Published in final edited form as:

J Neurol Sci. 2008 December 15; 275(1-2): 128–132. doi:10.1016/j.jns.2008.08.028.

Neonatal mitochondrial encephaloneuromyopathy due to a defect of mitochondrial protein synthesis

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Abstract

Mitochondrial diseases are clinically and genetically heterogeneous disorders due to primary mutations in mitochondrial DNA (mtDNA) or nuclear DNA (nDNA). We studied a male infant with severe congenital encephalopathy, peripheral neuropathy, and myopathy. The patient's lactic acidosis and biochemical defects of respiratory chain complexes I, III, and IV in muscle indicated that he had a mitochondrial disorder while parental consanguinity suggested autosomal recessive inheritance. Cultured fibroblasts from the patient showed a generalized defect of mitochondrial protein synthesis. Fusion of cells from the patient with 143B206 p⁰ cells devoid of mtDNA restored cytochrome oxidase activity confirming the nDNA origin of the disease. Our studies indicate that the patient has a novel autosomal recessive defect of mitochondrial protein synthesis.

Keywords

mitochondria; protein synthesis; autosomal recessive; mitochondrial disease; respiratory chain

1- Introduction

Mitochondrial disorders are highly heterogeneous diseases due to mitochondrial DNA (mtDNA) or nuclear DNA (nDNA) mutations that cause defects of oxidative phosphorylation. These disorders are generally multi-systemic and affect highly aerobic post-mitotic tissues, such as muscle and nerve.

MtDNA depends on nuclear genes for its biogenesis and maintenance. In fact, replication, transcription, and translation of mtDNA are entirely regulated by nDNA-encoded genes. Disruptions of these mitochondrial functions generally cause deficiencies of respiratory chain complexes I, III, IV, and V, all of which contain one or more subunits encoded by mtDNA,

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and have been classified as defects of intergenomic communication [1]. A growing number of autosomal recessive defects of mitochondrial protein synthesis have been identified [2,3] often as a consequence of consanguinity [4]. Here, we describe the clinical and biochemical features of an infant with a novel neonatal encephalomyopathy caused by a novel defect in mitochondrial protein synthesis.

2- Material and Methods

2.1 Cell lines and cultures

Experiments were performed with primary skin cell fibroblasts from the patient and controls. Cells were cultured in glucose Dulbecco's modified Eagle minimum medium supplemented with 15% fetal bovine serum, 1.2mM vitamin solution (D-Ca pantothenate 100 mg/L; choline 100mg/L; folic acid 100mg/L; inositol 200mg/L; nicotinamide 100mg/L; pyridoxal HCl 100mg/L; riboflavin 10mg/L; and thiamine-HCl 100mg/L), 0.6mM essential amino acids, 1.2mM non-essential amino acids, 2mM L-glutamine, 1.2mM sodium pyruvate, and 4.5µg/ml penicillin/streptomycin at 37 °C in 5% CO₂. The 143B206 ρ⁰ cell line was cultured in high-glucose Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum and 50µg/ml uridine [5]. All reagents were obtained from Invitrogen (Carlsbad, CA).

2.2 Cell Fusion

To confirm the nuclear origin of the disease, fibroblasts isolated from the patient were fused to the human osteosarcoma mtDNA-less derivative, 143B206 ρ⁰ cells, as described [6]. After fusion, hybrid cells were cultured for 48h in a glucose medium supplemented with 50µg/ml uridine. After this period, oxidative phosphorylation capacity was selected in a medium containing 5.5mM galactose, supplemented with 10% dialyzed serum and without supplemental uridine and pyruvate.

2.3 Biochemical analysis

Approximately 1×10⁸ cells cultured skin fibroblasts from the patient and controls were harvested by trypsinization, washed, and lysed in specific buffer solutions according to the biochemical assay for each mitochondrial complex. Respiratory chain enzyme activities for complexes I, III and IV were determined spectrophotometrically, as described [7,8] using citrate synthase activity as a marker of mitochondrial mass.

