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Defective mitochondrial fusion, altered respiratory function, and distorted cristae structure in skin fibroblasts with heterozygous OPA1 mutations

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ABSTRACT

Deleterious consequences of heterozygous *OPA1* mutations responsible for autosomal dominant optic atrophy remain a matter of debate. Primary skin fibroblasts derived from patients have shown diverse mitochondrial alterations that were however difficult to resolve in a unifying scheme. To address the potential use of these cells as disease model, we undertook parallel and quantitative analyses of the diverse reported alterations in four fibroblast lines harboring different *OPA1* mutations, nonsense or missense, in the guanosine triphosphatase or the C-terminal coiled-coil domains. We tackled several factors potentially underlying discordant reports and showed that fibroblasts with heterozygous *OPA1* mutations present with several mitochondrial alterations. These included defective mitochondrial fusion during pharmacological challenge with the protonophore carbonyl cyanide m-chlorophenyl hydrazone, significant mitochondrial elongation with decreased OPA1 and DRP1 proteins, and abnormal mitochondrial fragmentation during glycolysis shortage or exogenous oxidative stress. Respiratory complex IV activity and subunits steady-state were decreased without alteration of the mitochondrial deoxyribonucleic acid size, amount or transcription. Physical link between OPA1 protein and oxidative phosphorylation was shown by reciprocal immunoprecipitation. Altered cristae structure coexisted with normal response to pro-apoptotic stimuli and expression of Bax or Bcl2 proteins. Skin fibroblasts with heterozygous *OPA1* mutations thus share significant mitochondrial remodeling, and may therefore be useful for analyzing disease pathophysiology. Identifying whether the observed alterations are also present in ganglion retinal cells, and which of them underlies their degeneration process remains however an essential goal for therapeutic strategy.

1. Introduction

In 2000, heterozygous OPA1 mutations were found responsible for autosomal dominant optic atrophy (ADOA, OMIM 165500), one of the most common hereditary optic neuropathies [1,2]. These reports opened up a new field of diseases, due to factors involved in the mitochondrial membrane dynamics, which involves mitochondrial membrane fusion and fission events, the essential players of which are the four large GTPases: MFN1, MFN2 , OPA1 and DRP1 [3,4].

OPA1 is very well conserved among species. Its function has essentially been addressed by invalidation and overexpression approaches in yeast and in cultured mammalian cells. In both models it was shown essential for mitochondrial membrane fusion [4–8] and for mitochondrial DNA (mtDNA) maintenance [9,10]. Independent from its role in membrane fusion [11], OPA1 oligomerization has been shown to be involved in mammalian cells mitochondrial cristae structure and in protection against apoptosis [12–16].

Despite these recent developments in the field, the molecular consequences of disease causing-*OPA1* mutations remain the matter of much debate, for at least three very different reasons; i) the cellular consequences of *OPA1* invalidation or overexpression might significantly differ from those induced by heterozygous mutations, ii) the main target tissue, retinal ganglion layer, has provided little opportunity for direct analyses and iii) differences in the experimental protocols used for analyzing mitochondrial fusion and oxidative phosphorylation (OXPHOS) in the diverse models of heterozygous *OPA1* mutations have rendered difficult data confrontation. Cultured

Abbreviations: ADOA, autosomal dominant optic atrophy; AR, aspect ratio; cccp, carbonyl cyanide m-chlorophenyl hydrazone; $\Delta\psi$, mitochondrial inner membrane potential; mtDNA, mitochondrial DNA; OXPHOS, oxidative phosphorylation; SOD1, Cu,Zn-superoxide dismutase; SOD2, Mn-superoxide dismutase; t-BH, tert-butyl hydroperoxide; VO_2, oxygen consumption rate; TMRM, tetramethylrhodamine methyl ester

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primary fibroblasts derived from patients have been the most frequently analyzed model of heterozygous *OPA1* mutations [17–23]. They have shown diverse alterations, involving OXPHOS, mitochondrial dynamics and/or cell sensitivity to apoptosis or oxidative stress. However several discrepancies between reports have precluded drawing any firm conclusion.

In this paper we considered the hypothesis that primary skin fibroblasts derived from patients with ADOA were a relevant cell model for studying ADOA pathophysiology. To begin, we undertook the search for alteration(s) common to all *OPA1* heterozygous mutations and therefore, possibly responsible for the deleterious impact on ganglion retinal cells. Different degrees of alterations could correspond to clinical severity. Indeed ADOA typically presents in childhood with insidious loss of visual acuity due to atrophy of retinal ganglion layer with secondary loss of the optic nerve axonal fibers [24,25]. However, almost one-third of the patients present with phenotypes labeled "ADOA plus" including progressive appearance of additional symptoms such as sensorineural deafness, ataxia, ophthalmoplegia, and sensorimotor axonal polyneuropathy, as well as histological hallmarks of mitochondrial myopathy [26,27].

As there appears to be a lot of disagreement with regards to data in the literature, we undertook parallel analysis of the diverse parameters previously reported as abnormal, using quantitative assays when possible, and evaluating the overall impact on the cell life. Potential diversity due to mutation severity was evaluated by the parallel analysis of four primary skin fibroblasts harboring different types of *OPA1* mutations, missense or nonsense, in the GTPase or the C-terminal coiled-coil domains of the protein, one of which associated with an "ADOA plus" phenotype.

