THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 278, No. 6, Issue of February 7, pp. 4145–4150, 2003
Printed in U.S.A.

Leber's Hereditary Optic Neuropathy (LHON) Pathogenic Mutations Induce Mitochondrial-dependent Apoptotic Death in Transmitochondrial Cells Incubated with Galactose Medium*

Received for publication, October 8, 2002
Published, JBC Papers in Press, November 21, 2002, DOI 10.1074/jbc.M210285200

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Leber's hereditary optic neuropathy (LHON), a maternally inherited form of central vision loss, is associated with mitochondrial DNA pathogenic point mutations affecting different subunits of complex I. We here report that osteosarcoma-derived cytoplasmic hybrids (cybrid) cell lines harboring one of the three most frequent LHON pathogenic mutations, at positions 11778/ND4, 3460/ND1, and 14484/ND6, undergo cell death when galactose replaces glucose in the medium, contrary to control cybrids that maintain some growth capabilities. This is a well known way to produce a metabolic stress, forcing the cells to rely on the mitochondrial respiratory chain to produce ATP. We demonstrate that LHON cybrid cell death is apoptotic, showing chromatin condensation and nuclear DNA laddering. Moreover, we also document the mitochondrial involvement in the activation of the apoptotic cascade, as shown by the increased release of cytochrome *c* into the cytosol in LHON cybrid cells as compared with controls. Cybrids bearing the 3460/ND1 and 14484/ND6 mutations seemed more readily prone to undergo apoptosis as compared with the 11778/ND4 mutation. In conclusion, LHON cybrid cells forced by the reduced rate of glycolytic flux to utilize oxidative metabolism are sensitized to an apoptotic death through a mechanism involving mitochondria.

In 1988, Leber's hereditary optic neuropathy (LHON)[1] has been the first human pathology to be associated with a mitochondrial DNA (mtDNA) point mutation (1). However, its pathogenesis remains poorly understood (2–4). Currently, three most frequent (11778/ND4, 3460/ND1, 14484/ND6) (2–4) and other rare (14459/ND6, 10663/ND4L, 4171/ND1, 14482/ND6) (5–8) pathogenic mutations are found in the majority of patients affected with this maternally inherited form of optic neuropathy. LHON is due to a massive acute or subacute retinal ganglion cell death, characteristically leading to central vision loss (2–4). These pathogenic mutations invariably affect complex I subunits, which possibly interact with the quinone substrate (9, 10), and a combination of partial respiratory deficiency and increased oxidative stress is documented to be the pathological consequence in transmitochondrial cell systems (11–15). However, the mtDNA pathogenic mutations are a necessary, but not sufficient, condition to actually develop LHON, as suggested by the variable penetrance (2, 16). Thus, other mitochondrial or nuclear genetic factors, as well as environmental factors, are believed to be necessary for triggering the optic neuropathy. At the present time, most of these factors are not well defined. One potential modulator is possibly represented by the mtDNA background, as indicated by the association of some of the pathogenic mutations (11778/ND4, 14484/ND6, 10663/ND4L) with a specific mtDNA haplogroup characteristic of the European population, classified as J (2, 6, 16).

LHON has many other peculiar and a yet unexplained features. Among these, we emphasize the age-dependent, highly tissue-specific, mostly apoplectic, and wave of retinal ganglion cell death in the absence of classical signs of inflammation (16). Vascular signs, such as microangiopathy and small vessel tortuosity at the optic nerve head, usually precede the onset of the disease (16). Some of these features are thought to suggest a prevalent apoptotic mode of cell death (17), and by analogy with other pathological conditions, such as glaucoma, in which apoptosis has been directly documented (17, 18).

Growth impairment of transmitochondrial cytoplasmic hybrids (cybrids) carrying the most frequent LHON pathogenic mutation at position 11778/ND4 has been first reported by Hofhaus *et al.* (19) when cells were incubated in a medium containing galactose in place of glucose (19). This finding was in agreement with previous studies showing that cultured cells with defective mitochondrial metabolism often die in glucose-free galactose medium (20, 21). Indeed, in glucose medium, most of the total ATP yield of proliferating cells is produced by glycolytic glucose breakdown to lactate, and only a minimal amount of pyruvate is oxidized to CO_2 and water. Galactose can also enter the glycolytic pathway; however, the restricted flow of galactose to glucose-6-phosphate determines the formation of very little lactate since pyruvate, which is formed at a much slower rate, is further oxidized within the mitochondria (21).

