

Short Communication

X-Linked cardioskeletal myopathy and neutropenia (Barth syndrome): Respiratory-chain abnormalities in cultured fibroblasts

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X-Linked cardioskeletal myopathy, neutropenia and abnormal mitochondria (McKusick 302060; 3-methylglutaconic aciduria type 2; Barth syndrome) represents a rare metabolic disorder with a distinct clinical phenotype, reported so far from Europe (Barth et al 1983), North-America (Gibson et al 1991; Kelley et al 1991) and Australia (Adès et al 1993; Christodoulou et al 1994). It has the following clinical and biochemical profile: dilated cardiomyopathy; neutropenia due to maturation arrest; neuromuscular weakness; absence of cerebral involvement; and diminished statural growth. Clinical biochemical features include variable lactic acidemia and lactic aciduria (Barth et al 1983); increased urinary excretion of 3-methylglutaconic acid, 3-methylglutaric acid and 2-ethylhydracrylic acid (Kelley et al 1991; Gibson et al 1991); and low serum cholesterol (Kelley et al 1991). Linkage analysis assigned the disease to distal Xq28 (Bolhuis et al 1991; Adès et al 1993; Christodoulou et al 1994). Previous studies on the respiratory chain in skeletal muscle showed impaired oxidative phosphorylation at the level of complexes III and IV (Barth et al 1983) and decreased activity of complex IV (Christodoulou et al 1994). Results of clinical experiments pertaining to the organic acid abnormality and the low serum cholesterol included absent effect of leucine loading on the excretion of 3-methylglutaconic acid (Kelley et al 1991; Christodoulou et al 1994); normal activities of enzymes in the leucine pathway (3-methylglutaconyl-CoA hydratase (EC 4.2.1.18), 3-hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4), 3-hydroxy-3-methylglutaryl-CoA reductase); and normal *in vitro* biosynthesis of [¹⁴C]cholesterol from [¹⁴C]acetate (Gibson et al 1991).

By ascertaining respiratory-chain function in cultured fibroblasts we expected to

establish whether respiratory-chain abnormalities extend to other tissues beyond striated muscle. Furthermore, we wanted to exclude the possibility that such abnormalities were associated with mutated or depleted mitochondrial DNA (mtDNA) by studying the *in vitro* biosynthesis of mitochondrially encoded subunits of the relevant complexes.

MATERIALS AND METHODS

Fibroblasts were obtained from two patients from different pedigrees. Patient 1 belonged to an earlier described family (Barth et al 1983), patient 2 to a recently discovered new family.

Mitochondrial ATP synthesis was measured in fibroblasts from exponentially growing cultures, permeabilized with low concentrations of digitonin ($1\ \mu\text{g}/10^6$ cells). ATP synthesis was initiated by the addition of ADP to a final concentration of 10 mmol/L. Glutamate (10 mmol/L)+malate (2 mmol/L) or pyruvate (10 mmol/L)+malate (2 mmol/L) were used as substrates for oxidative phosphorylation via complexes I, III and IV. Succinate (10 mmol/L) (+2 μg rotenone/ml to inhibit complex I) was used as a substrate for oxidative phosphorylation via complexes II, III and IV.

Mitochondrial protein synthesis: After removing the normal culture medium, fibroblasts from exponentially growing cultures were rinsed twice with PBS and cultured in methionine-free DME containing 1 mmol/L pyruvate and supplemented with 10% heat-inactivated fetal calf serum in a humidified atmosphere of 10% CO₂ in air. After 1 h [³⁵S]methionine (specific activity >960 Ci/mmol) was added to a final concentration of 20 $\mu\text{Ci}/\text{ml}$. Emetine labelling was used to suppress cytoplasmic protein synthesis. Emetine was added 10 min before the addition of labelled methionine, to a final concentration of 100 $\mu\text{g}/\text{ml}$. Equal amounts of labelled cells were solubilized by lauryl maltoside treatment. The samples were centrifuged and the supernatants were subjected to electrophoresis as described (Schägger and Von Jagow 1987). The gels were dried under vacuum and labelled mitochondrial proteins were visualized by fluorography. The autoradiographic signals of the proteins were quantified on gels using a Phospho Imager.

The activity of individual respiratory-chain enzymes was determined in freshly prepared cell lysates, obtained by treatment with lauryl maltoside, according to standard methods (Nijtmans et al 1995).

PATIENTS

Patient 1: This patient, described previously (Barth et al 1983) (pedigree no. V, 12), had neonatal lactic acidosis (up to 13.9 mmol/L). On follow-up he had moderate muscle weakness, neutropenia, and dilated cardiomyopathy. He died from cardiac failure at 15 months. Myocardial mitochondria were abnormal in structure.

Patient 2: This patient was not related to patient 1. His maternal uncle died in infancy with a similar history. The proband had failure to thrive (statural length on the 3rd centile, weight below the 3rd centile), dilated cardiomyopathy, and increased excretion of 3-methylglutaconic acid and 3-methylglutaric acid. He died, probably from cardiac failure, at 13 months. Dilated cardiomyopathy was found at autopsy.

Table 1 ATP synthesis^a

Substrate	Patient 1 (n=4)	Patient 2 (n=2)	Control (n=10)
Glutamate/malate	18.1 (83%)	19.5 (88%)	22.0 (18.4–28.3)
Pyruvate/malate	15.5 (75%)	17.6 (85%)	20.7 (16.4–26.4)
Succinate (+rotenone)	11.2 (70%)	14.6 (91%)	15.9 (12.9–18.6)

^aActivity expressed as nmol ATP/mg protein per min; % is percentage of normal mean

Table 2 Mitochondrial enzyme activities^a

Enzyme	Patient 1 (n)	Patient 2 (n)	Control mean (range)	(n)
Complex I	3.0 (4)	3.4 (2)	3.6 (3.0–4.1)	(11)
Complex II	7.9 (4)	6.7 (2)	7.6 (5.6–7.8)	(11)
Complex III	3.2 (4)	3.7 (4)	11.1 (10.2–14.9)	(9)
Complex IV ^b	0.31 (10)	0.42 (8)	0.58 (0.45–0.62)	(53)
Citrate synthase	87 (6)	75 (4)	78 (58–86)	(53)

^aActivities expressed as nmol/mg protein per min

^bComplex IV: k (per min per mg)

RESULTS

Electrophoresis of polypeptides, labelled in the presence of emetine, showed no abnormalities in the mobility or the incorporation of radioactivity of mitochondrially encoded proteins. This suggests that the expression of mtDNA was normal in the fibroblasts from the patients.

Results of respiratory-chain studies are given in Tables 1 and 2.

CONCLUSIONS

The ATP production from glutamate + malate, pyruvate + malate, or succinate (+rotenone) was lower than the mean values found in control fibroblasts in patient 1 and in the low-normal range in patient 2. Decreased activities of complex III ($\pm 30\%$ of controls) and complex IV ($\pm 60\%$ of controls) are in agreement with the previous studies in muscle. These findings provide evidence that respiratory-chain dysfunction is an essential component of Barth syndrome. Results further indicate that the gene product is essential for the function, formation or stability of both complex III and complex IV. Both complex III and complex IV contain subunits that are encoded by mtDNA. The results of emetine labelling make it unlikely that the gene is involved in the expression of mtDNA. We furthermore suspect that 3-methylglutaconic aciduria in this syndrome represents an epiphenomenon which is as yet unexplained but has been described so far in patients with separate mitochondrial disorders (Gibson et al 1995).

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