

Superoxide-induced massive apoptosis in cultured skin fibroblasts harboring the neurogenic ataxia retinitis pigmentosa (NARP) mutation in the ATPase-6 gene of the mitochondrial DNA

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The oxidative stress resulting from the neurogenic ataxia retinitis pigmentosa (NARP) mutation in the mitochondrial ATPase 6 gene was investigated in cultured skin fibroblasts from two patients presenting an isolated complex V deficiency. Taken as an index for superoxide overproduction, a huge induction of the superoxide dismutase (SOD) activity was observed in these fibroblasts harboring >90% of mutant mitochondrial DNA. The oxidative stress denoted by the high SOD activity was associated with increased cell death. In glucose-rich medium, apoptosis appeared as the main cell death process associated with complex V deficiency. Complex V-deficient fibroblasts, which showed a high SOD induction and stained positive for all studied apoptosis markers, were successfully rescued by perfluoro-tris-phenyl nitrene, an antioxidant spin-trap molecule. This established that the superoxide production associated with the ATPase deficiency triggered by the NARP mutation could be sufficient to override cell antioxidant defenses and to result in cell commitment to die. The potential participation of superoxides and/or their derivatives in the pathogenic mechanism of specific respiratory chain disorders makes them a promising target for therapy.

INTRODUCTION

Mitochondrial respiratory chain (RC) deficiencies occur in a wide variety of both neurological and non-neurological diseases (1–3). Inherited deficiencies of the five RC complexes and ubiquinone, isolated or in association, have been reported (4–11). Yet, due to the many facets of the mitochondrial function in the cell, the actual consequences of RC defects and the relationship they bear with the clinical expression and course

of the disease are far from being understood. Indeed, the oversimplified view that the involvement of a given tissue essentially reflects its energy requirement gives no clue to understanding the striking heterogeneity of clinical presentation associated with these diseases. As long as mitochondrial diseases were restricted to those where mitochondrial DNA (mtDNA) mutations could be identified, the varying mutant to wild-type mtDNA load in tissues (heteroplasmy) was (and still is) widely advocated to account for tissue-specificity and clinical heterogeneity. However, recent reports of the clinical presentations associated with mutations in several nuclear genes encoding ubiquitously expressed mitochondrial proteins reveal a striking heterogeneity of tissue involvement and clinical presentation as well (6–11).

Indifferent to the complex being affected, RC deficiency should invariably tend to slow down mitochondrial ATP synthesis. Besides decreased ATP synthesis, an RC defect can result in blockade (or slowing down) of the intermediary metabolism, metabolic acidosis, perturbation of cell cation homeostasis and/or overproduction of free radicals (12,13). Additionally, as mitochondria play a prominent role in several forms of apoptosis (through free radical overproduction and/or inter-membrane space components release) and in necrosis (through cellular ATP control), mitochondrial RC deficiency can also be linked to increased cell death (14,15). All these factors can concur to hamper cell or tissue functioning, with differential sensitivity between tissues. The relative contribution of these factors in the development of the pathology is essentially unknown but, besides decreased ATP production, free radical generation is often given prime importance (16–18).

Numerous studies have described the *in vitro* generation of free radicals by isolated mitochondria under various conditions (19–21). In contrast, with few noticeable exceptions (22–24), only scarce reports can be found which actually produce direct or indirect evidences for free radical overproduction *in situ* in human cells with inherited RC deficiency, and increased

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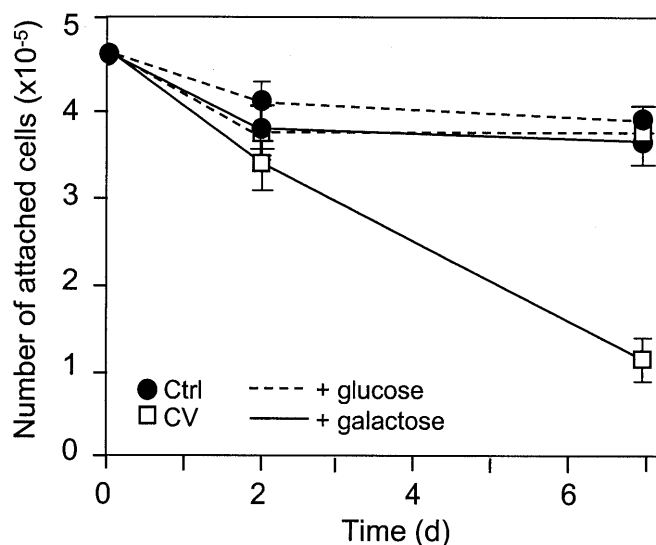


