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EVIDENCE FOR NUCLEAR MODIFIER GENE IN MITOCHONDRIAL CARDIOMYOPATHY

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Abstract

Mitochondrial DNA (mtDNA) inheritance and maintenance and function of the respiratory chain are the result of a synergistic action of the nuclear and the mitochondrial genomes. Mutations in either or both genomes can result in a wide range of multisystemic disorders. We have studied a homoplasmic mtDNA mutation in the tRNA^{Ile} gene that segregates exclusively with cardiomyopathy in two unrelated families. Cytochrome *c* oxidase (COX) deficiency was selectively observed only in the heart tissue and in patient's cardiomyocyte cultures and not in any other cell type, indicating that the defect is tissue specific. To understand the pathogenic mechanism of cardiomyopathy associated with a homoplasmic, tissue specific mtDNA mutation, we constructed transnuclear cardiomyocyte cell lines with normal or patient's nucleus and containing wild type or mutant mtDNA. Of the four cell lines analyzed, COX activity was low only in patient's cardiomyocytes illustrating that both the patient's nucleus and mitochondria are essential for expression of the phenotype. In cells with either wild type nucleus or wild type mtDNA, COX activity was normal. From these results it is evident that a tissue specific nuclear modifier gene may interact synergistically with the mtDNA mutation to cause COX deficiency.

Keywords

mitochondrial; cardiomyopathy; mtDNA; nuclear modifier; intergenomic interaction; cytochrome *c* oxidase; transnuclear cardiomyocyte culture; tissue specific; homoplasmic

Introduction

Mitochondrial diseases may be caused by a) primary defects of mtDNA, such as point mutations, large-scale deletions and rearrangements, b) defects of nuclear DNA-encoded proteins of the respiratory chain, c) defects of inter-genomic signaling, such as mutations in nuclear genes resulting in alterations of mtDNA [1,2] and d), due to a synergistic action of a mtDNA mutation with a nuclear modifier gene resulting in variable clinical phenotypes. The latter group includes mtDNA mutations in the ND genes (11778, 14484, 3460) associated with Leber's hereditary optic neuropathy (LHON), mutations in the 12S rRNA (A1555G) and two mutations, A7455G and T7511C in the tRNA^{Ser(UCN)} associated with sensorineural hearing loss (SNHL) caused by abnormalities of the hair cells in the cochlea, and the A4300G mutation

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in the tRNA^{Ile} gene associated with maternally inherited cardiomyopathy (MICM) associated with mutations in the mtDNA, [3-8]. The characteristics of this group of disease are: maternal inheritance, tissue specificity, variable penetrance, homoplasmic levels of the mtDNA mutation, and the requirement of a nuclear modifier gene in the expression of the clinical phenotype. Since mtDNA inheritance, replication, maintenance, and function are controlled by the nuclear DNA, a breakdown in the integrity of the cross talk between these two physically distinct genomes will lead to disruption in normal mitochondrial function. The large variability in clinical phenotype observed in pedigrees and the presence of tissue specific homoplasmic mtDNA mutations indicate that modifier genes which contain polymorphisms, may modulate the phenotypic expression of the disease. Modifier genes are neither necessary nor sufficient to cause the disease, but they affect the severity of the phenotype. This is evident in patients with hypertrophic cardiomyopathy, in whom a significant range of clinical manifestations of the cardiac hypertrophic response is observed [5]. The observed final phenotype may be a result of the interaction between the mtDNA mutation and the modifier genes, such as mutations associated with hearing loss and the Leber's optic atrophy [5,9-13]