In the present study, we have characterized the loss of cell viability of osteosarcoma-derived cybrids containing the three

* This work was supported by grants from PRIN 2001–2002: "Mitochondria in Cellular Pathology" (to M. R.); Progetto Dipartimentale "Approcci molecolari e genetici allo studio delle patologie" (to M. R.), "Progetto Giovani Ricercatori" (to A. G.); and from the University of Bologna; and Telethon Grant n.GGP02323 (to V. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: LHON, Leber's hereditary optic neuropathy; mtDNA, mitochondrial DNA; cybrid, cytoplasmic hybrids; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMEM, Dulbecco's modified Eagle's medium; TK, thymidine kinase.

TABLE I
Lhon mutations and mtDNA haplogroups of the cell lines utilized in the present study

Cell line	LHON pathogenic mutation	MtDNA haplogroup
Osteosarcoma parental cell line		
143B.TK$^-$	None (control cell line)	Haplogroup X
Cybrid cell line (multiple clones)		
HPC (control cell line)(15, 24, 25)	None	Haplogroup H
HGA (control cell line)(15, 24, 25)	None	Haplogroup J (4216/ND1, 13708/ND5)
HFF (LHON, affected)(11, 15, 24)	11778/ND4	Haplogroup U
HPE (LHON, affected)(11, 15, 24)	11778/ND4	Haplogroup J (4216/ND1, 13708/ND5)
HMM (LHON, affected)(15, 24)	3460/ND1	Haplogroup H
RJ206 (LHON, affected)(23, 24)	3460/ND1	Haplogroup TV
HBA (LHON, affected)(15)	14484/ND6	Haplogroup J (4216/ND1, 13708/ND5)
HL180 (LHON, affected)(15)	14484/ND6	Haplogroup J (4216/ND1, 13708/ND5)

main LHON pathogenic mutations (11778/ND4, 3460/ND1, 14484/ND6) grown in a medium in which glucose was replaced by galactose. We show that LHON cybrids, but not control cybrids or the parental osteosarcoma cell line (143B.TK$^-$), died of apoptosis. It is widely accepted that the apoptotic process is triggered through two major mechanisms, one involving the engagement of plasma membrane-associated death receptors and another involving the participation of mitochondria (reviewed in Ref. 22). Given that the only relevant difference between control and LHON cybrids is the presence of mtDNA mutations, our investigation focused on the possible direct involvement of these organelles in the apoptotic death triggered by a metabolic stress.

EXPERIMENTAL PROCEDURES

Materials—MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), orange acridine, ethidium bromide, and protease inhibitors mixture were purchased from Sigma. Hoechst-33342 was from Calbiochem. Anti-cytochrome *c* and secondary antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Lines and Culture Conditions—Cybrid cell lines were constructed using enucleated fibroblasts from two controls and six LHON probands as mitochondria donors and the osteosarcoma (143B.TK$^-$)-derived 206 cell line as acceptor rho^0 cell line (both 143B.TK$^-$ and rho^0 206 were kindly provided by Giuseppe Attardi and Michael King). Table I lists the cybrid cell lines used in this study and their origin (11, 15, 23–25). Both definition of the mtDNA haplogroup and identification of the LHON pathogenic mutations were performed by PCR/restriction fragment length polymorphism method carried out as reported previously (24, 25). Parental and cybrid cell lines were grown in DMEM medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.1 mg/ml bromodeoxyuridine. For the experiments, cells were seeded 4×10^5 cells/cm^2 and incubated in DMEM glucose-free medium supplemented with 5 mM galactose, 5 mM sodium pyruvate, and 5% fetal calf serum (DMEM galactose medium) at 37 °C in an incubator with a humidified atmosphere of 5% CO_2.

