient with DIDMOAD associated with ataxia.

CASE — The patient, a Caucasian woman 36 years of age at the time of investigation, developed insulin-dependent diabetes mellitus (IDDM) at 5 years of age. Her early development was normal, but at 11 years of age she was seen with visual deterioration associated with optic atrophy, and, by age 17, her vision was restricted to appreciation of light only. At age 15, diabetes insipidus was diagnosed and treatment with desmopressin commenced. Control of both diabetes mellitus and diabetes insipidus was good, and her condition remained stable until she was 30, when she began to complain of unsteadiness and dizziness. Gradually, she became severely disabled with dysphagia, dysarthria, and ataxia and died suddenly at age 36.

Examination 2 months before her death showed a short (150 cm), slightly obese woman with mild spinal scoliosis but no other dysmorphic features. She was confined to a wheelchair by severe ataxia. Speech was abnormal, with a cerebellar dysarthria. There was bilateral anosmia and optic atrophy, with no perception of light. The pupils were fixed. Eye movements were full, and there was no ptosis, but horizontal phasic nystagmus was present in all directions of gaze. Audiometry revealed a mild bilateral high-tone hearing loss. Coordination was mildly impaired in the arms and severely impaired in the legs. The reflexes were depressed in the arms and absent in the legs. Plantar responses were flexor.

The patient died suddenly at home, and a limited autopsy was done.

Family history

One sibling had DIDMOAD and died at 33 years of age from pyelonephritis secondary to renal tract abnormalities. She had no symptoms of ataxia at the time of her death, and no autopsy was done. Another sibling died at 3 years of age, shortly after diabetes mellitus was diagnosed. There was no other family history of DIDMOAD, but three distant maternal cousins had IDDM.

Investigation

A muscle biopsy was taken from the left quadriceps under spinal anesthesia 2 months before the patient's death; 3 g were used to prepare the mitochondrial fraction (17), and the remainder was used for histology and histochemistry (200 mg). Studies of oxidative flux through the respiratory chain were performed within 2 h of the mitochondrial preparation, using pyruvate and malate, succinate and oxoglutarate as substrates, and potassium ferricyanide as the electron acceptor (18). The mitochondrial cytochromes aa_3, *b*, and *c* were measured from the reduced minus oxidized spectra at the temperature of liquid nitrogen using the extinction coefficients given by Tervoort et al. (19). The remaining mitochondrial fraction was frozen at −85°C. Assays of the individual complexes of the respiratory chain were done after an interval of 1–6 weeks (20) and corrected for mitochondrial content by measurement of citrate synthase activity, an enzyme specific for mitochondria (21). The modified Lowry method was used for all measurements of protein concentration (22).

Molecular studies

Total DNA was extracted from muscle, brain, and pancreas using standard sodium dodecyl sulfate/proteinase K protocols.

Southern blot analysis. DNA (2.5 mg) was digested with *Pvu*II according to the manufacturer's protocol except that digestion was performed overnight at 37°C. The digested DNA was electrophoresed through an 0.8% agarose gel (30 V overnight) and then tranferred to a nylon membrane (GeneScreen, Du Pont-NEN, Boston, MA). The filter was probed with radioactively labeled whole mtDNA (23).

Polymerase chain reaction (PCR)/restriction digests and DNA sequencing. Point mutations and possible polymorphisms were identified either by PCR plus restriction endonuclease digest or by di-

rect sequencing. The 15904 C to T transition and 12308 A to G transition were screened for using the primers and restriction digests described by van den Ouweland et al. (24). Two mutations associated with Leber's hereditary optic neuropathy were studied: the 11778 G to A transition using Sfa*N1* digestion (25) and the 3460 A to G transition by *Afl*II restriction. DNA for sequencing was amplified by PCR using pairs of primers, one of which contained 5′-biotin. Single-strand DNA was generated using magnetic beads (DYNAL) and DNA-sequenced using a standard dideoxy protocol (Sequenase, United States Biochemicals, Cleveland, OH).

RESULTS— Full blood count, urea, and electrolytes were normal, as were fasting blood lactate (0.42 mM; normal, <1.7 mM) and pyruvate (0.04 mM; normal, <0.18 mM) concentrations. Cerebrospinal fluid protein was 0.22 g/l (normal, <0.5 g/l). A computed tomographic scan was normal at the onset of ataxia. Nerve conduction studies showed a mild axonal neuropathy in the lower limbs, and an electromyogram showed a slight excess of polyphasic motor unit potentials but no other myopathic changes.

Antemortem muscle biopsy

Histological examination was normal; in particular, no abnormalities in the succinate dehydrogenase and cytochrome oxidase preparations were seen, and no ragged red fibers or other morphological abnormalities of mitochondria were found. Electron microscopy showed no abnormality of mitochondrial structure.

Measurements of the activity of the individual complexes of the respiratory chain were normal (Table 1), as were studies of oxidative flux through the respiratory chain (Table 2) and cytochrome concentration (Table 3). Citrate synthase activity was 1.262 nmol 5,5′dithiobis (2-nitrobenzoic acid) reduced · min^{-1} · mg $protein^{-1}$ (control = 1.059, SD = 0.23, n = 30).