We studied two unrelated families with the A4300G mutation in the mitochondrial tRNA^{Ile} gene, which is a known hotspot for mutations associated with cardiomyopathy [4,8]. In both families, the clinical features and associated abnormalities were found exclusively in the heart. Biochemical analysis showed severely decreased Complex I and IV, comprised of both nuclear and mitochondrial subunits in heart mitochondria, with normal Complex II activity, which is exclusively nuclear encoded. The activities of the respiratory chain complexes were normal in skeletal muscle, cultured skin fibroblasts and myoblasts. Very low steady-state levels of the tRNA^{Ile} were observed in cardiac tissue on high resolution Northern blots. Maternal inheritance and respiratory chain defects strongly support a pathogenic role of the homoplasmic A4300G mtDNA mutation. Cybrid analysis is usually employed to determine pathogenicity of mtDNA mutations. By this technique, the mitochondria harboring the mtDNA mutation in question is transferred to a mtDNA-less (ρ^0) cell line with a neutral nuclear background. Analysis of cybrids harboring the A4300G mutation in the osteosarcoma nuclear background had normal respiratory chain function. This result gave a clue to the presence of a nuclear modifier gene, perhaps tissue-specific, that may act synergistically with the mtDNA mutation in the pathogenesis of cardiomyopathy.

If the affected tissues, such as the cochlear cells in sensory neural hearing loss (SNHL) associated with the A1555G mutation, or cardiac tissue from patients with maternally inherited cardiomyopathy (MICM) harboring the A4300G mutation, or optic nerve from LHON patients were readily accessible, analysis of the pathogenesis of homoplasmic tissue-specific mutations may be feasible. Analysis of patient's cardiomyocytes (CM) would be the most appropriate cellular model to study the intergenomic interaction. For this purpose, we developed a method to immortalize post-mitotic primary human CM cells that have exited the cell cycle [14]. Using this technique we generated proliferating cardiomyocyte cells from transplanted heart tissue from a patient with the A4300G mutation and control heart tissue with no mutation. We analyzed respiratory chain function in CM cultures from the patient and control, as well as in transnuclear cardiomyocytes with combinations of nucleus and mitochondria from patient and control cardiomyocytes generated in our laboratory. Cytochrome *c* oxidase (COX) activity was normal in all cell lines analyzed, except in the patient cardiomyocytes, indicating that the phenotype is expressed only in the presence of both the nucleus and mitochondria from the patient. Our results reveal evidence for a tissue-specific nuclear modifier gene present only in patient's CM cultures, which acts in concert with the A4300G mtDNA mutation to express the disease phenotype.

Materials and Methods

Patients

The clinical, histochemical, biochemical and molecular features of the two families harboring homoplasmic levels of the tRNA^{Leu} mutation have been reported earlier by us [4,8].

Cell lines

Primary fibroblasts: (FB) from a patient (IV-01, family 2), [4] with homoplasmic A4300G mtDNA mutation and normal control fibroblasts (ES), and normal proliferating cardiomyocyte cell line (AC16), previously described by us [14] were analyzed. Cybrids with the osteosarcoma (143B) nuclear background (206/FB109) containing homoplasmic A4300G mtDNA mutation were generated by our published methods [12].

AC16-4: ρ^0 cardiomyocyte cell line

(AC16-4) was obtained by completely depleting the mtDNA of AC16 cells with ditercalinium, a DNA intercalating reagent, by our method [15]. Several ρ^0 clones were isolated and characterized (Fig. 1A and 1B). The ρ^0 cardiomyocytes are auxotrophic for uridine and pyruvate and do not have a functional respiratory chain as shown by negative histochemical staining to complex IV of the respiratory chain, cytochrome *c* oxidase (COX), while complex II, succinate dehydrogenase (SDH), a nuclear-encoded enzyme is normal by histochemistry (Fig. 1A). mtDNA encoded subunits are absent by immunostaining with antibodies to COX subunit II (CII), while the nuclear-encoded subunit (CIV) is present (Fig. 1A). A ρ^0 fibroblast cell line used as negative control stains similar to AC16-4 cell line. The ρ^0 CM, AC16-4 cell line is completely devoid of mtDNA by long PCR of the entire mtDNA (16.5kb) as shown in Fig. 1B. Furthermore, the ρ^0 cardiomyocytes have retained their cardiac tissue phenotype as indicated by expression of CM markers, Connexin-43, β -Myosin heavy chain, Troponin I, α -cardiac actin, and atrial natriuretic peptide (data not shown). The AC-16-4 ρ^0 CM cell line was used to generate cybrids with the CM nuclear background.

