Ethambutol-induced optic neuropathy linked to OPA1 mutation and mitochondrial toxicity

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ABSTRACT

Ethambutol (EMB), widely used in the treatment of tuberculosis, has been reported to cause Leber's hereditary optic neuropathy in patients carrying mitochondrial DNA mutations. We study the effect of EMB on mitochondrial metabolism in fibroblasts from controls and from a man carrying an OPA1 mutation, in whom the drug induced the development of autosomal dominant optic atrophy (ADOA). EMB produced a mitochondrial coupling defect together with a 25% reduction in complex IV activity. EMB induced the formation of vacuoles associated with decreased mitochondrial membrane potential and increased fragmentation of the mitochondrial network. Mitochondrial genetic variations may therefore be predisposing factors in EMB-induced ocular injury.

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1. Introduction

Ethambutol (EMB) is one of the first-line antimycobacterial agents used for the treatment of tuberculosis. The precise molecular basis of the action of EMB is not known but it has been reported to inhibit RNA synthesis in mycobacteria (Forbes et al., 1965). EMB, which acts as a chelating agent, has been shown to interfere with the metal-containing enzyme systems essential to bacterial metabolism (Shepherd et al., 1966). Moreover, the target enzyme of EMB is an arabinosyl transferase involved in the biosynthetic pathway of the arabinogalactan cell wall of *Mycobacterium tuberculosis* (Belanger et al., 1996).

Since EMB was first used in the 1960s, ocular side effects manifesting as optic neuropathy have been described (Carr and Henkind, 1962). The classical symptoms of ocular toxicity are a progressive blurring of vision and decreased colour perception. Ophthalmologic examination reveals decreased bilateral visual acuity, central scotoma and dyschromatopsia (Chan and Kwok, 2006). Other more rare side-effects include peripheral neuropathy, cutaneous reactions, thrombocytopenia, and hepatitis (Chan and Kwok, 2006).

EMB toxicity is described as dose- and time-dependent but its reversibility remains controversial. EMB has been estimated to be responsible for 100,000 new cases of blindness each year since about 2% of patients treated with EMB develop an optic neuropathy (Lee et al., 2008; Sadun and Wang, 2008). This underscores the importance of studies aimed at elucidating the molecular mechanisms underlying the ocular toxicity induced by EMB.

The toxic effect of EMB on retinal ganglion cells has been confirmed *in vivo* and *in vitro* in rodents (Heng et al., 1999; Yoon et al., 2000). It was suggested that neuronal ganglion cell death may be mediated through EMB-induced glutamate excitotoxic pathway (Heng et al., 1999). However, Yoon et al. (2000) demon-

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strated that EMB induced the formation of vacuoles in neuronal retinal cells and that, contrary to the current theories, EMB-induced toxicity was not mediated by glutamate excitotoxicity or zinc chelation, but by a process involving intracellular zinc. In fact, this toxicity has recently been shown to be mediated by zinc and lysosomal membrane permeabilization (Chung et al., 2009). Moreover, it has been suggested that EMB may interact with mitochondrial cytochrome *c* oxidase (complex IV) activity through a copper-chelating action (Buyske et al., 1966).

The mitochondrial hypothesis has aroused interest since Dotti et al. (1998) first described a case of EMB-induced optic neuropathy in a patient carrying Leber's hereditary optic neuropathy (LHON) mitochondrial mutation at nucleotide 11778. This maternally-transmitted hereditary optic neuropathy is linked to mutations of mitochondrial DNA, affecting genes coding for subunits of mitochondrial complex I, and thereby reducing the specific activity of this complex (Carelli et al., 2004; Yu-Wai-Man et al., 2009). In fibroblasts from LHON patients, we found a 40% decrease of complex I activity, a decrease in ATP synthesis and an oxidative phosphorylation (OXPHOS) coupling defect (Chevrollier et al., 2008). The first report of a case of LHON related to EMB was followed by several others (De Marinis, 2001; Hwang et al., 2003; Ikeda et al., 2006), suggesting that EMB may be a pharmacological risk factor for the occurrence of LHON.

Autosomal dominant optic atrophy (ADOA) is another hereditary optic neuropathy related to a mitochondrial energetic defect. ADOA is linked to mutations in the *OPA1* gene (Delettre et al., 2000; Alexander et al., 2000) which encodes a GTPase, localized at the inner mitochondrial membrane, and involved in mitochondrial fusion and apoptosis (Olichon et al., 2003; Frezza et al., 2006), as well as mitochondrial DNA maintenance (Amati-Bonneau et al., 2008). We have shown that fibroblasts from ADOA patients present a 25% decrease in complex IV activity, and that LHON and ADOA fibroblasts share a common mitochondrial coupling defect (Chevrollier et al., 2008).

