Cells Bearing Mutations Causing Leber’s Hereditary Optic Neuropathy Are Sensitized to Fas-induced Apoptosis*

Three prevalent mitochondrial DNA pathogenic mutations at positions 11778, 3460, and 14484, which affect different subunits of Complex I, cause retinal ganglion cell death and optic nerve atrophy in Leber’s hereditary optic neuropathy (LHON). The cell death is painless and without inflammation, consistent with an apoptotic mechanism. We have investigated the possibility that the LHON mutation confers a pro-apoptotic stimulus and have tested the sensitivity of osteosarcoma-derived cybrid cells carrying the most common and severe mutations (11778 and 3460) to cell death induced by Fas. We observed that LHON cybrids were sensitized to Fas-dependent death. Control cells that bear the same mitochondrial genetic background (the J haplogroup) without the pathogenic 11778 mutation are no more sensitive than other controls, indicating that increased Fas-dependent death in LHON cybrids was induced by the LHON pathogenic mutations. The type of death was apoptotic by several criteria, including induction by Fas, inhibition by the caspase inhibitor zVAD-fmk (zVal-Ala-Asp-fluoro-methyl ketone), activation of DEVDase activity (Asp-Glu-Val-Asp protease), specific cleavage of caspase-3, DNA fragmentation, and increased Annexin-V labeling. These data indicate that the most common and severe LHON pathogenic mutations 11778 and 3460 predispose cells to apoptosis, which may be relevant for the pathophysiology of cell death in LHON, and potential therapy.

Leber’s hereditary optic neuropathy (LHON)1 is a maternally inherited disorder characterized by a primary degeneration of the retinal ganglion cells (RGCs) and atrophy of the optic nerve (1, 2). Central vision is mostly affected, due to a preferential death of the small nerve fibers of the papillomacular bundle (3). LHON is caused by three prevalent pathogenic mitochondrial DNA (mtDNA) point mutations at positions 11778, 3460, and 14484, which alter coding genes for complex I subunits in the mitochondrial electron transport chain (4–6). These three mutations account for greater than 90% of all LHON cases worldwide (1, 2).

Given the occurrence of LHON mutations in Complex I subunits, most studies and the primary hypotheses for the pathophysiological mechanism in LHON have focused on the possibility of a bioenergetic defect. There is agreement that isolated LHON mitochondria fed with Complex I substrates in vitro have reduced oxygen consumption relative to control mitochondria, whereas there is no such defect with substrates of Complex II (7–10). Thus, there is an overall defect in electron transport when Complex I substrates are used. However, when Complex I activity is measured directly in disrupted LHON mitochondrial membranes (i.e. submitochondrial particles), a decreased activity is consistently observed only in 3460 mitochondria (7, 10, 11), whereas the occurrence of an enzymatic Complex I defect in 11778 and 14484 is controversial (7–12). Thus, there is less consistent support for a common enzymatic defect in Complex I activity in LHON mitochondria with the three pathogenic mutations (13). However, analysis of the sensitivity of Complex I function of LHON versus control mitochondria with specific inhibitors, including rotenone (11, 12), indicates that the LHON functional alterations most likely affect the interaction of Complex I with quinone substrates (10–12).

In addition to their role in bioenergetics, mitochondria also have a central role in the process of apoptotic cell death (14). The degeneration of RGCs in LHON occurs over a period of a few months without pain or pathological evidence of inflammation, which are the hallmarks of an apoptotic process (1, 2, 15). Thus, the death of RGCs in LHON has been proposed to be apoptotic (16). RGCs are also thought to go through apoptotic death in glaucoma (17).

Perhaps the best understood pathway of apoptosis is the Fas pathway, in which mitochondria play a signal amplification role in the cell death program (18). Caspase-8 can cleave the cytosolic protein Bid, the resulting fragment tBid can then translocate to the outer mitochondrial membrane resulting in the release of cytochrome c from the mitochondria. Cytochrome c in the cytosol forms what has been termed the “apoptosome” complex by binding to the structural protein Apaf-1 and caspase-9. This causes the activation of caspase-9, which can then activate other “executioner” caspases, such as caspase-3, which begin to dismantle the cell. Thus, the Fas-induced apoptotic cascade provides a well-defined pathway that we could exploit to investigate any difference in cell death caused by LHON mtDNA mutations (14). In this study we have tested the

---

* This work was supported by United States Public Health Services Grants EY12245, AG11967, and AG16719, and by core support Grant F30ES05707 (to G. A. C.), and by Telethon Grant 792 (to Prof. Elio Lugaresi in support of 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.