Table 1—*Activity of respiratory chain complexes in skeletal muscle mitochondria*

	Patient	Control ± 1 SD	*n*
Complex I	190	243 ± 59	20
Complex II	221	304 ± 87	20
Complex III	2.44	2.35 ± 0.4	18
Complex IV	5.943	7.18 ± 1.0	9

Results are expressed as nmol of NADH oxidized · min^{-1} · mg $protein^{-1}$ (complex I); of dichlorophenolindophenol reduced · min^{-1} · mg $protein^{-1}$ (complex II); or of 5,5′-dithiobis (2-nitro benzoic acid) reduced · min^{-1} · mg $protein^{-1}$ (citrate synthase) or apparent first-order rate constant (s^{-1} · mg $protein^{-1}$) (complexes III and IV). Citrate synthase activity = 1.262.

Autopsy

Macroscopic examination of the brain was reported to show optic nerve atrophy and a reduction in cerebellar volume. Material available for microscopic examination was limited to the lower midbrain, the optic tract and anterior commisure, part of the basal ganglia, hippocampus, and cerebellum. The optic tracts showed severe demyelination and gliosis. In the cerebellum, marked Purkinje cell depletion with neuronal loss from the granular cell layer and diffuse white matter gliosis with loss of central cerebellar myelin was seen. Other areas of the brain examined showed no abnormality.

Molecular studies

No rearrangement of mtDNA was seen in skeletal muscle or pancreas. The patient was homoplasmic for the 12308 A to G transition in $tRNA^{Leu(CUN)}$ in all the tissues studied. There was no change in mtDNA sequence at positions 15904, 11778, or 3460 using restriction analysis. DNA sequencing of $tRNA^{Leu(CUN)}$ and $tRNA^{Leu(UUR)}$ showed no abnormality other than the transition at 12308.

Table 2—*Substrate oxidations by skeletal muscle mitochondria*

	Patient	Control ± 1 SD	*n*
Pyruvate/malate	243	221.5 ± 54.8	36
Succinate	266	316.6 ± 88.3	38
Oxoglutarate	198	165.1 ± 46.1	36

Results are expressed as nmol of ferricyanide reduced · min^{-1} · mg $protein^{-1}$.

Table 3—*Concentration of cytochromes in skeletal muscle mitochondria*

	Patient	Control ± 1 SD	*n*
Cytochrome aa_3	0.206	0.207 ± 0.073	27
Cytochrome *b*	0.101	0.149 ± 0.047	26
Cytochrome *c*	0.153	0.267 ± 0.077	27

Dithionite reduced. Results are expressed as nmol cytochrome/mg protein.

CONCLUSIONS— Wolfram syndrome and DIDMOAD are terms often used synonymously to describe the association of diabetes mellitus with optic atrophy and a variety of other multisystem abnormalities, usually diabetes insipidus and sensorineural deafness. Wolfram's original description only commented on the relationship between diabetes mellitus and optic atrophy (26). Later the term DIDMOAD assumed more general (although often incorrect) use, sometimes being applied to patients who have only diabetes and optic atrophy. Although diabetes insipidus and deafness are the features most commonly associated with diabetes mellitus and optic atrophy, many other associated abnormalities have been reported, including ataxia, ptosis, pigmentary retinopathy, seizures, elevated cerebrospinal fluid protein, aminoaciduria, ophthalmoplegia, sideroblastic anemia, small stature, hypogonadism, abnormalities of the urinary tract, anosmia, cataract, and goiter (26–28). The prevalence of these other features in Wolfram syndrome is uncertain. In the large study by Cremers et al. (28), 13% of patients were reported to have nystagmus and 7% ataxia, but many of these cases were not fully detailed, and the figures probably underestimate the true prevalence. Central nervous system abnormalities may be asymptomatic as demonstrated by the au-

topsies of two young adult patients with Wolfram syndrome who had no symptoms of ataxia but showed pathological changes in the brain stem and cerebellum similar to those found in olivopontocerebellar atrophy (29). There are few reports of neuropathological findings in Wolfram syndrome, and so, possibly, similar changes may be present in other asymptomatic patients and hence are more common than previously recognized.

Our patient fulfilled all the established criteria for DIDMOAD syndrome: she developed diabetes mellitus early in life (a common feature in this disorder [1]) followed by optic atrophy, diabetes insipidus, anosmia, short stature, sensorineural deafness (detected by audiometry), and, ultimately, ataxia. In common with many patients with Wolfram syndrome, despite having IDDM for >30 years, she had no retinopathy or other complications of diabetes. One (and possibly two) of her three siblings also had Wolfram syndrome, although her parents were normal, which is compatible with the autosomal recessive inheritance proposed for this disease (30). At 30 years of age, the patient developed the first symptoms of dizziness and unsteadiness, which were initially associated with few neurological signs, but her condition deteriorated inexorably until, just before her death, she was severely distressed by dysarthria, dysphagia, and ataxia. Our patient did not have a full neuropathological examination, but the findings were entirely consistent with the abnormalities reported by Carson et al. (29) in the most detailed neuropathological study of Wolfram syndrome.

