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Coenzyme Q_{10} Depletion is Comparatively Less Detrimental to Human Cultured Skin Fibroblasts than Respiratory Chain Complex Deficiencies

VANNA GEROMEL, AGNÈS RÖTIG, ARNOLD MUNNICH and PIERRE RUSTIN*

Unité de Recherches sur les Handicaps Génétiques de l'Enfant (INSERM U393) Hôpital Necker-Enfants-Malades, 149, rue de Sèvres, 75743 Paris cedex 15, France

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The oxidative stress possibly resulting from an inherited respiratory chain (RC) deficiency was investigated in a series of human cultured skin fibroblasts presenting either ubiquinone depletion or isolated defect of the various RC complexes. Taken as an index for superoxide overproduction, a significant induction of superoxide dismutase activity was observed in complex V-deficient fibroblasts harboring the NARP-mutation in the ATPase 6 gene. Superoxide dismutase induction was also noticed, albeit to a lesser extent, in complex II-deficient fibroblasts with a mutation in the nuclear gene encoding the flavoprotein subunit of the succinate dehydrogenase. No sign of oxidative stress could be found in ubiquinone-depleted fibroblasts. In all cases but complex IV-defect, increased oxidative stress was associated with increased cell death. In glucose-rich medium, apoptosis appeared as the main cell death process associated with all types of RC defect. However, similar to the great variations in oxidative stress associated with the various types of RC defect, we found that apoptotic features differed noticeably between defects. No indication of increased cell death was found in ubiquinone-depleted fibroblasts.

Keywords: Mitochondria; Respiratory chain defects; Coenzyme Q_{10}; Superoxide; Apoptosis

Abbreviations: CI–CV, the various complexes of the respiratory chain; RC, respiratory chain; ROS, reactive oxygen species; MnSOD, mitochondrial manganese superoxide dismutase; CuZn-SOD, cytosolic copper–zinc superoxide dismutase; PI, propidium iodide

INTRODUCTION

Due to the many facets of the mitochondrial function in the cell, the actual consequences of a mitochondrial respiratory chain (RC) defect and the relationship they bear with the clinical expression and course of the disease are far to be understood.[1] The oversimplified view that the involvement of a given tissue reflects its energy requirement gives no clue to understand the striking heterogeneity of clinical presentation associated with these diseases. In keeping with this, mutations in several nuclear genes encoding ubiquitously expressed mitochondrial proteins reveal a striking heterogeneity of tissue involvement and clinical presentation[1].

Besides a decreased ATP synthesis, a RC defect can result in a blockade (or slowing down) of the intermediary metabolism, a metabolic acidosis, a pertubation of cell cation homeostasis (calcium, iron, magnesium, etc) and/or an overproduction of free radicals. Additionally, as mitochondria play a prominent role in several forms of apoptosis and in necrosis,[2] a mitochondrial RC deficiency can also be linked to an increased cell death. 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. With few noticeable exceptions, only scarce reports can be found which

*Corresponding author. Tel.: +33-1-44-38-15-84. Fax: +33-1-47-34-85-14. E-mail: rustin@necker.fr.

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actually produce direct or indirect evidences for free radical overproduction *in situ* in human cells with inherited RC deficiency[3] and increased apoptotic features have been only occasionally described.[4]

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 with different types of RC deficiency, encompassing isolated defects of complex I–V and depletion of ubiquinone.

MATERIALS AND METHODS

Cell Culture

Fibroblast cultures were established from a skin biopsy from controls and patients presenting a RC deficiency. The clinical course and presentation of the six patients have been previously reported.[5–10] Cells were grown as described.[11] Glucose was changed to galactose (10 mM) in the selection medium for respiratory-competent cells.

Enzyme Assays

Enzyme measurements were performed as described[12] on freeze-thaw cell lysates re-suspended in 50 mM KH_2PO_4 buffer (pH 7.8). Proteins were measured using the Pierce method.