Patient's cardiomyocyte cell line (LV4B6)

Primary cardiomyocytes were cultured from the left ventricle of the transplanted heart of a patient (IV-09 from Family 2), [4]. Proliferating cardiomyocytes were subcloned as described [14]. The clones were screened for CM markers as described before, and one of the clones, LV4B6, which expressed the CM markers, was used in our analyses.

Transnuclear cardiomyocyte cell lines

1) Cardiomyocytes with patient nucleus and normal mitochondria (LV4B6-26)—

Patient cardiomyocyte cell line (LV4B6) was treated with rhodamine 6G (R6G), a mitotoxin which renders the mitochondria nonviable, and subsequently repopulated with normal mitochondria by fusion with enucleated cytoplasts from 143B osteosarcoma cells as described previously [16] and shown schematically in Fig. 2A. Cardiomyocytes are very sensitive to the toxic effects of R6G. We titrated down the concentration of R6G (0.75 μ g/ml) that eliminated the endogenous mitochondria of the LV4B6 cells without compromising cell viability by colony formation following test fusions. The fused cells were grown in regular growth medium and subcloned. Since the LV4B6 cells express the SV40 large T-antigen (T-Ag), clones that express large T-Ag as well as cardiomyocyte markers, Connexin-43 and β Myosin heavy chain by immunocytochemistry were selected and screened for COX activity by biochemical and histochemical analyses. Clone LV4B6-26 was selected for analyses.

Cardiomyocytes with normal nucleus and patient mitochondria (AC16-4-FB109)

Cybrids with normal cardiomyocyte nuclear background containing the A4300G mutation were generated by enucleating the A4300G cybrids in the 143B nuclear background, 206/FB109 with cytochalasin b followed by fusion of the cytoplasts with ρ^0 CM (AC16-4), as shown schematically in Fig. 2B. AC16-4 has a normal CM nucleus and is devoid of mtDNA. The fused cells were selected in minus uridine medium to eliminate the unfused AC16-4 cells and subcloned. Since the AC16-4 ρ^0 cells express the SV40 large T-antigen the clones were screened for T-ag and for cardiomyocyte markers by immunocytochemistry. One of the clones, AC16-FB109 that expressed T-Ag, connexin 43 and β myosin heavy chain was selected for analyses.

LV4B6-AC16-4-29

To determine if the COX deficiency in the patient's cardiomyocytes, LV4B6, can be rescued by wild type nucleus, LV4B6 cells were fused with ρ^0 cardiomyocytes (AC16-4) and grown in minus uridine selection to eliminate the unfused AC16-4 cells. Cells were subcloned and the clones were assayed for COX by histochemistry. Several COX positive clones were identified and LV4B6-AC16-4-29 was selected as a representative clone for further analysis.

Molecular genetic, biochemical, and morphological analyses

The levels of A4300G mtDNA mutation in all the cell lines included in this study were determined by quantitative PCR-RFLP analysis as described [4]. Basically, after twentyfive cycles of PCR amplification, dATP³² was added in the last cycle to prevent heteroduplex formation. The 263 bp PCR product was digested with HphI and the resulting 235 and 28 bp fragments were separated on a 12% non-denaturing polyacrylamide gel and exposed to X-ray film.

