Respiratory function in cybrid cell lines carrying European mtDNA haplogroups: implications for Leber’s hereditary optic neuropathy

Valerio Carelli a, Lodovica Vergani b, Barbara Bernazzi b, Claudia Zampieron c, Laura Bucchia a, Maria Lucia Valentin a, Chiara Rengod e, Antonio Torronid f, Andrea Martinuzzi c,*

aIstituto di Clinica Neurologica, Universita` di Bologna, Bologna, Italy
bDipartimento di Scienze Neurologiche e Psichiatriche, Universita` di Padova, Padua, Italy
cIRCCS “E. Medea” Scientific Institute, Conegliano Research Centre, via Costa Alta 37, 31015, Conegliano (TV), Italy
dDipartimento di Genetica e Biologia Molecolare, Universita` di Roma “La Sapienza”, Rome, Italy
eIstituto di Medicina Legale, Universita` Cattolica del Sacro Cuore di Roma, Rome, Italy
fDipartimento di Genetica e Microbiologia, Universita` di Pavia, Pavia, Italy

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Abstract

The possibility that some combinations of mtDNA polymorphisms, previously associated with Leber’s hereditary optic neuropathy (LHON), may affect mitochondrial respiratory function was tested in osteosarcoma-derived transmitochondrial cytoplasmic hybrids (cybrids). In this cellular system, in the presence of the same nuclear background, different exogenous mtDNAs are used to repopulate a parental cell line previously devoid of its original mtDNA. No detectable differences in multiple parameters exploring respiratory function were observed when mtDNAs belonging to European haplogroups X, H, T and J were used. Different possible explanations for the previously established association between haplogroup J and LHON 11778/ND4 and 14484/ND6 pathogenic mutations are discussed, including the unconventional proposal that mtDNA haplogroup J may exert a protective rather than detrimental effect.

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1. Introduction

Analysis of human mtDNA variation has identified specific combinations of polymorphisms that have been used to systematically classify mtDNAs into haplogroups, and to study origin, dispersal and evolution of human populations [1,2]. Some authors have further suggested that certain mtDNA population polymorphisms may interact with environmental factors and/or mtDNA pathogenic mutations, and therefore that mtDNAs belonging to different haplogroups could result in mitochondria with differential functionality [2]. In this regard, the most debated case is the plethora of mtDNA nucleotide changes that have been associated with Leber’s hereditary optic neuropathy (LHON) [2–10]. Three of these mutations, at positions 11778/ND4, 3460/ND1 and 14484/ND6, are now unanimously considered as pathogenic for most of the LHON cases worldwide [2,11]. At least five other transitions at positions 4216/ND1, 13708/ND5, 15257/cytb, 15812/cytb and 4917/ND2, defined as “secondary or intermediate mutations” [2], are polymorphisms of European populations [12]. Indeed, they occur in specific combinations within haplogroups J (4216/ND1 and 4917/ND2), T (4216/ND1 and 4917/ND2) and J/T (4216/ND1 and 4917/ND2) [12]. Both haplogroups J and T have diverged from a more ancient mtDNA lineage defined by the 4216/ND1 change only [12]. These five polymorphisms were initially associated with LHON on the basis of their more frequent recurrence in LHON pedigrees compared to the general population [3–6]. Subsequently, a more rational investigation was performed to test their association with LHON and three laboratories published systematic haplotype and phylogenetic analyses of LHON patients from the USA [13], Italy [14] and Germany [15]. These independent studies showed a preferential association of the pathogenic mutations 11778/ND4 and 14484/ND6 with haplogroup J.
This association was particularly striking with the 14484/ND6 mutation. On the other hand, LHON pedigrees carrying the 3460/ND1 mutation seemed widely distributed among all haplogroups [13,14]. The currently favored interpretation is that the haplogroup J may lead to increased penetration of the LHON pathogenic mutations 11778/ND4 and 14484/ND6 [13,14]. A more non-specific effect of the haplogroup J as a risk factor for neurodegenerative diseases in general, has also been suggested [9]. However, genetic and clinical investigations by other authors found no evidence for influence of the “secondary mutations”, including those clustered in haplogroups J and T, on the pathogenesis and clinical expression of LHON [7,8,10,16]. Intriguingly, haplogroup J may also be associated with successful aging, as a recent study carried out on Italian centenarians has suggested [17]. Moreover, another recent report described the high prevalence of haplogroup T in individuals with reduced spermatozoa motility and of haplogroup H in those with normal motility and these associations correlated with significant differences in haplogroup-specific respiratory chain performance [18].