** To whom correspondence should be addressed: Dept. of Molecular Biosciences, University of California Davis, School of Veterinary Medicine, Davis, California 95616, the Institute of Neurology, University of Bologna, 40123 Bologna, Italy, the Institute Eugenio Medea, Conegliano Research Center, 31015 Conegliano, Italy, and the Royal Free Hospital and University College Medical School, London NW3 2PF, United Kingdom

The abbreviations used are: LHON, Leber’s hereditary optic neuropathy; RGC, retinal ganglion cells; mtDNA, mitochondrial DNA; zVAD-fmk, zVal-Ala-Asp-fluoro-methyl ketone; Ab, antibody; DEVDase, Asp-Glu-Val-Asp protease; pNA, p-nitroanilide; FITC, fluorescein isothiocyanate; PI, propidium iodide.

---

Steven R. Danielson, Alice Wong, Valerio Carelli, Andrea Martinuzzi, Anthony H. V. Schapira, and Gino A. Cortopassi

From the Department of Molecular Biosciences, University of California Davis, School of Veterinary Medicine, Davis, California 95616, the Institute of Neurology, University of Bologna, 40123 Bologna, Italy, the Institute Eugenio Medea, Conegliano Research Center, 31015 Conegliano, Italy, and the Royal Free Hospital and University College Medical School, London NW3 2PF, United Kingdom
sensitivity of multiple LHON osteosarcoma-derived cybrid cell lines bearing the most common mutations 11778 and 3460 to Fas and characterized their death response.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—Control and LHON cybrid cell lines were obtained as described elsewhere (19), by fusing 206 rhô cells (a kind gift of G. Attardi) with enucleated fibroblasts from controls or LHON patients previously characterized for their mtDNA haplogroup and presence of LHON pathogenic mutations. After growth in selective medium (lacking uridine and with bromodeoxyuridine) the clones obtained underwent mtDNA investigation to confirm haplogroup definition and presence of LHON pathogenic mutations as described previously (20). All cell lines for experiments were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate, 50 μg/ml uridine, and 40 μg/ml gentamicin.

Osteosarcoma cell lines (143B) obtained from three different sources (ATCC Rockville, MD, A. Martinuzzi, and kind gifts from G. Attardi and M. King) were used as controls. They all carried an X haplogroup as mtDNA background. Moreover, three osteosarcoma-derived cybrid cell lines, constructed from control fibroblasts carrying a J haplogroup with no LHON pathogenic mutations, were also used as haplogroup-matched controls. Five LHON cybrid cell lines were constructed from four individuals and contained the 11778 double base-pair transition. Three carried the J haplogroup, one the U haplogroup, and one the H haplogroup (the latter was a kind gift from G. Attardi). Two more LHON cybrid cell lines derived from two different patients contained the 3460 mtDNA point mutation.

Cell Viability Assays—Cells were treated with 0, 0.5, 1, or 2.5 μg/ml an anti-Fas receptor antibody (anti-Fas, clone CH-11 Beckman Coulter, Fullerton, CA) for 24 h. The cells were then harvested by trypsinization and scored as alive or dead using the Trypan blue exclusion assay. Trypan blue is a membrane-impermeable dye, which is not taken up by viable cells with an intact plasma membrane. During the early stages of apoptosis the plasma membrane is normally intact and therefore impermeable to Trypan blue. However, cells grown in culture commonly experience a loss of plasma membrane integrity during the late stages of apoptosis and then become permeable to Trypan blue (21, 22).

For zVAD-fmk viability experiments, cells were treated with 50 μM zVAD-fmk 60 min prior to addition of 0, 0.5, 1, or 2.5 μg/ml anti-Fas and then treated with anti-Fas for 24 h. The cells were then harvested and scored as alive or dead using Trypan blue exclusion assay.