We report the case of a patient, carrying the OPA1_p.I382M mutation, who developed ADOA during EMB treatment, similarly to the LHON cases described previously. Despite strong suspicion of the mitochondrial toxicity of the drug, the direct biological impact of EMB on mitochondrial energetic metabolism has not yet been established. We therefore investigated the effect of EMB on mitochondrial structure and function in primary human skin fibroblast cultures from the ADOA patient, from control subjects, and in neuronal PC12 cells.

2. Patient, material and methods

2.1. Patient

A 62-year-old male patient was followed-up over a period of 30 years in the Department of Ophthalmology for bilateral loss of vision after treatment with ethambutol (EMB). No sign of visual trouble was described before EMB treatment. There was no family history of ocular or neurologic diseases. In 1974, the patient had received EMB (15 mg/kg), isoniazid and rifampicin at standard doses for pulmonary tuberculosis. Three months after the beginning of this treatment, he was admitted to the Department of Ophthalmology for sudden bilateral loss of vision to 3/10 in each eye. Fundus examination showed bilateral papillary pallor. There were no signs of hyperemia or capillary dilatation. Visual field tests revealed bilateral centrocecal scotoma. Colour vision was abnormal with bilateral deuteranopia. Considering the possibility of EMB-related optic neuropathy, the EMB treatment was immediately stopped. However, despite this, the visual acuity of the patient did not improve. The optic discs became paler with time, finally

presenting the aspect of bilateral optic atrophy. In 2005, genetic analysis led to the discovery of a heterozygous mutation in the *OPA1* gene (c.1146A > G; OPA1_p.I382M). Mitochondrial DNA sequencing revealed several polymorphisms excluding all pathological mutations (GenBank accession number: homosapiens GQ859272). This patient belongs to the mitochondrial European haplogroup U4b.

2.2. Sequencing

For the *OPA1* gene analysis, genomic DNA was amplified by PCR with specific primers designed to amplify all exons and flanking intronic regions (Amati-Bonneau et al., 2008). To exclude the presence of any rare mitochondrial DNA mutations, we sequenced entirely the mitochondrial genome. The mtDNA was PCR-amplified in eight fragments and sequenced as described elsewhere (Nochez et al., 2009).

2.3. Cell cultures

Fibroblast primary cultures were made from skin biopsies taken after obtaining written consent from control subjects and from the patient. They were grown in a 75 cm² flask, either in Dulbecco's minimum essential medium (Gibco, Cergy-Pontoise, France) containing 10% fœtal bovine serum (FCS) or in a medium supplemented with 1 mM EMB (Sigma Chemicals St. Louis, MO) for 28 days, or 1–5 mM EMB for 24 h. These concentrations were comparable to the approximate tissue level of EMB in patients with tuberculosis after an intravenous injection of 15 mg/kg EMB (Chung et al., 2009), and our cellular toxicity assay at 5 mM EMB showed no signs of toxicity (Resazurin reduction, CellTiter Blue Promega, Madison, WI, USA). According to manufacturer's instructions, 1000 cells per well (3 wells per sample) were cultured in 96-well plates and luminescence was recorded 10 min after addition of reagent on a multidetection reader for microplates Xenius XML (SAFAS, Monaco). Values are expressed in Relative Light Units (RLU). Results were expressed as the percentage of cell viability of EMB-treated cells to untreated cells. All experiments were conducted on cells with similar passage numbers, ranging from 5 to 20, to avoid artefacts due to senescence.

PC12 cells were grown in RPMI media (Lonza, Levallois-Perret, France), containing 10% FCS, 1% L-glutamine (Invitrogen, Cergy-Pontoise, France) (Letournel et al., 2006) and differentiated using NGF (150 ng/ml) for 10 days (Promega, Charbonnières, France).

2.4. Respiratory parameters in intact cells

To study the patient's mitochondrial metabolism we investigated the respiratory parameters in intact fibroblasts by polarography with a Clark-type oxygen electrode (Rank-Brothers, Cambridge, United Kingdom) as described elsewhere (Loiseau et al., 2007). The basal respiration rate of intact cells ($3 \cdot 10^6$–$5 \cdot 10^6$ cells placed in 500 µl Dulbecco's minimum essential medium at 37 °C) was determined by measuring the linear rate of oxygen consumption. Oligomycin (8 µg/ml) was then added, and the resting respiration rate, i.e. the non-phosphorylating respiration rate, was recorded. Next, the maximal uncoupled respiration rate was measured using 0.5–5.0 µM uncoupler carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP). Finally, respiration was inhibited by sequential addition of 1.25 µM rotenone and 3 µM antimycin. The mitochondrial uncoupling control ratio of the intact cells using substrates of the growth medium (UCRF/O: uncoupling control ratio FCCP/Oligomycin) was calculated as the ratio of oxygen consumed by the fibroblasts in the presence of the uncoupler FCCP (i.e. the response of respiration to the collapse of $\Delta \Psi m$) to that in the pres-