Short Communication

Mitochondrial DNA Depletion Syndrome is Expressed in Amniotic Fluid Cell Cultures

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Mitochondrial DNA depletion syndrome is an autosomal inherited disease associated with grossly reduced cellular levels of mitochondrial DNA in infancy. Most patients are born after a full and uncomplicated pregnancy, are normal at birth, but develop symptoms in the early neonatal period. These observations have led to the suggestion that the patients have a defect affecting the control of mitochondrial DNA copy number after birth. Using immunocytochemical techniques, we demonstrated that the disease is already expressed in amniotic fluid cells. Detection of mitochondrial DNA depletion in these fetal cells indicates that the defect may already be expressed early in embryological development. (*Am J Pathol* 1999, 155:67–70)

Mitochondrial DNA (mtDNA) depletion syndrome is a recently recognized disorder involving a quantitative defect of mtDNA. It presents in infancy with hepatorenal failure, myopathy, hypotonia, hypoglycemia, and lactic acidosis. Investigation demonstrates levels of mtDNA which may be less than 5% of control levels in the affected tissues of severe cases and less than 30% in more mildly affected patients. Infants with severe mtDNA depletion usually die within 12 months.^{1–6} Human mtDNA encodes 13 proteins, all of which are part of the oxidative phosphorylation system.⁷ The biochemical basis of organ failure in mtDNA depletion is therefore thought to be insufficient adenosine 5'-triphosphate (ATP) production for cellular requirements.

Although certain molecular events in the evolution of mtDNA depletion have been defined in cultured cells from patients,⁸ the primary molecular defect remains to be identified. A nuclear genomic etiology has been defined in two of our families by mtDNA transfer technology,^{8,9} indicating that the mtDNA depletion is secondary to a nuclear genetic defect. The fact that the affected children are born after uncomplicated pregnancies and are often normal at birth and during the early postnatal period^{1–6} has led to the suggestion that there may be a defect in a developmentally regulated switch controlling mtDNA copy number.¹ Here, we demonstrate mtDNA depletion in amniocytes from an infant who subsequently died from severe mtDNA depletion. These results suggest that the defect of mtDNA replication may be expressed early in embryological development.

Materials and Methods

Patient and Control Subjects

The female patient was the second child of healthy, unrelated Caucasian parents and was born at term after an uncomplicated pregnancy. She was well at birth but developed hypoglycemia during the first 24 hours. Later, she developed lactic acidemia (blood lactate 5.8 mmol/L) with progressive liver disease. Liver biopsy showed micronodular cirrhosis with proliferation of bile ducts and abundant neutral fat and bile pigment in hepatocytes. The activity of cytochrome-c oxidase was severely depleted in liver. Assay of phosphoenolpyruvate carboxykinase (PEPCK) in liver mitochondria revealed evidence of reduced mitochondrial PEPCK activity but PEPCK activity measured in amniotic fluid cells and fibroblasts was within normal limits. The patient died of progressive liver failure at the age of 6 months. Postmortem examination of

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skeletal muscle showed relatively uniform fibers with no evidence of ragged-red fibers with Gömöri trichrome staining. There was an excess of coarse lipid droplets in many muscle fibers. Cytochrome-c oxidase activity was normal and present in all fibers.

This patient's elder brother¹⁰ had been delivered at 42 weeks of gestation. He was well at birth but very soon developed symptoms compatible with liver failure. Blood lactate was variably increased (2.1–5.0 mmol/L). Assay of fibroblast PEPCK activity revealed marked deficiency. The child died, aged 7 weeks, of progressive liver failure.

Pediatric control skeletal muscle tissue was obtained, with informed parental consent, from children having orthopedic surgery, diagnostic biopsies, or autopsies, in whom no evidence of muscle or metabolic disease was found. Pediatric control liver specimens were derived from autopsies. Approval for study of this material was obtained from the ethical committees for Great Ormond Street Hospital and University College Hospital, both of London. Pediatric control fibroblasts were cultured from diagnostic skin biopsies of infants in whom no evidence of mitochondrial disease was found. Control amniotic fluid cell cultures were obtained from nine pregnancies with no family history of mitochondrial disease.