Cell Viability Assay—The tetrazolium salt MTT was used to determine the cell viability, as described in Ref. 26. Cells were seeded into 24-well dishes and incubated in DMEM galactose medium for different times, and then MTT was added to the final concentration of 0.5 mg/ml. After 3 h, 5% SDS and 5 mM HCl were added to solubilize the formazan salt crystals, and dishes were kept overnight in the incubator. Absorbance of solubilized formazan salts was measured with a Jasco spectrophotometer V-550 (Tokyo, Japan) at the wavelength of 570 nm. For each experiment, a calibration curve was constructed by seeding the cells at concentrations ranging from 10^4 to 10^5 and measuring the corresponding value of MTT absorbance.

Chromatin Condensation and DNA Fragmentation—Nuclear morphology was assessed in cells detached from the dishes at the times indicated, stained with 2 μg/ml Hoechst for 30 min at 37 °C, and fixed with 3% (w/v) paraformaldehyde. Nuclei were observed with a digital imaging system composed of an inverted epifluorescence microscope Nikon Eclipse 300 with a back-illuminated CCD camera (Princeton Instruments, Trenton, NJ) and acquisition/analysis software Metamorph (Universal Imaging Corp., Downingtown, PA) (27). Quantitative determination of apoptotic and necrotic cells was obtained by double staining with orange acridine and ethidium bromide. Both the dyes intercalate into DNA; however, only orange acridine can cross the plasma membrane and stain all the cells, whereas ethidium bromide is excluded from viable and early apoptotic cells, which have an intact plasma membrane (28). Briefly, cells were seeded in 6-well plates and incubated with DMEM galactose medium for different times and then incubated with 3 μg/ml orange acridine and 15 μg/ml ethidium bromide and examined with an inverted fluorescence microscope (Nikon, Tokyo, Japan). Early or late apoptotic cells exhibited green or orange nuclei with condensed chromatin, respectively. Necrotic cells showed orange nuclei with intact structure. For analysis of DNA fragmentation, cells were seeded into 25-cm^2 flasks in DMEM galactose medium for the times indicated and then harvested, and DNA was extracted as described in Ref. 29. DNA was separated by electrophoresis in 1% agarose gel and stained with ethidium bromide.

Subcellular Fractionation and Western Blot Analysis—After incubation in DMEM galactose medium, cells were harvested from five 75-cm^2 flasks, resuspended in 0.5 ml of 200 mM mannitol, 70 mM sucrose, 1 mM EGTA, 10 mM Hepes (pH 7.6), 100 μl/ml protease inhibitors mixture and homogenized for 30 strokes with a Dounce homogenizer. This and the subsequent steps were carried out at 4 °C. Samples were centrifuged for 10 min at $500 \times g$, and the resulting supernatant was centrifuged for 20 min at $10,000 \times g$. The supernatant (cytosolic fraction) was stored at −80 °C. Protein content of fractions was determined as described in Ref. 30. 80–100 μg of protein of cytosolic fraction were separated by 15% SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad). The membrane was treated with 5% non-fat milk in Tris-buffered saline-Tween 0.05% for 1 h and incubated with the cytochrome *c* primary antibody diluted 1:500 for 1 h at room temperature. Antigen-antibody complexes were detected by using horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 in Tris-buffered saline-Tween 0.05%, supplemented with 5% non-fat milk and incubated for 20 min at room temperature. The chemiluminescence signals were revealed using an ECL Western blotting kit (Amersham Biosciences) and measured with the Fluo-2 MAX Multimager system (Bio-Rad).

RESULTS

Growth Capability of Control and LHON Cybrids—Specific growth impairment has been reported previously in cells carrying the 11778/ND4 LHON mutation incubated in a medium containing galactose in place of glucose (19). Here, this observation has been extended to the other LHON pathogenic 3460/ND1 and 14484/ND6 mutations. The parental 143B.TK$^-$ cell line, two control cybrid cell lines, and two different cybrid cell lines for each of the three primary LHON mutations were incubated in galactose medium, and changes in cellular viability were determined. Results are shown in Fig. 1.

Fig. 1*A* shows that 143B.TK$^-$ cells and control cybrids exhibited a very similar behavior: after 1 day of incubation, a small decrease in the number of viable cells was detected, and then the number of cells remained steady or even increased up to 3 days of incubation, and then gradually decreased at longer times. During the time of the experiments shown in Fig. 1, the growth medium was never changed.