2.4 Histochemistry analysis

Fibroblasts cell lines and hybrid clones were stained histochemically for cytochrome *c* oxidase and succinate dehydrogenase as described [9].

2.5 Mitochondrial protein analysis

Total protein extraction from cellular homogenate—Fibroblasts cells from the patient and from one normal child control were grown in a 100mm Petri dish to 90% confluence, harvested by trypsinization and washed in 1×PBS pH 7.4. Mitoplasts, prepared by treatment of fibroblasts with 4mg/ml of digitonin [10], were solubilized with 1% lauryl maltoside.

2.6- Blue-native polyacrylamide gel, electrophoresis and immunoblotting

Blue-native polyacrylamide gel electrophoresis system (BN-PAGE) was used for separation of mitochondrial respiratory complexes on 5-13% polyacrylamide-gradient gels. 25µg of the total solubilized protein were electrophoresed for 20 minutes at 75V and 4 °C, followed by approximately 15 additional hours in a cathode buffer containing 50mM tricine, 150mM Bis-Tris, 0.02% coomassie G 250, pH 7.0 inside of the gel, and anode buffer containing 50mM Bis-Tris, pH 7.0, outside of the gel at 70V and 4 °C [11]. Subsequently, the cathode buffer was

changed to one without coomassie and electrophoresis was continued for 4 hours at 150V and 4°C. Proteins were transferred to nitrocellulose membrane for 14 hours at 4 °C. We used monoclonal antibodies for complex I (39kDa subunit), complex II (70kDa subunit), complex III (core 2 subunit), complex IV (COX II subunit) and complex V (α F₁F₀-ATPase) diluted 1:1000 (Molecular Probes, Invitrogen). The secondary antibody was monoclonal anti-mouse IgG horseradish (Amersham Biosciences) diluted 1:1000. ECL-Plus Western Blotting Detection Reagents (Amersham Biosciences) were used to detect the proteins according to the manufacturer's instructions, followed by X-ray exposure.

2.7 - Pulse labeling of mitochondrial translation products

Pulse labeling of mitochondrial translation in fibroblast cultures from the patient and controls was performed as described [12], with the following modifications: 1×10^5 cells were washed in methionine-free Dulbecco's essential medium and subsequently incubated in the same medium supplemented with 15% dialyzed fetal bovine serum, 1.2mM sodium pyruvate, and either glucose for 30 minutes or galactose for 1 hour. Cytosolic protein synthesis was inhibited by addition of 0.1 μ g/ μ l of emetine for 7 minutes at 37°C. The mitochondrial proteins were labeled with 50 μ Ci [³⁵S]-methionine –Redivue (Amersham Biosciences) in methionine-free medium, and incubated for 30 minutes and 1 hour at 37°C. After treatments, cells were collected by centrifugation and the reactions stopped by the addition of 75 μ l of Rödel mixture [13] and 500 μ l of 20mM methionine. Cellular proteins were precipitated by addition of 50% trichloroacetic acid, washed with water and dissolved in 20 μ l of 1X Laemmli buffer [14]. Samples were electrophoresed in a 12.5% polyacrylamide gel containing glycerol and urea [14] for 24 hours at constant 85V. Proteins were transferred to a nitrocellulose membrane and stained with 0.2% Ponceau solution to check the presence of proteins. The membrane was dried for 15 minutes at 80°C and exposed to an X-ray film.

2.8 Mitochondrial transcripts analysis

Approximately 1×10^8 cells cultured from skin fibroblasts of patient and controls were harvested by trypsinization and pellets washed with 1 $\times$ PBS, pH 7.4. Total cellular RNA was extracted from the pellets (Totally RNA isolation, Invitrogen) and quantitated by spectrophotometric absorbance at 260nm.