Cell Death

The immunohistochemical detection of apoptosis was achieved according to the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using an *in situ* cell death detection kit (Boehringer Mannheim). Fluorescent determination of phosphatidyl serine was performed using annexin V in cells counter-stained with propidium iodide (PI). Caspase-3 activity was measured using caspACE-3 colorimetric assay kit (Promega Corporation, Madison, USA) following the manufacturer's instructions. Approximately 4×10^5 cells (25–50 μg proteins) were used. The activity was spectrophotometically measured at 405 nm following the release of the P-nitro-analide from the caspase-3 substrate Ac-DEVD-pNA.

RESULTS

When cells were grown in a glucose-rich culture medium (RPMI 1640 added with pyruvate and uridine), no significant differences could be observed between control and RC-deficient cells (not shown). However, when galactose was substituted to glucose major differences in cell survival could be observed (Fig. 1). After 48 h-culture, CI-, CIII-, and CIV-deficient cells amounted to 76, 70 and 55% of control cells, respectively. Five days later, the difference was even more marked. With the exception of quinone-depleted fibroblasts, all the RC-deficient cells represented less than half of the control cells. Comparing the cell ability to survive in a glucose depleted medium with their residual respiration (Fig. 1, inset) revealed that the relatively unaffected survival of quinone-depleted fibroblasts corresponded to the highest residual cell respiration (about 60% of control respiration).

When grown in glucose, a reduced (always less than 1% of attached cells) but varying number of floating cells were observed in association with several RC deficiencies. From a three to four fold increased number of floating cells was found for CI-, CII-, CIII- and CV-deficient cells as compared to control. Increased floating cells should denote rapidly dividing cells and/or accumulation of dead cells. We therefore next estimated the proportion of floating dead cells by studying the cell permeability to PI and staining with annexin V (Fig. 2). A vast majority (between 70 and 80%) of the floating cells were stained with annexin V in the case of CI-, CII-, CIII- and CV-deficiencies (Fig. 2), suggesting that these cells were essentially dying through an apoptotic process. In the cases of quinone-depleted and CIV-deficient fibroblasts, the number of PI and/or annexin V-reactive cells was low as it was in control, indicating that most floating cells were dividing cells.

In glucose-rich medium, the number of TUNEL-positive attached cells increased dramatically, more than 30%, in both CI- and CV- deficient fibroblasts

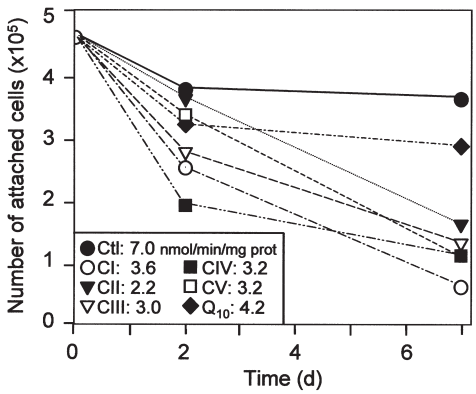

FIGURE 1 Survival of control and respiratory chain-deficient fibroblasts in glucose-free medium. The plots represent the number of attached cells grown in glucose-free RPMI1640 supplemented with 10 mM galactose. *Inset:* residual respiration (nmol O_2/mn/mg protein). CI–V: Complex I–V-deficient fibroblasts; Q_{10}: ubiquinone-depleted fibroblasts; Ctrl: control fibroblasts. Data points are means of three replicates on each patient or on three different controls.

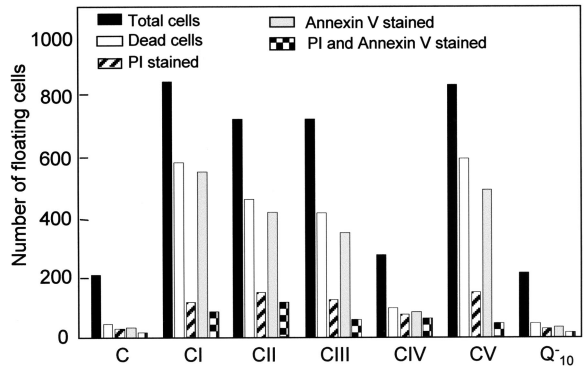

FIGURE 2 Annexin and propidium iodide (PI) staining of floating cells in control and respiratory chain-deficient fibroblasts. Annexin and PI staining as described under experimental procedures. CI–CV: Complex I–V-deficient fibroblasts; Q_{10}^-: ubiquinone-depleted fibroblasts. Ctrl: control fibroblasts. Values are means of three replicates on each patient or on three different controls.