Figure 1. Fate of confluent cultures from control and NARP fibroblasts grown in glucose and glucose-free medium. The plots represent the number of attached cells grown in glucose and glucose-free RPMI1640 supplemented with 10 mM galactose. CV, complex V-deficient fibroblasts harboring the NARP mutation; Ctrl, control fibroblasts. Values are means \pm SD obtained from triplicated experiments obtained on three control cell lines and the NARP fibroblasts from patient 1.

apoptotic features have been only described occasionally (25). Nevertheless, theoretical developments, mostly variations around the so-called vicious circle, have multiplied which give mitochondrial free radical production a central role in numerous diseases and aging as well (26–28). As a result, the idea has emerged that providing antioxidants should be beneficial to patients with an RC disorder (29). However, no conclusive evidence for the efficiency of free radical scavengers in patients with a primary RC deficiency has ever come from any randomized studies.

With the aim to investigate potential reactive oxygen species (ROS) overproduction and its consequences in RC deficiency, we have followed various markers for free radical generation and cell death in human cultured skin fibroblasts harboring the neurogenic ataxia retinitis pigmentosa (NARP) mutation and presenting an ATPase deficiency. For the sake of comparison, some of these parameters were also studied in cultured skin fibroblasts presenting other types of RC deficiency.

RESULTS

Mitochondrial ATP and growth rates of NARP [complex V (CV)-deficient] fibroblast

Because decreased mitochondrial ATP production might reduce cell growth and result in cell death, we first investigated the fate of subconfluent cultures of NARP (patient 1, CV-deficient) and control fibroblasts. When grown in glucose-rich culture medium (added with pyruvate and uridine), no significant differences could be observed between the fate of control and CV-deficient cells (Fig. 1). To figure out the dependence of human cultured skin fibroblasts on the mitochondrial ATP, we

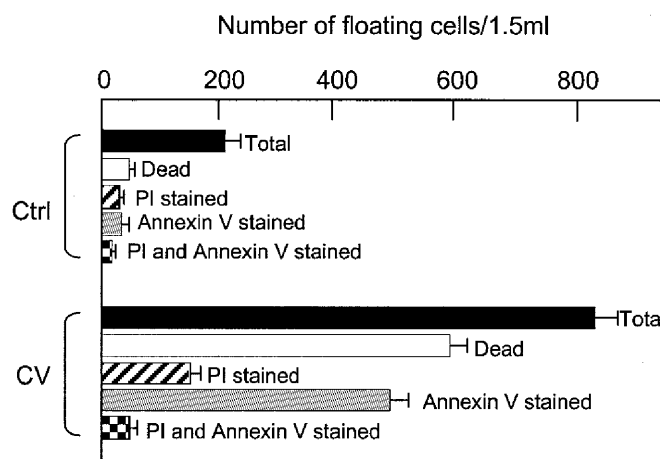


Figure 2. Annexin and PI staining of floating cells in control and NARP fibroblasts. Annexin and PI staining were as described in Materials and Methods. CV, complex V-deficient fibroblasts harboring the NARP mutation; Ctrl, control fibroblasts. Values are means \pm SD obtained from triplicated experiments on three control cell lines and the NARP fibroblasts from patient 1. For the sake of comparison, all studies were carried out on cells harvested after a similar number of population doublings and seeded with a similar density.

next attempted to estimate the ATP produced by the glycolysis and the oxidative phosphorylation in control fibroblasts grown in RPMI 1640. The comparison of the amount of lactate excreted (252 ± 52 nmol/min/mg prot) with the oxygen uptake (7.0 ± 0.3 nmol/min/mg prot) by intact fibroblasts provides a rough estimation of the ATP produced by the glycolysis (250 nmol/min/mg prot) and the mitochondrial respiration (~ 40 nmol/min/mg prot, based on an ADP/O value of 3), respectively. Calculation thus indicates that the glycolysis produces up to five times more ATP than the RC. Obviously, the ATP derived from the glycolysis was sufficient to ensure ATP supply to the RC-deficient cells. Accordingly, we did not observe changes in the ATP content in both patients' 1 and 2 NARP fibroblasts when grown in glucose-rich medium (data not shown).