Southern Blot Analysis

Total cellular DNA was extracted from tissue samples by proteinase K digestion and subsequent phenol:chloroform extraction and ethanol precipitation.¹¹ For Southern blot analysis of cultured fibroblasts, cells were harvested by trypsinization and washed twice in phosphate-buffered saline (PBS). Total cellular DNA was extracted with the Nucleon I kit (Scotlab, Strathclyde, UK), followed by ethanol precipitation.

Similar amounts (~3 µg) of DNA were digested with the restriction enzyme *PvuII*, resolved on a 0.8% agarose gel by electrophoresis, denatured, and blotted onto Hybond-N membrane (Amersham, Buckinghamshire, UK) as recommended by the supplier. Blots were hybridized according to standard conditions¹¹ with two probes simultaneously: a cloned 5.8-kb *EcoRI* fragment of the gene encoding 18S rRNA¹² and the entire mtDNA obtained by long-range polymerase chain reaction.¹³ Probes were labeled with [α -³²P]dCTP using the Rediprime random prime labeling system (Amersham). Signals on the blot were quantified by volume integration and corrected for background noise on a Phosphor Imager using ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Cell Culturing Conditions

Fibroblasts and amniotic fluid cells were maintained at 37°C in a humidified atmosphere of 8% CO₂ in air. Fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM) that included 25 mmol/L glucose and 4 mmol/L l-glutamine, supplemented with 10% fetal bovine serum, 2 µmol/L uridine, 1 µmol/L sodium pyruvate, 50 U/ml penicillin, and 50 µg/ml streptomycin. Amniotic fluid

cells were grown in a mixture of Hams F10 and Chang's medium supplemented with 2 µmol/L uridine, 1 µmol/L sodium pyruvate. Both pyruvate and uridine are essential nutrients for cells deficient in mitochondrial respiratory chain function.¹⁴

Immunocytochemical Staining

For immunocytochemical studies, cells were trypsinized and seeded at moderate density on sterile glass coverslips. All cells were grown for a further 24 hours; the culture medium for amniotic fluid cell cultures was changed to DMEM supplemented with 20% fetal bovine serum and 0.2% chick embryo extract in addition to pyruvate and uridine for this period to reduce nonspecific background staining. To stain the mitochondria, cells were cultured for 45 minutes in the presence of 2 µmol/L MitoTracker CM-H₂XRos (Molecular Probes, Eugene, OR) followed by culturing for 30 minutes in medium without this fluorescent dye. Cells were subsequently washed in PBS, fixed with 4% paraformaldehyde in PBS for 20 minutes, washed, permeabilized in methanol at -20°C for 15 minutes, and washed again. Protein binding sites were saturated with 10% normal goat serum in PBS for 30 minutes at 37°C in a humidified atmosphere. Next, coverslips were incubated with monoclonal antibodies to the mtDNA-encoded subunit I of cytochrome-c oxidase¹⁵ (10 µg IgG of monoclonal 1D6-E1-A8 per ml of PBS) or to the nuclear-encoded flavin protein subunit of succinate dehydrogenase¹⁶ (2 µg IgG of monoclonal 2E3-GC12-FB2-AE2 per ml of PBS) for 45 minutes at 37°C in a humidified atmosphere (monoclonals were kindly provided by Dr. R. A. Capaldi). After primary antibody incubation, coverslips were washed and incubated with goat anti-mouse IgG-fluorescein isothiocyanate conjugate (Southern Biotechnology Associates, Birmingham, AL; 100-fold dilution in PBS) for 45 minutes at 37°C in a humidified atmosphere. Finally, coverslips were washed once more in PBS and mounted onto glass slides in Citifluor-glycerol-PBS solution (Agar Scientific Ltd., Stansted, UK) supplemented with 1 µg/ml of 4,6-diamidino-2-phenylindole to reveal the nuclei. Fluorescence was inspected with a Zeiss Axiophot photomicroscope equipped with a 40X Plan-Neofluar lens. Photographs were taken on Kodak Ektachrome EPL 400X film.