(Fig. 3A). A lower increase (about 15%) was found in CII-deficient cells. Other RC-deficient fibroblasts displayed a low number of TUNEL-reactive cells comparable to control (Fig. 3A). Accordingly, the number of annexin V-positive cells was significantly increased only in the cases of CI-, CII-, and CV-deficient fibroblasts (Fig. 3B). Finally, a significant increase of caspase 3 activity was noticed in the case of CIII–V-deficient fibroblasts (Fig. 3C).

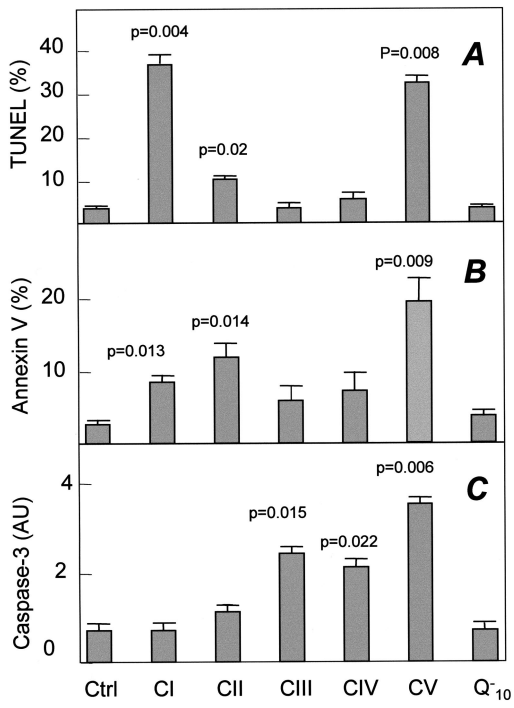

FIGURE 3 Cell death in attached fibroblasts from control and patients with respiratory chain-deficiency. A, B: TUNEL-positive (A) and annexin-positive (B) cells as a percent of total cells (at least 500 cells were counted per each condition). C: Caspase-3 activity was measured as described under Experimental procedures. Values are means of three replicated on each patient or on three different controls.

Noticeably, CIII- and CIV-deficient attached cells showed a significant caspase-3 activation, although annexin-positive cells were not significantly increased in these two cultures.

Superoxides overproduced by a deficient RC are known to possibly trigger cell death.[12] With the aim to detect a potential increased production of superoxides, we investigated the activity of the inducible-superoxide dismutase in our series of RC-deficient fibroblasts. The activity of both mitochondrial and cytosolic superoxide dismutases was spectacularly increased in the case of CV-deficiency and to a lesser extent in the case of CII-deficiency (Fig. 4A and B). Because the activation of the antioxidant systems evidenced in some RC-deficient fibroblasts denotes an increased load of pro-oxidants, we next looked for the consequences of such an overload in these cells. We studied the activity of aconitase, an iron–sulfur protein exquisitely sensitive to superoxides as a marker for oxidative degradations. No specific decrease of this latter enzyme activity (ratioed to the isocitrate dehydrogenase) was observed in any of the attached RC deficient fibroblasts, suggesting that mitochondrial manganese superoxide dismutase (MnSOD) induction was sufficient to cope with the