When galactose, which enters glycolysis slowly compared with glucose, was substituted for glucose, major differences in growth of NARP fibroblasts could be observed (Fig. 1). After 7 days of culture, these cells amounted to less than half of control cells. Noticeably, decreased growth was paralleled with a quantitative increase in the number of floating cells in the culture medium.

Increased cell death is associated with the NARP mutation

While similar growth rates were measured in glucose-rich medium for NARP and control fibroblasts, a 4-fold increased number of floating cells was, however, found in the former. This nevertheless represented a reduced amount of cells (always <1% of attached cells). Increased floating cells should denote rapidly dividing cells and/or accumulation of dead cells. Therefore we next estimated the proportion of floating dead cells by studying their permeability to propidium iodide (PI) and staining with annexin V (Fig. 2). A vast majority ($\sim 80\%$) of the floating cells were stained with annexin V

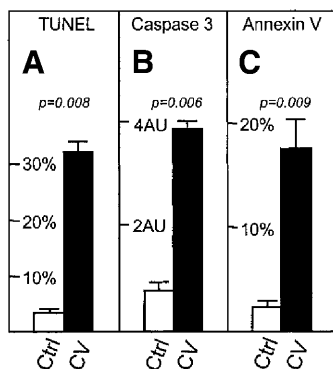


Figure 3. Cell death in attached fibroblasts from controls and patients with NARP mutation. (A) TUNEL-positive cells as a percentage of total cells (≥ 500 cells were counted per condition). (B) Caspase-3 activity measured as described in Materials and Methods. (C) Annexin V-positive cells as a percentage of total cells. *P* values were from *t*-test analyses. CV, complex V-deficient fibroblasts harboring the NARP mutation; Ctrl, control fibroblasts; AU, arbitrary unit. Values are means \pm SD obtained from triplicated experiments on both control and NARP fibroblasts from patient 1.

(Fig. 2), suggesting that these cells were essentially dying through an apoptotic process.

We next studied the initial steps of the death process occurring in NARP mutant cells grown in glucose-rich medium by focusing our analyses on the attached cells. Transferase-mediated dUTP nick-end labeling (TUNEL) staining was first used to reveal DNA fragmentation (Fig. 3A). The number of TUNEL-positive cells increased dramatically in NARP fibroblasts to $>30\%$ compared with the control. Accordingly, a significant increase of caspase 3 activity was noticed (Fig. 3B). Finally, the number of annexin V-positive cells was also significantly increased in NARP fibroblasts (Fig. 3C). A similar number of TUNEL-positive cells was observed in the cultured fibroblasts from patient 2 with the NARP mutation.

A drop of mitochondrial membrane potential ($\Delta\Psi_m$) studied by using different probes, including MitoTracker CMXRos and rhodamine 123, has been shown to be involved at early stages in different apoptotic processes (15). To evaluate $\Delta\Psi_m$ in NARP fibroblasts grown in RPMI plus glucose, we used two approaches and two different probes. On one hand, we carried out an *in situ* observation of attached cells stained with the fluorescent MitoTracker CMXRos probe to possibly observe cell-to-cell differences. On the other hand, we quantitatively studied rhodamine 123 fluorescence in trypsinized fibroblasts after permeabilization with digitonin (0.004% final concentration) supplemented with 10 mM succinate and 20 mM glycerol-3-phosphate as mitochondrial substrates. Neither of these approaches allowed us to provide evidence for any large decrease of $\Delta\Psi_m$ in NARP fibroblasts, suggesting that the predictable loss of $\Delta\Psi_m$ ultimately resulting from cell death would be a late event in this particular apoptotic process.