2.9 - RT-PCR

2 μ g of total RNA after treatment with DNase for 10 minutes at 25°C were reverse transcribed as described by the manufacturer's protocol (Super Script One Step, Invitrogen). In the final reaction, cDNA was treated with RNase H for 20 minutes at 37°C. Mitochondrial gene transcripts *12S rRNA*, *ND2*, *CYTB* and the cytosolic β -actin gene were analyzed by serial dilutions of the initial cDNA product. Initially, 1 μ l of cDNA from each sample was used in the first PCR reaction. The second and third reactions used 1:10 and 1:100 cDNA dilutions for the amplification of the genes. Nuclear controls primers in the intronic region of the *NDUFV1* and *NDUFS4* genes were used to confirm that the PCR products were not derived from amplification of genomic DNA. The PCR conditions were the following: 94°C for 3 minutes; followed by 35 cycles of 94°C $\times$ 30 seconds, 57°C $\times$ 45 seconds, 72°C $\times$ 1 minute; and one cycle of 72°C for 10 minutes. The PCR products were analyzed in a 1% agarose gel and stained with ethidium bromide.

2.10 - Northern Blot

Thirty μ g of total RNA from patient and control samples derived from cultured skin fibroblasts were prepared as described elsewhere [15], and submitted to electrophoresis in a 1% agarose gel prepared in an RNase-free water containing 0.02 M Mops, 8mM sodium acetate, 1 mM EDTA pH 7.0 and 0.22M formaldehyde for approximately 1 hour at 80V. The RNA was

transferred to a nitrocellulose membrane (Hybond+, Amersham Biosciences, Piscataway, NJ) by capillary action for 14 hours and then UV crosslinked to the membrane. Mitochondria probes for *12S rRNA*, *ND2*, *CYTB* and for the cytosolic β -*actin* genes were amplified by PCR using a control DNA, and the following oligonucleotide primers: *12SrRNA* forward 5'-AGGTTTGGTCCTAGCCTTTC, reverse 5'-CTTTGAATTCCCAGCTTCCA; *ND2* forward 5'-ACACTCATCACAGCGCTAAG, reverse 5'-GCGTAAGGATGATGAGTTGA; *CYT b* forward 5'-TGATGAAACTTCGGCTCACT, reverse 5'-TGTTTGATCCTCCGCAGGAA; and β -*ACTIN* forward 5'-AACACCCCAGCCATGTACGT, reverse 5'-AGAAGTCGGAAGGAAGGAC. The PCR products were purified (GENECLEAN II, BIO 101 Systems, MP Biomedicals, Irvine, CA). Probes were labeled as described in the protocol of the Gene Image™ Random Prime Labeling Module kit (Amersham Biosciences) and hybridized to the membrane at 63°C for 14 hours. The subsequent steps were performed as described by the manufacturer's protocol for Gene Images™ for Southern and northern blots (Amersham Biosciences).

2.11- DNA sequencing

All of the exons and flanking intronic sequences were sequenced for the following genes: *TSM*, *MTIF*, *MRPS16*, and *MRPS22* using BigDye 3.1 with an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City CA).

2.12- Microsatellite analysis

Fluorescently labeled microsatellite markers flanking *EFG1* (D3S1569 and D3S1565) and *PUS1* (D12S86 and D12S1638) were PCR amplified and analyzed with an ABI-310 Genetic Analyzer (Applied Biosystems).

3- Case Report

This 18 day-old male infant of consanguineous Saudi Arabian parents presented with severe neonatal encephalopathy. At birth, the infant was unresponsive, but was successfully resuscitated and intubated. He had little spontaneous limb movement and required mechanical ventilation. Examination revealed prominent tongue fasciculations, bilateral equinus deformities of the feet, and profound hypotonia of arms and legs. Tendon reflexes were absent. On the third day of life, he developed myoclonic jerks. Family history was notable for an older sister with congenital arthrogryposis, short neck, breathing problems, breech presentation, and hypoventilation requiring intubation and mechanical ventilation.