Fig. 1, *B–D*, shows that the metabolic stress provoked a different effect in all cybrid cell lines bearing LHON mutations. In particular, a similar reduction of viable cells was observed in the cybrids bearing the 3640/ND1 and 14484/ND6 mutations, which were alive in an average of 31 and 37% of cells, respec-

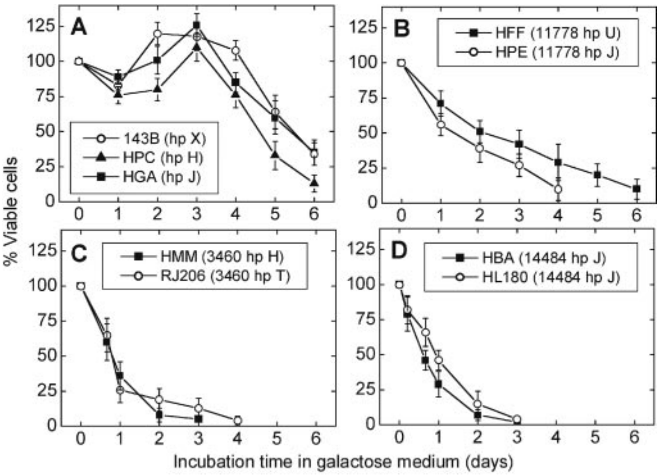

FIG. 1. **Decreased cell viability of cybrid cell lines bearing LHON mutations incubated in galactose medium.** Control and LHON cybrids were incubated for the times indicated in galactose medium and then treated for 3 h with MTT and overnight with SDS and HCl, as described under "Experimental Procedures." The mtDNA haplogroups (*hp*) of parental, control, and LHON cybrid cell lines (Table I) are indicated in the *inset* of each *panel*. The MTT absorbance value at time 0 was considered as the 100% value of viable cells. Each data point is means ± S.E. of 3–5 determinations.

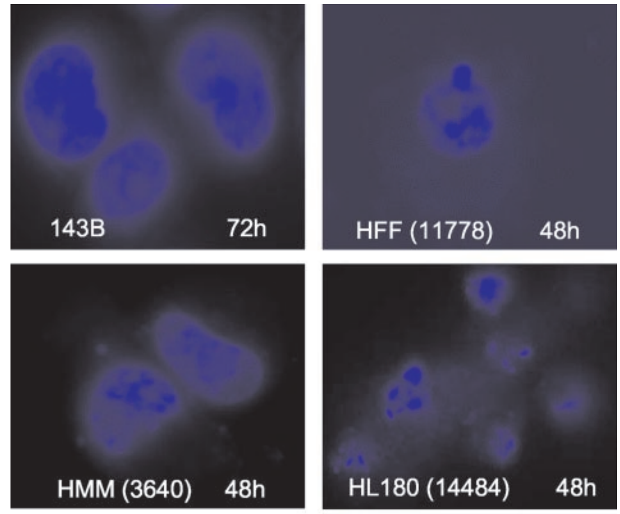

FIG. 2. **Nuclear morphology of control and LHON cybrids incubated in galactose medium.** 143B.TK$^-$ cells and LHON cybrids were incubated for the times indicated in galactose medium and loaded with Hoechst, as described under "Experimental Procedures." Images of cells, captured by a digital imaging system, are representative of most of cells in the same coverslip.

tively, after 1 day of incubation with galactose medium, whereas the effect of the 11778/ND4 mutation was less severe, found in an average 65% of cells that were viable after 1 day and 20% of cells that were viable after 4 days. It seems, therefore, that all cybrids carrying LHON pathogenic mutations are more sensitive to metabolic stress induced by growth in galactose medium than control cybrids, although some differences among the three mutations are also apparent.

Haplogroup Influence on Cell Death—Both control and LHON cybrid cell lines were characterized for their mtDNA background, as shown in Table I. The differences in the mtDNA haplogroup did not induce any observable variability in growth capabilities of control cybrids and the 143B.TK$^-$ parental cell line in galactose medium, as already reported (25). Moreover, the presence of different mtDNA haplogroups in the LHON cybrids, in particular haplogroup J, which has been associated with the 11778/ND4 and 14484/ND6 mutations (16), did not show any clear-cut influence on cell death. We refer in particular to the direct comparison of HPE/HFF cybrids (Fig. 1*B*) carrying both the 11778/ND4 mutation but differing in their haplogroup, J and U, respectively. Both HBA and HL180 cybrids carrying the 14484/ND6 mutation and haplogroup J seemed to present the highest sensitivity to cell death in galactose medium. However, in this case, we unfortunately lack the direct comparison with a further cell line carrying the same pathogenic mutation with a different haplogroup.