To evaluate respiratory chain function, in the various cell types, oxygen consumption was determined by our published method [17]; complex I activity was assayed by the method of Birch-Machin [20]; activities of citrate synthase, COX biochemistry and histochemistry were performed as reported [18]. All spectrophotometric measurements were performed with a Cary UV 100 spectrophotometer (Varian Inc., Walnut Creek, CA, USA). Steady-state levels of mitochondrially-encoded COX subunit II and nuclear-encoded pyruvate dehydrogenase (PDH) subunits were analyzed by immunocytochemistry [18]

Results and Discussion

RFLP analysis

All the cell lines were checked for the presence of the A4300G mtDNA mutation by RFLP analysis after repopulation with appropriate controls. As shown in Fig. 3, cell lines with WT mtDNA (lanes 2, 5, 6 and 8) had a homoplasmic WT band corresponding to 263 bp and those with mutant mtDNA (lanes 3, 4, 7 and 9) had a 235 bp band diagnostic of the A4300G mutation, at *homoplasmic levels*, similar to the patient fibroblasts, (lane 7) from which the mitochondria harboring mutant mtDNA was transferred to the various cell lines in one or more steps. The RFLP data confirm that the intended mitochondrial swaps during repopulation were successfully accomplished and genotypes were quantitatively transferred to the target cells and that the WT and mutant cells contain the respective genotypes.

Analysis of primary fibroblasts and cybrids

We analyzed fibroblasts from the proband of family 2 (IV-01) and a cousin (IV-09) [4]. No abnormalities in respiratory chain function were noted as compared with normal fibroblasts (Fig. 4a and 4b). This was not surprising because in previous studies biochemical defects were

found only in heart tissue of the patient. To determine the pathogenicity of the mtDNA mutation, we studied cybrids in a neutral background of 143B cells. COX activity was essentially normal in cybrids (206/FB109), generated by fusion of enucleated fibroblasts from the proband with ρ^0 osteosarcoma cells (143B206) (Fig 4c). Therefore, we concluded that the mtDNA mutation may not be the exclusive cause for the disease, but additionally a nuclear modifier gene may act in concert with the mtDNA mutation to result in the observed phenotype. It was clear from our histochemical data that the nuclear modifier, if present, is not expressed in the primary fibroblasts or in cybrids. Based on the fact that the homoplasmic mtDNA mutation is exclusively associated with heart disease in the two families studied, and causes COX deficiency only in the heart and normal in all other cell types studied, we entertained the idea of a nuclear modifier gene that may be tissue specific and therefore, the patient's cardiomyocytes may be the more appropriate cell type to be studied.

Analysis of patient's cardiomyocytes, LV4B6

Proliferating CMs were established from the left ventricle of a transplanted heart from a patient with the A4300G mtDNA mutation (IV-9 from Family 2), [4]. COX histochemistry of the patient's CM cell line, LV4B6, showed severely decreased staining for COX (Fig 4d). This was confirmed by biochemical analysis of complex IV (COX) normalized to a mitochondrial matrix enzyme citrate synthase (CS), routinely done in the evaluation of respiratory chain function. The ratio of COX to CS was only 0.4 in LV4B6 similar to that in AC16-4 ρ^0 cells. Additionally complex I activity also was low, the ratio of Complex I to CS was 0.3 similar to AC16-4 (0.1), (Table 1). The data obtained in cultured cardiomyocytes corroborate our previous report of more severe complex I and IV (COX) deficiency in the heart than in any other tissue [4]. Oxygen consumption, another index of respiratory chain function was measured in the patient's CM cultures and was found to be only 16.6% of normal (AC16) and comparable to mtDNA-less ρ^0 CM (24.9, Table 1). These results demonstrate that the biochemical and histochemical defects in the respiratory chain are expressed only in the presence of the patient's nuclear background. We thus provide evidence that the 4300 mutation in the presence of the presumed nuclear modifier segregates with respiratory chain defects in CM cultures from the patient.

Analysis of transnuclear cardiomyocytes

We analyzed transnuclear cardiomyocyte cultures generated in our laboratory containing wild type or patient's cardiomyocyte nucleus in combination with normal or patient's mitochondria harboring the A4300G mutation to provide evidence for our hypothesis that both the nuclear modifier and the mtDNA mutation act in concert to cause respiratory chain defect.