The proband's venous lactate was elevated, 3.2mM (normal 0.5-2.2), on two measurements but both measurements of venous pyruvate were normal. Cerebrospinal fluid (CSF) showed disproportionately elevated lactate (5.9 mM; normal 0.5-2.2) relative to pyruvate (0.2 mM; normal 0.06-0.13). CSF protein was elevated (166 mg/dl; normal 15-45). EEG was severely abnormal with diffuse signal attenuation, low amplitude, and slow activity consistent with post-anoxic encephalopathy. MRI of the brain revealed signs of immaturity with prominence of the cortical sulci, possible pachygyria of the anterior frontal and temporal lobes, and hypomyelination.

Muscle biopsy revealed diffuse cytochrome *c* oxidase (COX) deficiency. Biochemical measurements of mitochondrial enzymes in muscle demonstrated severe COX deficiency (0.47μM/min/gram; normal 2.80±0.52), with low normal activities of succinate cytochrome *c* reductase (complexes II+III; 0.48 μM/min/gram; normal 0.7±0.23), NADH cytochrome *c* reductase (complexes I+III; 0.55 μM/min/gram; normal 1.02±0.38), and succinate dehydrogenase (SDH or complex II; 0.76 μM/min/gram; normal 1.0±0.53). Sural nerve biopsy revealed sparse myelinated nerve fibers, indicating severe loss of fibers and endoneurial fibrosis

consistent with a peripheral neuropathy. Based on these findings, the initial diagnosis was profound neonatal encephalomyopathy with COX deficiency, probably due to an autosomal recessive mutation.

4- Results

4.1- Characterization of the molecular defect and etiology of the disease

Histochemical staining of the patient's fibroblasts revealed severe uniform reduction of COX activity, while SDH activity was normal compared to control cells, indicating that the COX deficiency was probably due an nDNA defect (Fig 1A). Biochemical analysis of the patient's fibroblasts showed marked reduction of complex IV activity (0.106 nmol/min/mg protein) corresponding to 11% of activity in normal controls while complex I activity (4.8 nmol/min/mg protein) was 68% and complex III activity was 149% of normal control means.

To confirm that the defect was of nuclear origin, patient's fibroblasts were fused with human ρ^0 cells, which are devoid of mtDNA but contain a uniform nuclear genome. The COX activity increased to 92 nmol/min/mg protein, an 87% increase over the COX activity in the patient's fibroblasts before fusion. Histochemical analysis confirmed COX restoration. These two set of data indicated that the nuclear genome from human ρ^0 cells complemented the nuclear defect of patient's fibroblasts and confirmed that the oxidative phosphorylation deficiency was due to a recessive nuclear allele (Fig 1B). Genomic DNA sequences of *TSM*, *MTIF*, *MRPS16*, and *MRPS22* were normal. Homozygosity mapping using microsatellite markers excluded defects of *EFG1* and *PUS1*.

4.2-Mitochondrial protein analysis

Immunoblots using a non-denaturing polyacrylamide gel, demonstrated that the patient's fibroblasts contain reduced steady-state levels of fully assembled complexes I, IV and V, normal levels of complex II, and a distinct increase of complex III (Fig 2). Because the combined defects of mitochondrial complexes suggested an impairment of mitochondrial protein synthesis, we performed pulse labeling experiments to assess mitochondrial translation, using fibroblasts of the patient in the presence of [35 S]-methionine. These showed a generalized decrease of all mitochondrial protein products compared to control fibroblasts (Fig 3). To see if these findings might reflect impaired mtRNA transcription or processing, we determined the level of mitochondrial transcripts in total RNA from fibroblasts using labeled polymerase chain reaction products of the *12SrRNA*, *ND2*, *CYTb* and β -actin gene as probes. RT-PCR and Northern blotting analysis showed normal levels of all analyzed transcripts in the patient's fibroblasts (Fig 4, 5).

5- Discussion

A growing number of patients show multiple respiratory chain enzyme deficiencies, but lack mitochondrial genome mutations and some of these patients have evidence of autosomal inheritance. Obvious "suspects" for these disorders are nDNA-encoded components of the mitochondrial protein-synthesis machinery, which is required for expression of the mtDNA-encoded subunits of mitochondrial complexes I, III, IV, and V [4].