Chromatin Condensation and Nuclear DNA Laddering in LHON Cybrids—The type of cell death, apoptosis *versus* necrosis, induced in LHON cybrids by metabolic stress was first evaluated by detection of nuclear morphology of cells stained with Hoechst. In fact, cells undergoing apoptosis are characterized by the dramatic change that occurs in the nucleus, *i.e.* chromatin condensation and nuclear DNA fragmentation (28).

Fig. 2 shows that after 72 h of incubation in galactose medium, the nuclei of 143B.TK$^-$ cells showed a diffuse fluorescence. Conversely, in cybrids bearing the pathological LHON mutations, the nuclear chromatin of most cells was condensed and showed highly fluorescent dense aggregates after 48 h of incubation. Furthermore, quantitative data on the percentage of apoptotic and necrotic cells were obtained by the acridine orange/ethidium bromide uptake. Cells with bright green or orange nuclei with condensed chromatin were identified as early or late apoptotic cells, respectively. As illustrated in Fig. 3, after 2 days of incubation in galactose medium, the percentage of apoptotic cells was negligible for 143B.TK$^-$ parental cells and for control cybrids, whereas it was about 40% in cybrids carrying the 11778/ND4 mutation and higher than 60 and 90% in cybrids carrying the 3460/ND1 and 14484/ND6, respectively. The percentage of necrotic cells (cells with orange nuclei with intact structure) was negligible (less than 3%, result not shown).

The nuclear morphology characterized by chromatin condensation is caused by specific digestion of internucleosomal DNA, leading to the typical fragmentation or laddering of DNA into small molecular weight bands, which can be easily evaluated after isolation of nuclear DNA and separation with agarose gel electrophoresis. In Fig. 4, the *three upper panels* show representative gels of DNA isolated from the parental 143B.TK$^-$ cell line and from two control cybrid cell lines incubated for the times indicated in galactose medium. No significant DNA laddering was observed up to 72 h of incubation. Conversely, a significant DNA fragmentation was apparent after 48–72 h of incubation with galactose in the two cybrid cell lines bearing the 11778/ND4 mutation and already after 24 h in the cybrids with the 3460/ND1 and 14484/ND6 mutations.

Galactose Medium-induced Release of Cytochrome c in LHON Cybrid Cells—To understand whether mitochondria are involved in the apoptotic process induced in LHON cybrids by metabolic stress, the release of cytochrome *c* from mitochondria to cytosol has been detected as a function of incubation time in galactose medium. No significant release of this protein was observed in the cytosolic fractions obtained from subcellular fractionation of homogenates from the parental 143B.TK$^-$ cells (Fig. 5). Conversely, a significant cytochrome *c* release was determined in cybrids with the 11778/ND4 mutation starting from 24 h and maximal after 48 h. In cybrids with the 3460/ND1 and 14484/ND6 mutations, a remarkable release of cytochrome *c* was already apparent after 16 h of incubation in galactose medium (Fig. 5).

DISCUSSION

Galactose Medium Induces Cell Death in LHON Cybrids—The first important result of this study was the significant

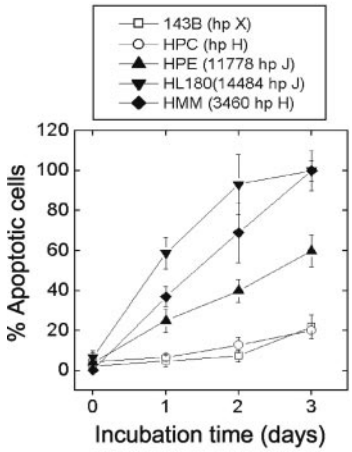

FIG. 3. **Quantification of apoptotic cells in cybrids incubated in galactose medium.** Control and LHON cybrids were maintained in galactose medium for the times indicated and then incubated with orange acridine and ethidium bromide and examined with an inverted fluorescence microscopy. Data are means ± S.D. of at least three determinations. The percentage of apoptotic cells in all LHON cybrids was significantly different from control or parental cells ($p < 0.05$ after 1 day and $p < 0.005$ after 2 and 3 days respectively, calculated using the paired Student's *t* test).