Oxidative stress induced by the NARP mutation

Superoxides overproduced by a deficient RC are known to possibly trigger cell death (17,25). Such superoxide overproduction can be readily detected by using the naturally

occurring sensor for superoxides, namely the inducible superoxide dismutases (SODs) (30). Noticeably, any artificial probe able to trace superoxides would have to compete with these SODs and might be less specific for the superoxide radicals than the SODs. With the aim to detect a potential increased production of superoxides, we therefore investigated the activity of the inducible SODs in NARP fibroblasts. The activity of both mitochondrial (Fig. 4A) and cytosolic (Fig. 4B) SODs was quite significantly increased. The fibroblasts of a second patient harboring the NARP mutation (nucleotide 8993 in ATPase6 gene; $>90\%$ mutant mtDNA) displayed a quite similar induction of SOD activities [mitochondrial manganese SOD (MnSOD), 47.4 ± 7.7 U/mg prot; cytosolic copper-zinc SOD (CuZnSOD), 19.6 ± 5.7 U/mg prot]. The increased activity of the mitochondrial MnSOD activity was paralleled by an increased amount of its mRNA (Fig. 5A). For the sake of comparison, SOD activities were measured in several other fibroblasts with RC-deficiency (Fig. 4A and B). The SOD activities were not increased in any of the cases except in CII-deficient fibroblasts harboring a mutation in the Fp subunit (R544W) (5).

To complete our survey of the status of the cell antioxidant defenses in the NARP mutant fibroblasts of patient 1, we also studied the expression of Bcl-2, a multifunctional protein with antioxidant properties that might be involved in the control of oxidative stress (31). Western blot analysis revealed that, compared with control, Bcl-2 (ratioed to actin) was moderately increased (~ 3 -fold) in NARP mutant fibroblasts where maximal SOD induction and numerous apoptotic cells could be observed (Figs 3 and 4). As a comparison, Bcl-2 (ratioed to actin) in CII-deficient fibroblasts with very few detectable apoptotic cells and superoxide overproduction was strongly increased (>8 -fold). This indicated that no correlation exists between Bcl-2 expression and superoxides produced by the RC.

We next determined the glutathione level, glutathione-related enzyme activities and catalase activity in the fibroblasts of three controls and of both NARP patients (Fig. 4C–E). Neither glutathione level nor enzyme activities were found significantly changed compared with the control. Similar results were obtained for the different RC-deficient fibroblasts (data not shown).

Finally, to establish that an increased free radical production by the RC might indeed act as an early signal in the cell death cascade without significant loss of $\Delta\Psi_m$ or cytochrome *c* leakage (no decrease of substrate oxidation rates measured), we investigated the effect of perfluoro-tris-phenyl nitron (TAPBN), a spin-trap molecule derived from *N*-ter-butyl- α -phenylnitron (32). A 48 h treatment of NARP fibroblasts with 50 or 100 μ M TAPBN resulted in a significant, although partial, decrease in SOD activities (Fig. 6A). The resulting SOD activities plus the TAPBN antioxidant effect were then sufficient to protect NARP fibroblasts from the oxidative stress. Accordingly, cell death as studied by the TUNEL reaction was strongly decreased in the attached cells, up to a point where dead cells were virtually absent in the culture flask (Fig. 6C–E).