In the present study, we used the transmitochondrial cytoplasmic hybrid (cybrid) technology [19] to compare, in the nuclear background of the osteosarcoma 143B.TK-parental cell line, the respiratory chain function of mtDNA-less rho0 cell lines (206) repopulated with mitochondria from control fibroblasts with different mtDNA haplogroups.

2. Materials and methods

2.1. Cells

Fibroblast cell lines have been obtained, after informed consent, from skin biopsies of normal subjects. Fibroblast cell lines were then enucleated and fused with the mtDNA-less 206 rho0 cells following the described method [19]. Positive mtDNA re-population was identified by picking up growing clones (a minimum of four successful clones for each fusion) in selective medium: normal DMEM supplemented with 5% dialyzed fetal bovine serum (dFBS) (Seromed-Biochrom, Berlin), 0.1 mg/ml bromodeoxyuridine and no uridine. The re-population of the 206 rho0 cells with the fibroblast mtDNA was confirmed by screening for the diagnostic mutations (Table 1) identified in the mtDNA of the fibroblast cell line. The human cell line 143B.TK- (obtained from G. Attardi as kind gift), from which the 206 rho0 line was developed [19], was used as control. To ensure complete stabilization of mtDNA amount, functional assessment of selected clones was carried out only after at least 3 months of clone cycling.

2.2. High resolution RFLP analysis and haplogroup classification of mtDNAs

To determine high resolution RFLP haplotypes, the entire mtDNA from the fibroblast cell lines and the osteosarcoma 143B.TK-parental cell line and control fibroblasts

<table>
<thead>
<tr>
<th>Sample</th>
<th>Haplogroup</th>
<th>RFLP Haplotype±b</th>
</tr>
</thead>
<tbody>
<tr>
<td>143B.TK-PC</td>
<td>X</td>
<td>$-1715c; +14465c; +15397j; +16517e$</td>
</tr>
<tr>
<td>143B.TK-PS</td>
<td>H</td>
<td>$-6920c; -7025a; -14766u; -16303k$</td>
</tr>
<tr>
<td>143B.TK-GA</td>
<td>T</td>
<td>$+4210a; +4914c; +10727c; +13366m; -13367b; +13367j$</td>
</tr>
<tr>
<td>143B.TK-</td>
<td>J</td>
<td>$+15606a; +16025; +16157e$</td>
</tr>
</tbody>
</table>

* Sites are numbered from the first nucleotide of the recognition sequence. A “+” indicates the presence of a restriction site, a “−” indicates the absence. The explicit indication of the presence/absence of a site implies the absence/presence in haplotypes not so designates. The restriction enzymes used in the analysis are designated by the following single-letter codes: a, AluI; b, Avall; c, Ddel; d, HaeII; g, HinfI; i,MspI; j, MboI; k, Rsal; m, BamHI; n, NalIII; t, BfAl; s, AccI; r, BsrFI; u, MseI. An oblique separating states indicates the simultaneous presence or absence of restriction sites that can be correlated with a single nucleotide substitution.

b Sites diagnostic of haplogroups are underlined.

143B.TK-parental cell line was amplified in nine overlapping fragments by the use of PCR and the primer pairs previously described [14]. Each of the nine PCR segments was then digested with 14 restriction endonucleases (AluI, Avall, BamHI, Ddel, HaeII, HaeIII, Hhal, HinfI, HpaI, MspI, MboI, Rsal, TaqI) (Promega, Madison, WI, USA). In addition, mtDNAs were screened for the presence/absence of the BstOI site at np 13704, the AccI sites at nps 14465 and 15254, the BfAl site at np 4914, the NalIII sites at np 4216 and 4577, and the MseI site at 14766. The A → G sequence polymorphism at np 12308 in the tRNA⁴⁰⁰ gene was also tested by using a mismatched primer, which generates a HinfI site when the A12308G mutation is present [14].