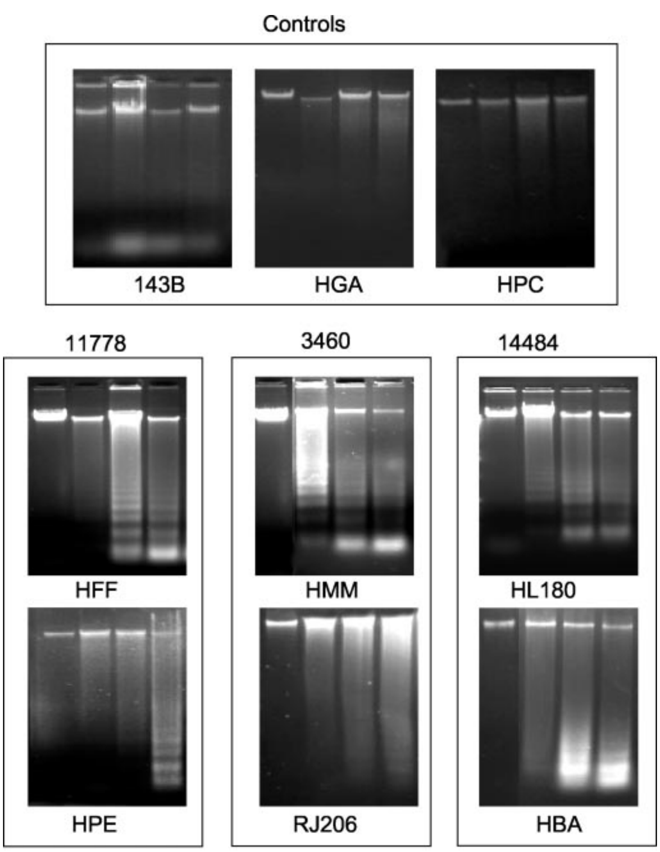

FIG. 4. **Time-course of DNA fragmentation of cybrid cell lines with LHON mutations.** Nuclear DNA was isolated from cells incubated in galactose medium for the times indicated, as described under "Experimental Procedures." DNA was separated in 1% agarose gel and stained with ethidium bromide. In each *panel*, the *lanes* were: time 0, 24, 48, and 72 h of incubation in galactose medium. The results are representative of experiments run at least in triplicate.

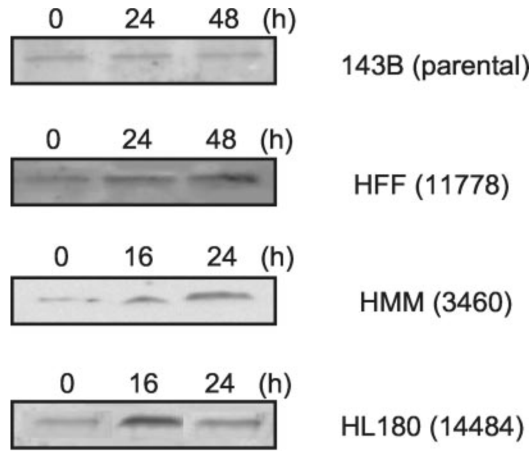

FIG. 5. **Release of cytochrome *c* in the cytosolic fraction in cybrid cell lines with LHON mutations.** Control and LHON cybrids were incubated in galactose medium for the times indicated and then harvested, homogenized, and centrifuged, as detailed under "Experimental Procedures." 50–70 μg protein from the cytosolic fractions were separated by SDS-PAGE, and Western blotting was performed with specific antibody against cytochrome *c*. Data are representative of three similar experiments.

growth impairment documented in cybrids carrying the LHON pathogenic mutations when incubated in a glucose-free/galactose medium. Under this experimental condition, the reduced rate of the glycolytic pathway causes the forced oxidation of pyruvate through the mitochondrial respiratory chain. This experimental condition has been used previously to identify an impaired respiratory function (20, 21), and Hofhaus *et al.* (19) did report a reduced ability of LHON/11778 cybrids to grow in galactose medium (19). Our study extended this seminal observation to the whole range of the main LHON pathogenic mutations, also showing that growth impairment was somewhat milder in 11778 cybrids as compared with those carrying the 3460 and 14484 mutations.