Western Blot Analysis—Cells extracts for Western blot analysis were prepared as previously described (23). Briefly for each cell line, 1 × 10⁶ cells were washed with phosphate-buffered saline, harvested, and then lysed in cell extraction buffer (100 mM HEPES, pH 7.5, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride) 10 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 mM EDTA) for 30 min at 4 °C with brief (2 s) vortexing every 7 min, the lysate was then centrifuged at 12,000 × g for 10 min at room temperature, and counterstained with propidium iodide (final concentration 1 μg/ml). After the incubation period, the cells were then treated with 400 μl of Annexin-V binding buffer. Cells were analyzed by flow cytometry using a Becton Dickinson FACScan flow cytometer with Cell Quest software (Becton Dickinson, Mountain View, CA).

RESULTS

Cells Containing Mitochondria with LHON Mutations Are Sensitive to Fas-induced Death—The ability to construct trans-mitochondrial cell lines, i.e. cell lines with a common parental nucleus but with different exogenous mitochondrial genomes, allows the determination of which cellular phenotypes are the result of mitochondrial genotypes (24, 25). Seven control lines, five 11778 and two 3460 LHON cell lines, were treated with 1 μg/ml anti-Fas for 24 h. Cells were then harvested and scored as alive or dead by Trypan blue exclusion assay. We observed that LHON cybrid cells bearing the 11778 and 3460 mtDNA mutations exhibited substantially more death than controls (Fig. 1, A and B). The death was dependent on both dose and time of exposure to anti-Fas. Similar experiments with staurosporine exhibited no increased death in LHON versus control cells (data not shown).

Haplogroup Independence of Cell Death—Because the LHON mutant cybrids carried the identical nuclear background as the controls, the difference in viability is most simply explained by mtDNA variation. mtDNA variation between control and LHON cells includes the pathogenic mutations 11778 and 3460 and the mtDNA background (mtDNA haplogroup), i.e. variations at other positions than the pathogenic sites. Most of the cybrids carrying the 11778 mutation were haplogroup J. Thus, to test the possible haplogroup dependence of the increased cell death, control cybrids with J haplogroup were also exposed to anti-Fas antibody. In contrast to the 11778 and 3460 lines these haplogroup-matched controls displayed only a minor Fas-dependent reduction in viability (Fig. 1C) indicating that the excess death observed in LHON cybrids is not dependent on mtDNA haplogroup.

Rho0 osteosarcoma cells lack mtDNA. These cells have a deficient mitochondrial membrane potential, and a defective electron transport chain and ATPase because several subunits are encoded by the mtDNA (24). We observed that rho0 cells, which completely lack electron transport, only exhibited a slight decrease in viability when treated with anti-Fas (data not shown), consistent with earlier reports in which staurosporine was used to induce apoptosis (26, 27). Thus, a complete deficit in bioenergetic function provides no major stimulation of Fas-induced apoptosis.

Caspase Dependence of Fas-dependent LHON Cell Death—Caspases play critical roles in the initiation and execution of apoptosis (28–30). Pre-treating LHON and control cells with the caspase inhibitor zVAD-fmk completely rescued LHON cybrids from anti-Fas-induced death. As shown in Fig. 2, addition of zVAD-fmk to cells containing the 11778 (Fig. 2A) or 3460 (Fig. 2B) mutations restored viability to levels seen in control cells after treatment with anti-Fas. These results indicate that the excess Fas-dependent death observed in LHON cybrids is caspase-dependent.

Increased Fas-dependent Caspase-3 Activity and Cleavage in LHON Cybrids—The zVAD-fmk-dependent inhibition of cell death in the earlier experiment indicated that Fas-induced
death was caspase-dependent. Caspases have multiple specificities and the most important “effector caspase” in most cells is caspase-3, which cleaves the oligopeptide substrate DEVD (28). Extracts were prepared from LHON and control cells 6 h after Fas treatment, and DEVDase activity was assayed (Fig. 3A). There was a mean increase of ~335% in DEVDase activity in the LHON versus control extracts treated with anti-Fas (p < 0.05). Control DEVDase values were not significantly different in extracts from Fas-treated versus untreated cells. There was a clear time- and dose-dependent increase of DEVDase activity in the Fas-treated LHON extracts compared with controls (data not shown).