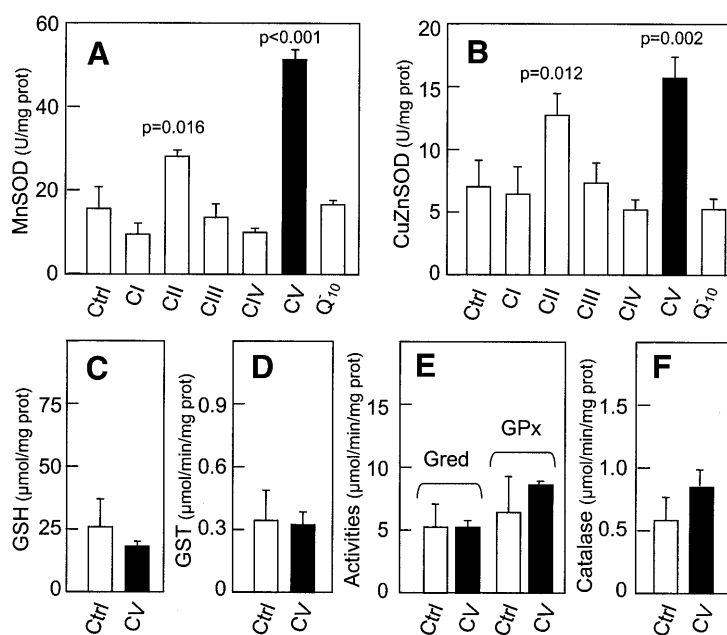


Figure 4. Measurement of antioxidant and marker enzymes in control and RC-deficient fibroblasts. Activities were measured and expressed as described in Materials and Methods. MnSOD, mitochondrial SOD; CuZnSOD, cytosolic SOD; Gred, glutathione reductase; GST, glutathione-S-transferase; GSH, reduced glutathione; CI–CV, Complex I–V deficient patient fibroblasts. Values are means \pm SD obtained from triplicated experiments on three control cell lines and the NARP fibroblasts from patients 1 and 2.

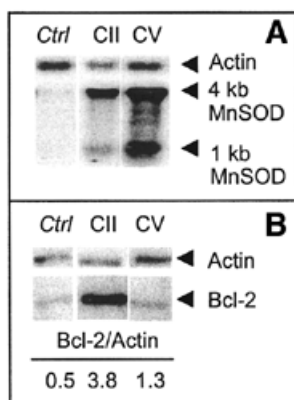


Figure 5. MnSOD mRNA (A) and Bcl-2 protein (B) in control and RC-deficient fibroblasts. (A) Total RNA hybridized with a 32 P-dCTP human MnSOD probe. (B) Western blot analysis of Bcl-2 in RC-deficient fibroblasts. Numbers below the blot represent the amount of Bcl-2 ratioed to actin. CII and CV, complex II and V-deficient fibroblasts harboring the NARP mutation; Ctrl, control fibroblasts.

DISCUSSION

This study was carried out with the aim to examine whether the ROS production potentially associated with the NARP mutation represents a major challenge for cultured skin fibroblasts and could constitute a target for therapy. In humans, cultured cells constitute the only available material allowing extensive experimentation and their study presents obvious drawbacks. In particular, when dealing with disorders showing a pronounced tissue-specificity such as RC deficiencies, conclusions that

may be drawn from such studies should not be over-rated. A second limitation comes from the specificity of each RC defect; any conclusion from this study should be restricted to the particular cases of the CV deficiency associated with the NARP mutation we have studied. Indeed, different defects, even in one given complex, can have different consequences according to their precise location in the complex. For example, the inhibition of electron flow through complex III at the cytochrome *b* donor or acceptor sites does not result in similar superoxide production (33).

First we have shown that the glycolysis generates enough ATP for the RC-deficient fibroblasts to grow and divide if glucose is made available. Moreover, we found no detectable changes in the ATP content in NARP fibroblasts when grown in glucose-rich media (V. Geromel, unpublished data). This implies that any phenomenon observed under this condition has a limited chance to originate from decreased ATP availability. This also makes cultured cells different from several human tissues where mitochondrial ATP is the major source of cellular ATP.

Because the striking variability of clinical involvement observed in inherited mitochondrial disorders cannot simply result from an ATP depletion presumably associated with most RC deficiencies, we have to consider alternative mechanisms and, in particular, superoxide overproduction, as additional factors in the pathogenesis. Under normal physiological conditions, 2–4% of the oxygen consumed by mitochondria is converted to superoxide anions as by-products of the electron transport chain (34). Production of superoxide radicals is known to occur at sites within RC complexes, which implies stabilization of flavin- or semi-quinone radicals (CI–CIII) being increased under conditions of RC over-reduction and