An apparent discrepancy between our data and those reported by Hofhaus *et al.* (19) concerns the behavior of the 143B parental cell line and the other control cybrids. In fact, control cells were reported to have a substantial growth rate in galactose medium, although reduced as compared with their growth in glucose medium (19). Conversely, we report here that the viability of these cells decreased after 1 day of incubation and then slightly increased up to 4 days to further decrease at longer times. This late loss of viability is likely due to nutrient shortage since it was abolished after replacing the growth medium (result not shown). We believe that the slow proliferation rate at the early times might be due to the density of cell seeding because we observed a significant growth of control cells in galactose medium when seeded at a lower density ($1–2 \times 10^4/cm^2$ instead of $4 \times 10^4/cm^2$, result not shown).

The growth impairment of LHON cybrids with a different mtDNA haplogroup did not show any clear variations that could be related to the mitochondrial genome background. However, we cannot exclude that some differences could become observable, increasing the number of cell clones investigated and properly comparing different haplopgroups within the same mutational category.

The Type of LHON Cybrid Cell Death Is Apoptotic—The second novel result presented in this study is that the type of cell death occurring in LHON cybrids incubated in galactose medium is apoptotic. In fact, we show, for the first time, that LHON cybrids, but not control cybrids and the 143B.TK$^-$ parental cell line, exhibited some typical hallmarks of apoptosis, such as the changes in nuclear morphology, chromatin condensation, and DNA laddering. From these results, it is clear that cybrids with the 11778/ND4 mutation were, again, less sensitive to the metabolic stress in comparison with the 3460/ND1 and 14484/ND6 mutations.

Glucose is an essential energy source, and its deprivation or treatment with the glucose antimetabolite 2-deoxyglucose can lead to arrest in G_0/G_1 phase non-transformed cells (31) or to apoptosis in Myc-transformed cells (32). Furthermore, it has been shown that restriction of the glycolytic rate by reducing the glucose concentration in the medium or increasing glucose uptake by over-expression of GLUT1 promotes or delays, respectively, the apoptosis induced by growth factor withdrawal (33). Therefore, changes in the cellular metabolism may be sufficient to cause the commitment to programmed cell death (34).

However, in our experimental cell system, only LHON mutant cybrids did undergo apoptosis in galactose medium. We assume that the glycolytic flux reduction in our system is still compatible with an adequate availability of metabolites needed for mitochondrial synthesis and substrates for electron transport sustaining cell survival and eventually lower rates of growth capabilities. Given that the only difference between control and LHON cybrids is the presence of the 11778/ND4, 3460/ND1, and 14484/ND6 pathogenic mutations, known to affect complex I function (3, 9, 10, 12), we believe that this genetic difference is responsible for the inability of the LHON cybrids to cope with the metabolic stress.

Mitochondrial Involvement in Apoptosis—The third relevant finding emerging from this study is the evidence of mitochondrial involvement in the apoptotic pathway activated in the death of the LHON cybrids. This has been clearly demonstrated by the significant release of cytochrome *c* from mitochondria to cytosol documented in all LHON cybrids. Different forms of cellular stress (*e.g.* DNA damage, cytokine deprivation, exposure to cytotoxic drugs) have been reported to promote a pathway for apoptosis typically regulated by these organelles. In this pathway, the engagement of death-promoting members of the Bcl-2 family of proteins (Bax, Bid, Bad, Bak, Bok, etc.) induces their translocation to mitochondria and the subsequent release of proapoptotic molecules, such as cytochrome *c*, apoptosis-inducing factor, and Smac/Diablo (22, 35). This effect is counteracted by the Bcl-2 survival family of proteins (Bcl-2, Bcl-X_L, etc.), which are anchored to the outer membrane of mitochondria and which function, at least in part, by blocking the release of cytochrome *c* (22, 36, 37). It is of interest that glucose deprivation during hypoxia of cultured kidney cells has been reported previously to result in the translocation of Bax from the cytosol to mitochondria (38). We failed to observe detectable levels of Bax in cell lysates from 143B.TK$^-$ ostosarcoma cell line and LHON cybrids[2]; therefore, studies are in progress to identify the proapoptic Bcl-2 protein involved in the process of cytochrome *c* release during galactose medium-induced cell death.