2.3. Oxygen consumption and rotenone sensitivity

Oxygen consumption was measured in intact cells (samples of 5 × 10⁶ cells in 2 ml of DMEM lacking glucose, supplemented with 5% dialyzed FBS at 37 °C) with a YSI 5300 oxy meter (YSI Instruments, Yellow Springs, OH) [19,20]. Each clone was assayed in at least three independent experiments, and results are expressed as mean ± standard deviation. The sensitivity of the lines carrying the different haplogroups to the complex I inhibitor rotenone was assessed as described [21]. After testing the effects of a wide range of rotenone concentrations (5 nM–0.1 mM), we chose the concentration range of 5–500 nM for the subsequent experiments: the cells were treated with different concentrations (5, 10, 20, 40, 100 and 500 nM) of rotenone for 4 h, then harvested and the endogenous cell respiration was measured in whole cells as detailed above. Results are expressed as percentage of remaining cell respiration.

2.4. Growth curves

Cell growth was assessed in glucose-free/galactose + medium (Gibco) [22]. Multiple series of 60 mm Petri dishes
were seeded each with a constant number of cells \(10^5\) in glucose-free/galactose medium: DMEM with L-glutamine without D-glucose, 0.9 mg/ml galactose, 10% dFBS. Duplicate dishes were trypsinized and counted daily for 7 days. Values obtained from fibroblast derived cybrid clones were compared to those for the parental 143B.TK-cell line. Measurements for each haplogroup were repeated at least in quadruplicate, and results are expressed for each time point as mean ± standard deviation.

2.5. Enzymatic activities

Cytochrome c oxidase (COX) and citrate synthase activities were assayed spectrophotometrically on freshly harvested and sonicated cells as described elsewhere [20]. COX activity was divided by the citrate synthase activity of that sample (citrate synthase ratio) to minimize the effects of variability in mitochondrial mass in the various cell culture samples.

2.6. Statistical analysis

One-way analysis of variance (ANOVA) was used to test for statistically significant differences \((P < 0.05)\) between cybrid cell lines with different mtDNA haplogroups or between cybrids and the 143B.TK-parental cell line.

3. Results

On the basis of RFLP analysis (Table 1), we selected three fibroblast cell lines: one belonging to haplogroup J (HGA-haplo J), the second belonging to haplogroup T (HPS-haplo T), and the third belonging to haplogroup H.
(HPC-haplo H), the most common (about 40%) haplogroup in Europe. The RFLP analysis showed that the 143B.TK-parental cell line harbored a mtDNA belonging to haplogroup X. All polymorphisms, including those putatively LHON-related, were found to be homoplasmic as expected. The three fibroblast-cell lines selected were then used to construct the cybrid cell lines. Four clones from each cybrid cell line were chosen for further investigations (HPC1, 5, 7, 10; HPS1, 3, 6, 11; HGA1, 2, 4, 13). Each of these clones was maintained for several months and haplogroup-specific RFLP markers were repeatedly checked to confirm that the mitochondrial re-population was with the expected mtDNAs.

The results of oxygen consumption studies are shown in Fig. 1. There were no significant differences in respiratory capacity among fibroblast-derived cybrid cell lines or between any of them and the parental 143B.TK-cell line (Fig. 1A). Although we observed some interclonal variability (Fig. 1B), we found no significant differences by clone-to-clone comparison, except for one single clone with haplogroup T (HPS11, Fig. 2B). The interclonal variability is a known phenomenon in cybrid studies even in control cell lines. It has been suggested that it probably relates to some nuclear variability of the recipient 206 rho0 cell line, and it is not correlated to mtDNA content or growth rate, thus does not affect the general interpretation of the mean values obtained [22,23].

In order to explore even slight differences in complex I function that might not be reflected in its specific enzymatic activity, as happens for 11778/ND4 and 14484/ND6 LHON pathogenic mutations [24,25], we chose to test cybrid respiration sensitivity to the complex I specific inhibitor rotenone. Treatment of the cybrid cell lines with rotenone caused a dose-dependent inhibition of endogenous cell respiration in all tested lines (Fig. 2). The inhibition was slightly more marked in the parental 143B.TK-line (32.6% of baseline oxygen consumption with no inhibitor) compared to the fibroblast derived cybrids (40–46%). There was no difference in rotenone sensitivity amongst the fibroblast-derived lines carrying the three different haplogroups. These results suggest that complex I function was not significantly affected by the different sets of polymorphisms in ND subunits defining the three haplogroups here investigated.