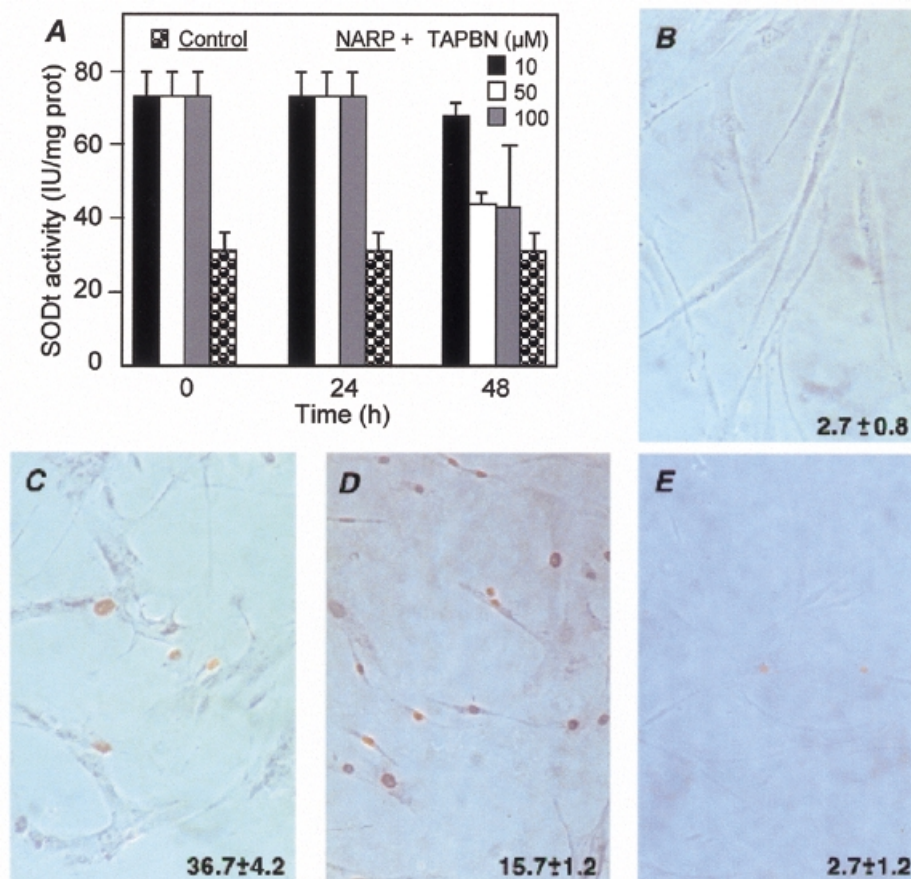


Figure 6. Effect of TAPBN on SOD activity and cell death in CV-deficient fibroblasts. (A) Total superoxide activity (SODt) was measured as described in Materials and Methods before and after 24 and 48 h incubation with the indicated concentration of TAPBN. (B–E) TUNEL staining of control fibroblasts (B) and CV-deficient fibroblasts-harboring NARP mutation (C–E). TUNEL reaction was performed on CV-deficient fibroblasts before (C) or after 24 (D) or 48 h (E) treatment with 50 μM TAPBN. Numbers of TUNEL-positive cells expressed as percentage of total cells (500 cells counted) are indicated in (B–E). Values are means ± SD obtained from triplicated experiments on NARP fibroblasts from patient 1.

high membrane potential (35–38). At variance with most other RC deficiencies, a blockade of the ATPase presumably results in a high membrane potential, a condition known to favor the production of superoxides by the electron transfer chain (39).

To date an elevation in the MnSOD protein—denoting an increased rate of superoxide production—has been reported in fibroblasts from a limited number of CI-deficient patients (17,22). In addition, increased SOD has been immuno-cytochemically detected in the ragged-red-fiber rich regions of muscle section in patients harboring deletions of mtDNA (23,24). We have shown here that in cultured fibroblasts with NARP mutation, both MnSOD and CuZnSOD are strongly induced. We also observed massive apoptosis in these ATPase-deficient cells. Both SOD induction and apoptosis were readily abolished by using TAPBN, an efficient spin trap (32), thus providing strong evidence for a primary role of superoxides or their derivatives in both processes.

Our results suggest that superoxide overproduction associated with the NARP mutation can be sufficient to override cell antioxidant defenses and trigger apoptosis. This superoxide-induced apoptosis might bear some relationship with the degenerative lesions observed in the brain of the NARP

patients who presented a typical Leigh syndrome characterized by symmetrical bilateral necrotic lesions in the brain stem, basal ganglia, thalamus and spinal cord (1) and thus represent a promising target for therapy. We also observed quite different consequences, e.g. variable SOD induction and cell death, associated with different RC deficiencies. Delineation of the mechanisms leading to cell dysfunction or death specifically associated with each type of RC defect may provide new clue to devise therapeutic strategies, as recently illustrated in the case of Friedreich's ataxia (40).