2. Material and methods

2.1. Materials and cells

All reagents were of the highest purity and purchased from Sigma-Aldrich (France) unless otherwise stated. Control cell lines were obtained from the tissue repository of AFM (Association Française Contre les Myopathies). Primary skin fibroblasts were obtained from four patients with heterozygous *OPA1* mutations. Three of these patients presented with isolated ADOA: OPA1-1 and OPA1-1′ were siblings with mutation c.2846T>C (p.Leu949Pro); OPA1-2 had mutation c.1833_1836delTACA (p.Thr612GlnfsX20). The fourth patient (OPA1-3) had AOAD with deafness and mutation c.1334G>A (p.Arg445His). OPA1-1, -1′ and -2 cells were derived from patients' skin biopsies using standard procedures while the fibroblasts OPA1-3 were kindly provided by Pr Pascal Reynier, Angers, France. Alteration of the *OPA1* gene was verified by direct sequencing of the fibroblasts genomic DNA. The cell growth rate was normal (doubling time 2.9 ± 1.8 days, range 1.9 to 4.3 for mutant *OPA1* cells and 2.6 days ± 0.9 for controls, $p = 0.86$). Absence of mycoplasma contamination was systematically verified at each passage.

Fibroblasts were cultivated at 37 °C in 5% CO_2 in a medium consisting of D-MEM (Dulbecco's modified Eagle's medium) (LifeTech, Invitrogen) supplemented with 10% fetal calf serum, 200 μM uridine, 50 IU/mL penicillin and 50 μg/mL streptomycin. Although the cells were initially amplified in culture medium with high glucose (4.5 g/L), their analyses were always performed after at least 3 days culture in medium with 1 g/L glucose.

2.2. Morphological analyses

For immunofluorescent labeling of the mitochondrial compartment, the cells were plated at subconfluence on glass coverslips at least 72 h before rapid washing in warm PBS followed by fixation in 4% paraformaldehyde and 0.1% glutaraldehyde. Mitochondrial compartment was visualized using polyclonal antibodies against cytochrome c oxidase subunit 2 (anti-CO2) [28]. The coverslips were mounted in Mowiol containing 0.15 μg/mL DAPI. Fluorescent images were acquired on an Axiophot 2 microscope (Zeiss, Jena, Germany) with a charge-coupled device camera and the images were processed with Metaview [29]. Two different protocols were used for quantitative analysis of mitochondrial morphology. One was based on visual classification of at least 100 individual cells per coverslip into three categories according to the pattern of their mitochondrial morphology: filamentous, punctate or intermediate [30]. The second protocol used Image J software analysis to obtain quantitative measurement of the mitochondrial compartment of at least 30 individual cells per cover slip as described by Koopman et al. [31]. This technique delimits each mitochondrion within an ellipse whose axes, area and perimeter are measured allowing calculation of diverse parameters among which two have been considered most informative: aspect ratio (AR) and form factor [31]. AR is a ratio between the long and short axes of the mitochondrial ellipses and thus measures mitochondrial elongation while the form factor is a ratio between the perimeter and area of the ellipses and thus measures mitochondrial branching [31]. In our experiments, AR and form factor always followed the same trend. AR showed the least variation among control cells and was therefore chosen for comparison between control and mutant *OPA1* cells.

For ultrastructural analysis of the mitochondria cells were plated on plastic coverslip (Thermanox, Nalge Nunc, Rochester, NY, USA) at least 72 h before their fixation in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4 and post-fixation in 2% OsO_4. Gradual dehydration in acetone, including a 2% uranyl staining step in 70% acetone, preceded final embedding in Epon resin (EMS, Fort Washington, PA, USA). After uranyl and lead citrate staining, ultrathin sections were examined with a Philips CM120 electron microscope and photographed with a SIS Morada digital camera. Quantification of mitochondrial morphology was performed by classification of at least 40 mitochondrial profiles into three categories: normal, altered and intermediary, by three independent examiners, blind to the genotype of the cells.

2.3. Molecular analyses

Total DNA was extracted from cells using standard techniques based on proteinase K and SDS extraction followed by isopropanol precipitation. Multiple deletions of the mtDNA were searched for with the long range PCR kit Expand™ Long Template PCR System (Boehringer) and mitochondrial DNA copy number was quantified by real-time PCR amplification on Light Cycler (Roche Diagnostics) as described [32].

Total RNA was extracted from cells using the RNeasy kit from Qiagen (Courtaboeuf, France), reverse transcribed with random hexamers and SuperScript™ First-Strand Synthesis System for RT-PCR kit (Invitrogen) according to the manufacturer's guidelines. Expression of *ACTB*, *OPA1*, *DNML1*, *CS*, *MT-CO2*, *COX4I1*, *NRF1*, *PPARGC1A*, and *SOD2* genes was quantified by real-time PCR amplification of the cDNAs using LightCycler 480 Sybr Green I Master kit (Roche). All results were expressed as % of controls after their normalization to β-actin gene (*ACTB*) expression and to one single control. Official gene symbols, primers, standards and conditions of the amplifications are summarized in Supplemental Table 1.

2.4. Cellular metabolic and oxidative stress

Cells were seeded at subconfluence at least 72 h before the cellular challenge, on glass coverslips for subsequent morphological analyses and in well dishes for cytotoxicity assays. Cell survival was analyzed by staining with 34 μg/mL Neutral red for one hour, followed by two washes with warm PBS, neutral red extraction with 50% ethanol, 1% acetic acid and read on a microplate spectrophotometer at 540 nm [33].