Cardiomyocytes with patient nucleus and normal mitochondria (LV4B6-26)

We replaced the A4300G mitochondria in the LV4B6 cell line with normal mitochondria from 143B cells by R6G treatment followed by fusion with enucleated 143B cytoplasts containing wildtype mitochondria. 18/20 clones picked were COX positive indicating that the endogenous A4300G mitochondria that were COX-negative in LV4B6 cells were replaced by wild type mitochondria from 143B cells which rendered the cells COX-positive. One of these clones, LV4B6-26 was selected for representation. Fig 5 shows the results of histochemical staining of the transnuclear cardiomyocytes. Normal CM (AC16) stains positive, while the ρ^0 CM, (AC16-4) is clearly negative due to the absence of mtDNA and therefore absence of a functional respiratory chain (Fig.5a, 5b). LV4B6-26, which contains patient's CM nucleus and normal mitochondria exhibits normal COX staining (Fig.5c). This clearly indicates that the nuclear modifier present in the patient's CM nucleus *per se* does not cause COX deficiency, but interacts with the A4300G mtDNA mutation to result in the respiratory chain defect. Further

evidence for this interaction is provided by the CM cell line containing normal nucleus and patient's mitochondria.

Cardiomyocytes with normal nucleus and patient mitochondria (AC16-4-FB109)

AC16-4-FB109 is a cybrid with the normal CM nuclear background and patient's mitochondria. This cell line was obtained by direct fusion of ρ^0 CM (AC16-4) with enucleated cytoplasts from A4300G cybrids with the 143B nuclear background (Fig 2). ρ^0 CM (AC16-4) has normal nucleus and is devoid of mtDNA. It is COX-negative because it lacks a functional respiratory chain (Fig.5d). When fused with cytoplasts from A4300G cybrids, the cells become COX-positive demonstrating that the mtDNA mutation does not cause COX-deficiency in the absence of the nuclear modifier from the patient's nucleus.

The nuclear modifier is recessive

The recessive nature of the nuclear modifier was evident when the patient cardiomyocyte cell line (LV4B6) was fused with a ρ^0 human cardiomyocyte line (AC16-4). Clones were grown in minus uridine selection to eliminate the AC16-4 cells. This selection medium will permit the growth of cells that have a functional respiratory chain. Both AC16-4 and LV4B6 are COX-negative (Fig. 6a and 6b). Several clones were picked and screened for COX histochemistry. More than 90% of the clones selected were COX positive. Clone LV4B6-AC16-4-29 was selected for representation. COX deficiency in LV4B6 cells was restored by complementation with the normal CM nucleus from the AC16-4 cells, indicating that the nuclear defect is autosomal recessive (Fig. 6c). Therefore, a single copy of a normal allele should rescue the COX deficiency at least partially.

Our cell culture studies demonstrate that neither the nuclear modifier nor the mtDNA mutation by itself is pathogenic. In both the families maternal inheritance of the A4300G mutation, and its association with respiratory chain dysfunction, indicate that the A4300G may be the primary mutation, necessary for the expression of the phenotype, but not sufficient to be pathogenic. However, it is clear that the nuclear gene defect is also a requisite for pathogenesis, but does not induce the phenotype *per se* but contributes to the pathogenic effect of the A4300G mutation. The interdependence of two genomes in the evolution of the disease illustrates the two locus genetic model.

In most pathological mtDNA mutations, mitochondrial function is compromised when the percentage of mutated molecules exceeds a threshold. However, the homoplasmic A4300G mtDNA mutation *per se* seems to be less harmful than pathogenic heteroplasmic mtDNA mutations. It is therefore possible that the nuclear modifier acts synergistically with the homoplasmic mtDNA mutation and the additive effect may cause phenotypic expression. In the two families harboring the A4300G mutation homoplasmy has been observed in several generations, even though a wide range of clinical severity of heart involvement in individual members has been observed [19]. Variable clinical symptoms may also be explained based on the differential interaction of the two genomes, and may sometimes be influenced by other factors, such as the mtDNA haplotypes, or environmental factors [3].