Potential Mechanisms for Increased Apoptosis in LHON Cybrids—The biochemical consequences of LHON pathogenic mutations have been incompletely characterized (3). However, we have enough data indicating that a variable respiratory defect is associated with these mutations with the possibility of a partial decrease of ATP synthesis. This may be either due to a reduced release of quinol or due to a less efficient energy conservation at complex I level, which in turn may be reflected in changes of membrane potential ($\Delta\psi$). However, it is not clear whether compensatory mechanisms, such as complex II upregulation, may actually maintain the ATP synthesis or whether unknown tissue-specific regulatory mechanisms may differentially influence the level of ATP defect.

On the other hand, the complex I dysfunction in LHON seems mainly characterized by the common feature of affecting the interaction with the quinone substrate, and an increase of reactive oxygen species has been predicted as a direct consequence (9, 10). Some recent studies are now supporting this hypothesis. A cellular model developed by Barrientos and Moraes (39), in which a partial complex I impairment is obtained both genetically or by rotenone inhibition, showed that apoptotic cell death was positively correlated with reactive oxygen species production rather than with a decrease in respiratory chain function. Moreover, a significant increase of reactive oxygen species production was observed in a neuronal (NT2) cybrid cell model carrying the 11778/ND4 and 3460/ND1 LHON mutations only after retinoic acid-mediated differentiation (14). Data from our own laboratories confirm these same observations in the osteosarcoma (143B.TK$^-$)-derived cybrids with LHON mutations (15). Thus, under certain conditions, the increase of production of reactive oxygen species could be implicated in triggering a mitochondrial-mediated apoptotic cell death in addition to the hypothesis of exclusive bioenergetic impairment.

A different line of recent experimental evidence may possibly be relevant for linking the LHON mutations, affecting different NADH dehydrogenase subunits, with apoptosis. A regulatory role for complex I and ubiquinone analogs has been implicated in the modulation of the mitochondrial permeability transition pore (40, 41). It is tempting to speculate that the LHON pathogenic mutations, which affect the interaction of complex I with the ubiquinone substrates, might also influence the pore opening with the consequent release of cytochrome *c* and activation of the apoptotic cascade.

The findings of the current study are in agreement with and complement those recently reported using the very same LHON cybrid cell lines but exposed to a different model of apoptotic activation (24). In fact, Danielson *et al.* (24) recently showed that these LHON cybrids were more sensitive to Fas-induced apoptosis (24). In this apoptotic pathway, the death receptor-dependent autocatalytic activation of caspase 8 can directly cleave and activate the downstream caspases, such as caspase-3, -6, and 7 (42). In addition, caspase-8 also activates these downstream caspases indirectly by inducing cytochrome *c* release from mitochondria, as a consequence of cleavage of the cytosolic protein Bid, generation of truncated Bid, and its subsequent translocation into the outer mitochondrial membrane (43, 44). Although the contribution of these two pathways to Fas-induced apoptosis in LHON cybrids has not been investigated, nevertheless, the increased sensitivity of these cells to both Fas- and metabolic stress-induced apoptosis suggests that the complex I mutations can strongly influence cell survival.

The further characterization of the exact steps of the apoptotic pathway involved in LHON will provide more details on the pathophysiology of this disease and more details in general on the link between mitochondrial dysfunction induced by complex I mutations and cell death. This issue is of general interest for a wider category of neurodegenerative diseases and for possible therapeutic pharmacological strategies aimed to inhibit or reverse the apoptotic cascade. This therapeutic approach would provide a hope for those LHON patients undergoing the acute phase of the disease, when the retinal ganglion cell loss is thought to occur, thus limiting the retinal ganglion cell death and the consequent visual loss.

Acknowledgments—We are grateful to Drs. Gino Cortopassi and Steven Danielson, University of California Davis, for providing some cybrids cell lines and to Dr. Rosario Rizzuto, University of Ferrara, for making available the Cell Imaging Facility.

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J. Biol. Chem. 2003, 278:4145-4150.
doi: 10.1074/jbc.M210285200 originally published online November 21, 2002