Diabetes mellitus is now known to be associated with mitochondrial disease. Abnormalities of mtDNA identified in patients with diabetes mellitus include rearrangements and point mutations. Poulton et al. (31) described two patients with Kearns-Sayre syndrome who developed diabetes mellitus early in their lives; both had duplications of mtDNA (31). Subsequently, Rötig et al. (32) described two children with renal dysfunction and diabetes mellitus who also had a duplication of mtDNA; an identical abnormality was found in their mother, who was only mildly affected by the disease. Maternally inherited diabetes and deafness associated with a large rearrangement of mtDNA has also been described (15). More recently, Rötig et al. (33) reported a large heteroplasmic deletion of mtDNA in association with early-onset IDDM, optic atrophy, short stature, retinal pigmentation, deafness, and a cerebellar syndrome. No rearrangement of mtDNA was identified in our patient by Southern blotting in either skeletal muscle or pancreas. We did not screen further using PCR because we felt that any significant pathogenic rearrangement should be detected by Southern blotting.

The association between point mutations of mtDNA and diabetes mellitus is also recognized. Van den Ouweland et al. (34) and, subsequently, Reardon et al. (35) described a family with maternally inherited diabetes mellitus and a mutation at position 3243 in $tRNA^{Leu(UUR)}$. This point mutation was first identified in association with the mitochondrial disease MELAS (mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes) (36). Interestingly, we found that two patients having this mutation who had neurological symptoms also had glucose intolerance. Single-base substitutions (at positions 12308 and 15904 on the mitochondrial genome) have also been found in patients with Wolfram syndrome (17).

Studies of mtDNA in our patient showed that she had the 12308 A to G transition in $tRNA^{Leu(CUN)}$. One of the difficulties in the study of mtDNA, however, is establishing whether an abnormality in the mitochondrial genome is pathogenic or simply polymorphic. mtDNA is highly polymorphic, fixing base changes at a rate estimated to be 10 times that of nuclear (chromosomal) DNA (37). When abnormalities of mt DNA are found, the following criteria are usually applied to determine whether the abnormality is pathogenic or merely an incidental polymorphism: the change should not be present in the general population; it should involve a conserved site and affect function; and, if involving a tRNA, it should be heteroplasmic. The 12308 transition has a population frequency reported as 0.16 (24), and our studies confirm this. Moreover, although little is known about the function of the different parts of the tRNA molecule, the high population frequency suggests that the base change does not critically affect function. Lastly, in both our patient and those reported earlier, the base change is homoplasmic, in our case in all three tissues studied. These features suggest that the 12308 A to G transition is a polymorphism. No other change in mtDNA was identified, although the whole mitochondrial genome was not sequenced. Because point mutations in protein-coding genes do not cause changes in mitochondrial morphology and do not always cause detectable biochemical abnormalities, we have not totally excluded this genome as the site of abnormality in this patient. Nevertheless, we think that our studies are most compatible with the previous suggestion that a chromosomal (nuclear) DNA abnormality inherited in an autosomal recessive fashion is the cause of this disorder.

In addition to abnormalities of mtDNA, disorders of mitochondrial function have recently been described in Wolfram syndrome. A case report by Bundey et al. (16) suggested that DIDMOAD is associated with minor morphological abnormalities of mitochondria on electron microscopy and an abnormality of glutamate dehydrogenase (a mitochondrial enzyme not part of the respiratory chain). The patient reported by Rötig et al. (33) as having a deletion of mtDNA in association with Wolfram syndrome (see above) was also found to have a generalized decrease in the activity of respiratory chain complexes. Our patient showed no such histological abnormalities either on light or electron microscopy; furthermore, we found no evidence of respiratory chain dysfunction on detailed biochemical

analysis. One possible explanation for the apparent discrepancy in these findings is that different, albeit phenotypically similar, diseases were being studied. The patient of Bundey et al. had only diabetes mellitus and optic atrophy and hence does not fulfill all the criteria for DIDMOAD. The patient of Rötig et al. had diabetes mellitus, optic atrophy, and deafness but did not have diabetes insipidus; she also fulfilled all the criteria for Kearns-Sayre syndrome, a multisystem disease characterized by progressive ophthalmoplegia and retinal pigmentation (3), which is known to be due to a rearrangement of mtDNA. Patients with Kearns-Sayre syndrome may develop diabetes early in life (31). Our patient had diabetes insipidus, diabetes mellitus, optic atrophy, deafness, and a cerebellar syndrome and is quite distinct clinically from the other two patients reported to have mitochondrial abnormalities associated with DIDMOAD. The relationships between different syndromes that include diabetes mellitus and optic atrophy and mitochondrial function will only be established by further detailed clinical, genetic, and metabolic studies of patients with such syndromes. The term DIDMOAD should not be loosely applied to any patient with diabetes mellitus and optic atrophy. Our studies in this patient, with all the diagnostic criteria for DIDMOAD, do not lend any support to the suggestion that the full syndrome of DIDMOAD is due to an abnormality of respiratory chain function.

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