Approximately 150 nDNA genes encode proteins that participate in mitochondrial translation [4,16], and mutations have already been identified in six genes: *EFG1*, *MRPS16*, *MRPS22*, *PUS1*, *TSM*, and *TUFM* [2,3,17-20]. *EFG1*, *TUFM*, and *TSM* encode three of the four elongation factors required for mitochondrial protein synthesis in humans. A homozygous missense N174S mutation in *EFG1* was identified initially in two siblings, who were born of consanguineous parents and died in infancy of severe hepatocerebral syndrome. Partial defects

of mitochondrial respiratory chain complexes I, III, IV, and V with normal complex II were observed in these patients. Compound heterozygous *EFG1* mutations were then identified in two children with similar hepatocerebral syndromes and in a child with early-onset Leigh syndrome [18,19]. Mutations in two genes, *MRPS16* and *MRPS22*, encoding polypeptide components of the mitochondrial small ribosomal subunit caused devastatingly severe fatal neonatal encephalopathy and antenatal mitochondrial disease [3,21]. In contrast, mutations in *PUS1* encoding pseudouridine synthase, an enzyme required for post-transcriptional modification of transfer RNAs, been associated with myopathy, lactic acidosis, and sideroblastic anemia (MLASA) [17,22]. Curiously, the same homozygous *TSFM* mutation (R333W) has been identified in two patients who presented with different phenotypes: one with encephalomyopathy and the other with hypertrophic cardiomyopathy [18]. Finally, a homozygous *TUFM* mutation (R339Q) was reported in a patient with severe infantile macrocystic leukodystrophy [19].

Our patient, born of consanguineous parents, presented with a neonatal encephalomyopathy, lactic acidosis, and his muscle biopsy revealed severe COX deficiency with low normal activities of complexes I+III and II+III, suggesting an autosomal defect of intergenomic communication. In support of this concept, fibroblasts from the patient showed: (i) generalized and severe defect in mitochondrial protein synthesis; (ii) reductions in the steady-state levels of complexes I, IV and V; (iii) normal complex II and a distinct increase of complex III. Assays of mitochondrial protein synthesis in cultured fibroblasts showed a generalized reduction of all mitochondrial-translated products, but the translation defect cannot be ascribed to abnormal transcription or RNA processing because reverse-transcriptase PCR and northern blot assays for three mtDNA-encoded genes showed normal transcriptional levels. Based on these findings we excluded autosomal recessive defects in five nDNA-encoded candidate genes required for mitochondrial translations by DNA sequencing or homozygosity mapping.

Curiously, despite reduced synthesis of all mitochondrial-translated polypeptides including cytochrome *b*, the patient's fibroblasts, but not muscle, showed increased activity of complex III and decreased complex I activity. This abnormal biochemical profile correlated with the results of BN-PAGE protein analysis revealing normal to increased levels of assembled complex III and very low levels of complexes I, IV and V. Similarly, in fibroblasts of patients with mutations in *TSFM*, BN-PAGE demonstrated that complex III was less affected than other respiratory chain complexes [18], while fibroblasts of patients with *PUS1* or *TUFM* mutations showed normal complex III activity [19,22]. These findings suggest that complex III is a stable enzyme relative to the other mitochondrial respiratory chain components. Furthermore, Rana and colleagues reported a *CYTB* 4-base-pair deletion that was pathogenic but did not cause reductions in complex III activity or assembly indicating that this enzyme complex may be stable despite defects of cytochrome *b* [23]. Alternatively, the increased activity and amount of complex III in the patient's fibroblasts could be related to decreased assembly or active degradation of other respiratory chain complexes in the setting of defective mitochondrial translation. Further elucidation of the mechanism responsible for increased complex III is necessary to understand the cell-specificity of this phenomenon, which was not observed in the patient's muscle.