MATERIALS AND METHODS

Cell cultures

Fibroblast cultures were established from a skin biopsy from six controls and seven patients presenting a RC deficiency. Two patients (patients 1 and 2) presented a Leigh syndrome associated with the NARP mutation (nucleotide 8993 in the ATPase6 gene, >90% mutant mtDNA). Complex II deficient patient presented a R544W mutation in Fp subunit, complex III deficient patient presented a cytochrome *b* mutation (nucleotide

15246, >99% mutant mtDNA). Three additional patients presenting complex I, IV or quinone deficiency (of yet unknown molecular origin) were also studied for the sake of comparison. The clinical presentation of the seven patients has been previously reported (4,5,41–44). Cells were grown in RPMI 1640 (Life Technologies SARL) supplemented with glutamax (446 mg/l), 10% undialyzed fetal calf serum, 100 µg/ml streptomycin, 100 IU/ml penicillin, 200 µM uridine and 2.5 mM sodium pyruvate, at 37°C under standard conditions (45). Glucose was changed to galactose (10 mM) in the selection medium for respiratory-competent cells (46). For the sake of comparison, all studies were carried out on cells harvested after a similar number of population doublings. Viability was estimated from cell counting at days 2 and 7.

Lactate and oxygen uptake determination

Excretion of lactate by intact fibroblasts was spectrofluorimetrically recorded in a magnetically-stirred 3 ml cell maintained at 37°C by measuring the reduction of a large excess of both exogenously added NAD⁺ (1 mM final concentration) and porcine muscle purified L-lactic dehydrogenase (20 U/ml; Sigma-Aldrich Chimie) in 1 ml of medium containing 0.5 M glucose, 10 mM KCl, 5 mM MgCl₂, 1 mg/ml bovine serum albumin and 10 mM KH₂PO₄ (pH 7.4). Oxygen uptake by intact fibroblasts was simultaneously measured under the same conditions, as described previously (47).

Enzyme measurements

Enzyme measurements were performed on freeze-thaw cell lysates re-suspended in 50 mM KH₂PO₄ (pH 7.8). CuZnSOD plus MnSOD (EC 1.15.1.1) activities were determined by monitoring the autoxidation of pyrogallol at 420 nm (48). Pre-incubation of the sample with 2 mM cyanide for 30 min at room temperature was used to inhibit CuZnSOD. Activities were expressed as IU/mg protein. Glutathione peroxidase (GPx; EC 1.11.1.9) activity was spectrophotometrically determined by following NADPH oxidation in a coupled assay with glutathione reductase (GRed) using t-butyl hydroperoxide as substrate (49). The reaction medium consisted of 0.5 mM reduced glutathione (GSH), 1 mM t-butyl hydroperoxide, 100 U/l yeast GRed (Type III; Sigma Chemical), 75 µM NADPH and 0.5 mM KCN in 1 ml of 0.1 mM Na₂HPO₄ (pH 7.0). GRed (EC 1.6.4.2.) activity was spectrophotometrically assayed in 1 ml of 0.1 M Na₂HPO₄ (pH 7.4) by following the oxidation of 75 µM NADPH in the presence of 0.5 mM oxidized glutathione (GSSG) (50). Glutathione-S-transferase (GST; EC 2.5.1.18) activity was measured using 30 mM 1-chloro-2,4-dinitrobenzene as substrate (51,52). The S-conjugate accumulation was spectrophotometrically followed at 340 nm. Assays were performed in duplicate on three samples for each control or patient on an automatic Cobas-Bio centrifugal analyzer (Hoffman-La Roche) (53). Cyanide-sensitive catalase (EC 1.11.1.6) activity was polarographically measured at 37°C in 500 µl of 0.1M KHPO₄ (pH 7.0) using a Clark oxygen electrode (Hansatech Instrument) (54). All enzyme activities were measured at 37°C and expressed as µmol/min/mg protein. Protein was measured by the Pierce method (55). Chemicals were of the highest purity available from Sigma-Aldrich Chimie.