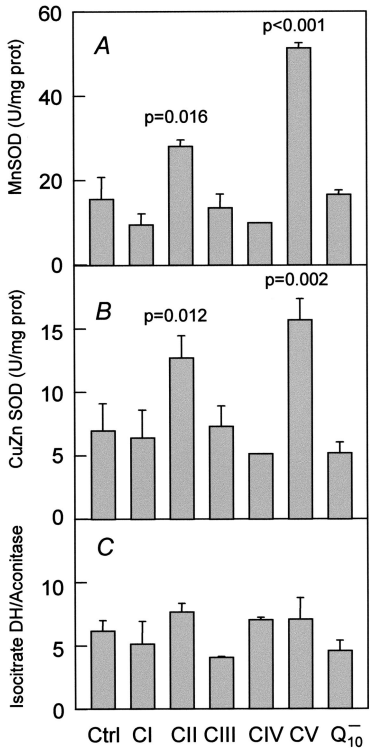

FIGURE 4 Superoxide dismutase activity and isocitrate dehydrogenase to aconitase ratio in fibroblasts from control and patients with respiratory chain deficiency. A, B: Mitochondrial (A) Mn- and cytosolic (B) CuZn-superoxide dismutase activity. C: Isocitrate dehydrogenase to aconitase activity ratio. Enzyme activities were measured as referred under experimental procedures. Values are means of three replicates on each patient or on three different controls.

increased superoxide production by RC-deficient fibroblasts (Fig. 4C).

DISCUSSION

This study was carried out with the final aim to examine if the ROS production triggered by inherited RC-deficiencies represents a major challenge for the cells and might constitute a promising target for therapy. Because of the well-established tissue specificity of RC diseases, conclusions drawn from this study should be restricted to fibroblasts and to the particular cases of the molecularly defined deficiencies we have studied. 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,[1] we have to consider alternative mechanisms and, in particular, superoxide overproduction which might act as an additional factor in the pathogenesis.[12]

We have shown here that the nature of the deficient complex largely determines the extent of the SOD induction. In cultured fibroblasts with CV- or CII-deficiency, both MnSOD and cytosolic copper–zinc superoxide dismutase (CuZnSOD) were induced. The induction of apoptosis associated with increased SOD activity suggests a deleterious effect of superoxides and/or their derivatives. Ubisemiquinone represents the major site of superoxide production in the RC. Accordingly, no induction of SODs was evidenced in ubiquinone-depleted cells.[13]

In cultured cells grown in a glucose-rich medium, we have found increased cell death associated with CI, CII, CIII and CV deficiencies. Increased cell death was always associated with typical features of apoptosis. Apoptosis can be induced by a number of oxidative stresses.[14] Here, we have looked for overproduction of superoxides (as detected by SOD induction) and have observed striking differences according to the type and location of the defect in the chain. CV deficiency associated the highest rate of cell death to the highest SOD induction. A lesser increase of SOD activity, particularly MnSOD, was observed in CII-deficient fibroblasts harboring a mutation in the gene encoding the flavoprotein of the SDH. This was too associated with increased cell death. CI- and CIII-defective fibroblasts showed increased cell death without clear-cut evidence in support of any defined cell death-inducing pathway. If most floating dead cells stained with both Pi and annexin V suggestive of an apoptotic process, caspase-3 activity was only increased in attached CIII-deficient fibroblasts, while no significant induction of SOD could be detected in association with these two RC defects. In fact increased superoxides and apoptotic features have been inconsistently found in CI-deficient fibroblasts.[15] In the absence of defined molecular bases, it is difficult to further interpret these differences that may simply reflect various locations of electron flow blockade into the complex. CIV-deficient fibroblasts showed a dramatic decrease of cell growth in glucose-free medium, which was not associated with increased cell death. In this case, a decreased mitochondrial ATP production might account for slow growth, since normal growth was observed in glucose-rich medium. Finally, to our surprise, the consequence of the ubiquinone-depletion appears even milder since growth remained essentially unaffected even in glucose-free medium. In this latter case, a higher residual respiration might be sufficient to support sub-normal ATP generation and therefore cell growth in glucose-free medium. Neither induction of SOD nor sign of fibroblasts suffering from ubiquinone-depletion was noticed in either the presence or absence of glucose.

In this study, we have shown that superoxide overproduction by a defective RC can be sufficient to override cell antioxidant defenses and therefore might be part of a pathogenic mechanism. 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.[16]

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