Results

Total genomic DNA was isolated from liver and skeletal muscle tissue of the patient and age-matched controls. DNA was restricted with the endonuclease *PvuII*, which linearizes the covalently closed circular mtDNA molecule by cutting at a unique site. A Southern blot of the digested DNA was cohybridized with a probe for the entire 16.6-kb mtDNA molecule and a probe for the multicopy nuclear gene for 18S rRNA to assess the amounts of DNA loaded in each lane. The experiment demonstrated severely depleted steady-state levels of mtDNA relative to the nuclear DNA signal in both liver and skeletal muscle tissue of the patient compared to the control specimens

Figure 1. Autoradiograph of Southern blot of total genomic DNA isolated from biopsied liver (P_L) and skeletal muscle (P_M) specimens of the patient and age-matched controls. The blot was hybridized simultaneously with ^{32}P -labeled probes for mtDNA and the nuclear gene for 18S rRNA (nDNA).

(Figure 1). Quantification of the signals by phosphorimaging revealed that mtDNA levels in the patient's liver were 7% of mean control values, whereas mtDNA levels in the patient's skeletal muscle were 18% of mean control values. Cultured skin fibroblasts of the infant also exhibited a marked decrease in mtDNA levels (not shown).

Expression of the mtDNA-encoded subunit I of cytochrome-c oxidase was investigated in both cultured fibroblasts and amniotic fluid cells of the patient and compared to control cultures by immunocytochemistry. Cells were cultured in the presence of MitoTracker CM- H_2XROS to label the mitochondria fluorescent red. After fixation, cells were immunostained fluorescent green for cytochrome-c oxidase subunit I, followed by a fluorescent blue staining of nuclear DNA. In these experiments, all fibroblasts of the patient contained mitochondria (red) but subunit I of cytochrome-c oxidase (green) was only expressed in 30% of the cells (red + green = yellow) (Figure 2A). Also, patient amniotic fluid cells negative for subunit I of cytochrome-c oxidase were readily and consistently identified in all preparations (Figure 2B). Immunostaining for the flavin protein subunit of succinate dehydrogenase showed, however, that this nuclear-encoded polypeptide was present in mitochondria of all cultured cells of the patient (not shown). Control fibroblasts (not shown) and control amniotic fluid cell cultures ($n = 9$) showed a uniform expression of cytochrome-c oxidase subunit I in the mitochondria of all cells (Figure 2C).

Discussion

To date, we have reported seven neonates in five pedigrees with mtDNA depletion syndrome.^{5,8,9} These children were all born following normal pregnancies and died before the age of 2 years with features of mtDNA depletion. The diagnosis was confirmed by demonstrating low levels (1–25% of age-matched controls) of mtDNA in liver, skeletal muscle, and/or cultured skin fibroblasts or myoblasts. We report here a new patient with grossly reduced levels of mtDNA in liver and skeletal muscle tissue (Figure 1). The elder brother of the patient died in infancy with acute liver failure originally ascribed to PEPCK deficiency.¹⁰ PEPCK activity in the amniotic fluid cell culture of the patient was normal and the pregnancy was allowed to proceed. Following delivery, this

Figure 2. Patient's fibroblasts (A) and amniotic fluid cells (B) and control amniotic fluid cells (C) triple-stained for mitochondria (red fluorescence), subunit I of cytochrome-c oxidase (green fluorescence), and nuclear DNA (blue fluorescence).

second child also developed liver failure and died at 9 months.

Both fibroblasts and amniotic fluid cells of the second child were studied with immunocytochemical techniques. These experiments showed that all cultured cells contained mitochondria and all mitochondria contained the nuclear-encoded flavin protein subunit of succinate dehydrogenase, but the mitochondrially encoded subunit I of cytochrome-c oxidase was present only in a subset of the patient fibroblasts and amniotic fluid cells (Figure 2). These results demonstrate that despite the apparently normal fetal development during pregnancy, fetal amniotic fluid cell cultures expressed mtDNA depletion.

It is interesting that such early embryological cells as amniocytes express mtDNA depletion. It is possible that mtDNA depletion in the amniocytes developed during culture. Cultured cells in general express fetal isoforms of proteins and so the expression of mtDNA depletion in this patient's amniocytes and fibroblasts, and in the fibroblasts and myoblasts of other patients,^{8,9} suggests that failure of a developmental switch is unlikely to explain presentation after birth. Analysis of fresh amniotic fluid cells for mtDNA depletion may provide a further clue to the timing of onset of mtDNA depletion during fetal development. The limited quantity of tissue available will require such analysis to be undertaken by immunocytochemical techniques similar to those described here.

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