In summary, in an infant with a severe neonatal encephalomyopathy and consanguineous parents, we have observed defects of mitochondrial respiratory chain complexes with mtDNA-encoded subunits in the patient's muscle and cultured skin fibroblasts. Although levels of mtDNA transcripts were normal in fibroblasts, we observed a generalized decrease of mitochondrial protein synthesis. Restoration of COX activity by fusing the patient's fibroblasts with cells lacking mtDNA indicates that the patient's disease is due to a defect of nDNA;

however, screening for mutations has not yet revealed the etiology. Taken together, our results indicate that this patient has a novel autosomal recessive disorder of mitochondrial translation.

Acknowledgements

This research was supported by National Institutes of Health grants NS11766 and HD32062 (M.H), FAPESP (CFB, CHT and MHB), CNPq (CHT), and Marriott Mitochondrial Disorders Clinical Research Fund (MMDCRF)(CFB, CK, SD, MH). We are in debt to Dr. Alexander Tzagoloff (Columbia University) for the *in vivo* mitochondrial translation labeling experiment.

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Figure 1.

Histochemistry analysis for COX and SDH in patient's fibroblasts before and after fusion with human 143B206 ρ^0 cells. Panel A: The patient's fibroblasts show COX deficiency and normal SDH activity. Panel B: After fusion with human ρ^0 cells, the patient's fibroblasts had normal COX activity. Panel C: control fibroblasts stained for COX and SDH show normal activity.

Figure 2.

Analysis of the assembly status of all OXPHOS complex in patient's fibroblasts. Fibroblasts from the patient and a control were analyzed with the use of blue-native polyacrylamide gel electrophoresis in the first dimension. The gel were immunoblotted with monoclonal antibodies against complex I (CI- 39 kDa), II (CII-70kDa), III (CIII- core 2), IV (CIV-COXII) and V (CV- α F1F0-ATPase) to assess the amount of fully assembled oxidative phosphorylation complexes. The samples are control (C) and patient (P) and the respective mitochondrial complexes are indicated.

Figure 3.

Analysis of mitochondrial translation products. Mitochondrial translation products in fibroblasts from the patient (P) and control (C) were submitted to electrophoresis in 12.5% polyacrylamide gel. To assess the rate of mitochondrial translation, cells were labeled with [³⁵S]methionine in the presence of an inhibitor of cytoplasmic protein synthesis (emetine) for 30 minutes in glucose-containing medium or 1 hour in a glucose-free medium with galactose. The patient's fibroblasts showed a severe defect in mitochondrial translation products: COXI, COXII and COXIII subunits I, II and III of complex IV; *ND1*, *ND2*, *ND3*, *ND4*, *ND4L*, *ND5* and *ND6* subunits of complex I; *ATP6* and *ATP8* subunits of complex V; and *CYTb*, apocytochrome *b* subunit of complex III are indicated. The molecular weight are presented in kDa.

Figure 4.

Mitochondrial transcripts analysis by semi-quantitative reverse transcriptase PCR (RT-PCR). Two µg of total RNA were used to synthesize cDNA from patient (lane 1), child control (lane 2), and adult control (lane 3). A. PCR for mitochondrial genes *ND2*, *CYT B*, 12S *rRNA* and *β-actin*. B. PCR using cDNA diluted 1:10. C. PCR using cDNAs diluted 1:100. D. PCR using control primers (*NDUFV1* and *NDUFS4*) annealing to intronic regions of complex I ND genes. Patient (lanes 1 and 6), child control (lanes 2 and 7), adult control (lanes 3 and 8), control DNA (lanes 4 and 9) and adult control (lanes 5 and 10). The patient's fibroblasts showed normal levels of mitochondrial transcripts for all analyzed genes.

Figure 5.

Northern Blotting analysis for mitochondrial transcription products. Total RNA was extracted from fibroblasts of the patient (P) and one child control (CC) by Trizol method. After electrophoresis and transfer, the hybridization was done separately with probes labeled with chemiluminescence for mitochondrial genes *ND2*, *CYT B* and *12SrRNA*. We observed normal levels of mitochondrial transcripts for all the analyzed genes.