There was also no difference in citrate synthase normalized COX activity among cybrid clones belonging to the various mtDNA haplogroups and between them and the parental line 143B.TK (Table 2). COX activity was the only respiratory complex previously reported to be clearly affected by haplogroup differences [18].

In order to disclose more subtle defects of respiratory function among the cybrid cell lines under investigation, growth experiments were carried out in a glucose-free/galactose + medium which is a reliable way of testing the respiratory chain-dependent metabolic capacity of cells [22]. Cybrid cell growth in glucose free/galactose + medium (Fig. 3) also failed to show any substantial difference among the clones investigated. However, the haplogroup T growth capacity was at the lower end of the range.

<table>
<thead>
<tr>
<th>Cytochrome c oxidase</th>
<th>143B.TK-</th>
<th>HPC-haplo H</th>
<th>HPS-haplo T</th>
<th>HGA-haplo J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytochrome c oxidase</td>
<td>0.98 (0.25)</td>
<td>0.99 (0.37)</td>
<td>1.06 (0.46)</td>
<td>1.10 (0.47)</td>
</tr>
</tbody>
</table>

Enzyme activity is expressed as citrate synthase ratio. Values are mean of at least three separate experiments ± (S.D.).

Fig. 2. Effect of complex I inhibition on endogenous cell respiration: values are expressed as percentage of baseline (no rotenone) cell O2 consumption. The degree of endogenous cell respiration in presence of growing concentration of the complex I inhibitor rotenone is not different among the different lines.
Fig. 3. Cell growth in glucose-free/galactose+medium. In each graph, the parental 143B.TK-line is compared with cybrids belonging to a different haplogroup. Each point corresponds to the mean of cell counts ± S.D. at that time after seeding. All the cell lines were able to increment their number in this medium, with a pattern which did not significantly differ from the parental 143B.TK-line.
4. Discussion

Our study, using a cybrid cellular model, does not reveal any significant overall respiratory defect in both haplogroups J and T cybrids. Few scenarios may be considered.

The first is that more subtle functional changes are induced by specific haplogroups, and these may in turn become relevant and observable only through the interaction with particular environmental conditions and/or nuclear background or tissue-specific nuclear expression. Wallace et al. [2] recently speculated that haplogroup J might induce a partially uncoupling effect on oxidative phosphorylation. This may be difficult to demonstrate using the experimental model of cybrid cells. However, others and we have been recently able to demonstrate a clear growth impairment, using the galactose medium, of cybrid cells harboring each of the LHON pathogenic mutations 11778/ND4, 3460/ND1 and 14484/ND6 [22] (Carelli V, Vergani L, Martinuzzi A, unpublished data). A defective oxidative phosphorylation has been difficult to demonstrate in LHON-derived cybrid cell lines, particularly for the 14484/ND6 mutation that showed only 10–15% reduction in respiration rate [26]. Thus, growth in galactose medium proved to be sensitive enough to unmask the defective phenotype induced by LHON pathogenic mutations.

A second scenario is that haplogroup J polymorphisms are functionally neutral unless the superimposition of a LHON pathogenic mutation occurs. In a previous study with LHON-derived cybrid cell lines, we showed that clones carrying the 11778-haplo J genotype had lower oxygen consumption and higher doubling time than those with the 11778-non J mtDNA [20]. Multiple cybrid cell clones, derived from a further individual with the same 11778-haplo J mitochondrial genome, are currently under investigation and they showed a milder defect, more similar to the defects observable in 11778-non J subjects (Carelli V, Vergani L, Martinuzzi A, unpublished data). A defective oxidative phosphorylation has been difficult to demonstrate in LHON-derived cybrid cell lines, particularly for the 14484/ND6 mutation that showed only 10–15% reduction in respiration rate [26]. Thus, growth in galactose medium proved to be sensitive enough to unmask the defective phenotype induced by LHON pathogenic mutations.

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8:1), an indirect indication of lower penetrance in females, was reported for the 14484/ND6 mutation [35–37]. This mutation may also be persistent in the population, as shown was reported for the 14484/ND6 mutation [35–37]. This consequence, the 11778/ND4-haplo J and 14484/ND6-haplo J LHON families should be more frequently large and homoplasmic rather than small, heteroplasmic pedigrees or sporadic cases. The unexpectedly high percentage of sporadic cases reported in some populations for the most frequent 11778/ND4 mutation provides a good opportunity to test this hypothesis [14,35,36,38]).

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References