Determination of glutathione levels

Glutathione was fluorimetrically measured on aliquots of the supernatant (10 000 g × 5 min) of freeze-thaw cell pellets treated with Triton X-100 (0.4% final concentration) using 100 µM mono-chlorobimane and 1 U GST in 1 ml of oxygenated Krebs-Henseleit buffer (120 mM NaCl, 5 mM KCl, 1 mM KH₂PO₄, 1.2 mM MgSO₄, 25 mM NaHCO₃, 2.5 mM CaCl₂ and 25 mM HEPES pH 7.4) (56).

Mitochondrial membrane potential

Membrane potential was spectrofluorimetrically monitored using 1 µM rhodamine 123 (excitation, 525 nm, 5 nm band-pass; emission, 558 nm, 5 nm band-pass; LS 50B spectrofluorimeter; Perkin-Elmer), in 2 ml of a medium containing 0.5 M glucose, 10 mM KCl, 5 mM MgCl₂, 1 mg/ml BSA, 10 mM KH₂PO₄ (pH 7.4, 37°C) and 0.01% digitonin to allow exogenous reducing substrates (succinate, glycerol-3-phosphate) to be oxidized by the mitochondria (57). Alternatively, attached fibroblasts were labeled for 5 min with 1 µM MitoTracker CMXRos probe (Molecular Probes) and examined for fluorescence (Leitz DM IL; Leica Microscopy and Scientific Instruments Group).

Cell death and death factors

The immunohistochemical detection of DNA fragmentation was performed on fibroblasts grown on slides at a density of ~20 000 cells/chamber using an *in situ* terminal deoxynucleotidyl TUNEL assay (cell death detection kit; Boehringer Mannheim). After chromogenic substrate reaction, apoptotic and non-apoptotic cells were counted under a light microscope (Leitz DM IL, Leica Microscopy and Scientific Instruments Group).

Fluorescent determination of phosphatidyl serine was performed using annexin V in cells counter-stained with PI. Cell pellet (~10⁶ cells) were re-suspended in 100 µl of a reaction medium containing annexin V, PI and HEPES buffer following the manufacturer's instructions (annexin V-fluorescence staining kit, Boehringer Mannheim), incubated in the dark for 15 min and examined for fluorescence.

Caspase-3 activity was measured using caspACE-3 colorimetric assay kit (Promega) following the manufacturer's instructions. Approximately 4 × 10⁵ cells (25–50 µg proteins) were used. The activity was spectrophotometrically measured at 405 nm following the release of the p-nitro-analide from the caspase-3 substrate Ac-DEVD-pNA.

RNA analysis

For northern blotting, total RNA (10 µg) was prepared as described by Chomczynski and Sacchi (58), electrophoresed for 12 h through a 1.4% agarose/16.2% formaldehyde gel and transferred onto a nylon membrane (Hybond N+) by upward capillary transfer. The membrane was hybridized with a ³²P-dCTP MnSOD probe (nucleotides 98–782) in ExpressHyb hybridization solution (Clontech) according to the manufacturer's recommendations.

Protein analysis

For western blot analyses, subconfluent cultured cells were scrapped, resuspended in PBS and centrifuged for 10 min at 3000 g. Lysis buffer (150 mM NaCl, 50 mM Tris-HCl pH 7.5, containing 0.25% sodium deoxycholate, 10 µg/ml aprotinine, 50 µg/ml leupeptine, 1 mM phenylmethylsulfonyl fluoride and 1mM NaF) was added to the cell pellet (100 µl/10⁶ cells) (59). After centrifugation (15 000 g × 30 min), pelleted protein was electrophoresed on a SDS-polyacrylamide gel (12% acrylamide, 0.12% bis-acrylamide) and transferred onto a nylon membrane (Immobilon, Millipore). Blots were sequentially hybridized overnight at 4°C in 5% non-fat milk and 0.1% Tween 20 with mouse monoclonal antibody raised against Bcl-2 (1:200 dilution; Dako) and with mouse monoclonal anti-actin antibody (1:4000 dilution; Chemicon International) for quantification.

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