In the three cell types studied, (cybrids, fibroblasts and cardiomyocytes), complex I and IV deficiencies are expressed only in the cardiomyocytes and only when both the patient nucleus and mitochondria with the A4300G mutation are present. We report for the first time evidence for a nuclear modifier that acts in concert with the homoplasmic A4300G mtDNA mutation to cause a tissue-specific disease phenotype. The cardiomyopathy associated with these two unrelated families is unique. It is clear that the mtDNA mutation is causal since the A4300G mtDNA mutation segregates with respiratory chain deficiency, which is tissue-specific and its synergistic interaction with a nuclear modifier gene may be the basis for pathogenesis of

mitochondrial cardiomyopathies. The identity of the nuclear modifier and the pathogenic mechanism by which it causes cardiomyopathy will shed light on the complex interaction and interdependence of the functionality of the two genomes.

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Abbreviations

mtDNA	mitochondrial DNA
CM	Cardiomyocytes
COX	Cytochrome <i>c</i> oxidase
SDH	Succinate dehydrogenase
T-Ag	Large T-antigen
MICM	maternally inherited cardiomyopathy
LHON	Leber's hereditary optic atrophy
SNHL	sensorineural hearing loss
R6G	rhodamine 6G

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Fig. 1.

A: Immunocytochemistry with antibodies to COX II subunit (a) and COX IV (b), and histochemistry for COX (c) and SDH (d) of AC16 (ρ^+), AC16-4 (ρ^0) and ρ^0 fibroblasts (negative control).

B: Long PCR of AC16 ρ^0 clones (1-11), AC16 ρ^+ (12), 143B (13, WT), 143B206 (14, ρ^0), Human genomic DNA, (15), showing absence of mtDNA in clones 1-11.

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Fig.2.

A, B: Scheme-Generation of transnuclear cardiomyocyte cell lines. (see Materials and Methods).

Fig.3.

RFLP analysis of the A4300G mtDNA mutations in the cardiomyocyte cell lines. WT band: 263 bp; Mutant band: 235 bp (see methods). M; DNA size marker, 1: Uncut, 2: AC16, 3: AC16-4-FB109. 4: LV4B6, 5: LV4B6-26, 6: Control fibroblasts, 7: Patient fibroblasts (FB), 8: 143B WT (osteosarcoma), 9: 206/FB109 (143B ρ^0 -patient cybrid).

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Fig.4. COX histochemistry of a: control fibroblasts (ES), b: patient's fibroblasts (FB), c: cybrids with 143B nuclear background and A4300G mitochondria (206/FB109) and d: patient's CM (LV4B6), shows COX deficiency only in patient's CM, indicating cell-type specificity of the respiratory chain defect.

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Fig.5.

COX histochemistry of a: normal CM (AC16), b: ρ^0 CM (AC16-4), c: transnuclear CM (LV4B6-26), d: transnuclear CM (AC16-4-FB109) and e: patient's CM (LV4B6). COX deficient phenotype is expressed only in the presence of patient's nucleus and mitochondria.

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Fig. 6.

COX histochemistry of a: patient's CM (LV4B6), b: ρ^0 CM (AC16-4) and c: fusion product of a and b (LV4B6-AC16-4-29), shows restoration of COX by complementation, indicating that COX deficiency is autosomal recessive.

Table 1
Respiratory chain activity in the cardiomyocyte cultures

Cell type	COX	Complex I	CS	COX/CS	Complex I/CS	O2	% of Normal
AC 16	76.2	29.8	42.9	1.8	0.7	6.61	100
AC16-4 (p ⁰)	6.1	3.4	40.0	0.2	0.1	1.65	24.9
AC16-4-FBI09	126.6	87.2	17.2	7.4	5.1	5.25	79.4
LV4B6-26	151.7	11.5	17.4	8.7	0.7	4.67	70.7
LV4B6	6.45	4.7	18.4	0.4	0.3	1.1	16.6

COX and citrate synthase activity in nmol/min/mg protein
Complex I activity in mM/min/mg protein; and O2 consumption in fmol/cell/minute
Values are mean of 3 assays in duplicate.