However, other caspases in addition to caspase-3 may have some affinity to cleave the DEVD oligopeptide (31). Therefore, to address the specificity of DEVDase activity, a Western blot of caspase cleavage was carried out, using an antibody specific to caspase-3 as a probe, and lanes were loaded with equivalent amounts of total protein (Fig. 3B). Caspases are synthesized as inactive zymogens and then undergo proteolytic cleavage to become active. Thus activation of caspase-3 can be determined by assaying for the cleavage of the caspase (28). Much more cleaved caspase-3 was observed in the Fas-treated LHON extracts compared with controls (Fig. 3B).

Fas-dependent DNA Fragmentation of LHON Cybrid Cells—A characteristic of apoptosis is the fragmentation of DNA into small molecular weight bands that resemble a ladder of DNA, which can be readily visualized by gel electrophoresis (32). Chromosomal DNA isolated from LHON and control osteosarcoma cells showed DNA fragmentation upon treatment with anti-Fas for 24 h (Fig. 4), consistent with the other characteristic features of apoptosis described in preceding and following paragraphs.

LHON Cells Exhibit Fas-dependent Annexin-V Staining—To further delineate the morphology of the cell death, we stained treated (anti-Fas) and untreated control and LHON cybrids with Annexin-V. In normal healthy cells, phosphatidylserine residues in the plasma membrane are oriented toward the cytoplasm, however, an early event of apoptosis is the translocation of these residues to the extracellular environment (33). Annexin-V is a human anticoagulant that has high affinity for binding to phosphatidylserine residues. Therefore, Annexin-V, in combination with the cell-impermeable nucleic acid stain propidium iodide (PI), can be used to differentiate apoptotic, necrotic, and normal cells. Apoptotic cells will be positive for Annexin-V staining and negative for PI staining, whereas necrotic cells will stain positively for PI and normal cells will stain negatively for PI and Annexin-V. In multiple experiments LHON (11778) cells treated with 1 µg/ml anti-Fas for 18 h exhibited a significant (p < 0.005) increase in the percentage of apoptotic cells as measured by Annexin-V-FITC/PI staining as compared with untreated LHON cells (one representative experiment is illustrated in Fig. 5A; the mean for all experiments is represented in Fig. 5B). There was also a significant difference (p < 0.05) between the percentage of apoptotic cells be-
between LHON and control cells after treatment with anti-Fas (Fig. 5B). However, control cells exhibited no significant ($p = 0.7$) difference in apoptotic cells upon treatment with anti-Fas (Fig. 5B). These data support the results seen in Fig. 3 (A and B) in which only LHON cells exhibited a significant increase in DEVDase activity and activation of Caspase-3, respectively. This indicates that LHON cells are primed for apoptotic cell death. Only cells that were Annexin-V-positive and PI-negative were considered apoptotic.

Normal Fas Receptor Expression of Control and LHON Cybrids—As described above, LHON cybrids were more sensitive to Fas-dependent death than controls, and this increased death was time- and Fas-dependent. This increased cell death: 1) was inhibitable by a caspase inhibitor (zVAD-fmk); 2) resulted in increased DEVDase activity and caspase-3 cleavage; 3) occurred with nuclear DNA laddering; and 4) resulted in increased Annexin-V staining. Each of these factors is consistent with apoptotic cell death. One possible explanation for the increased sensitivity of the cells containing LHON mutations to anti-Fas would be an LHON-dependent increase of the Fas receptor. Therefore, we isolated protein from two LHON cell lines and three control cell lines and utilized Western blot analysis to determine if there was a difference in the relative level of Fas receptor expression that could be the result of the LHON mutation. We found no significant difference in the amount of Fas expression between LHON and control cells (Fig. 6). There was also no difference in the level of total Fas RNA transcript as measured by reverse transcription-PCR (data not shown) in control versus LHON cells.

DISCUSSION

Increased Fas-dependent Death in LHON Cells—We investigated the hypothesis that the mtDNA LHON pathogenic mutation sensitizes LHON cells to Fas-dependent cell death. We found that LHON cells exhibited an increased DEVDase activity and activation of Caspase-3 as compared to control cells upon treatment with anti-Fas. This increased DEVDase activity and Caspase-3 activation was inhibited by a caspase inhibitor, indicating that it was mediated by caspase-dependent pathways. Additionally, nuclear DNA laddering and increased Annexin-V staining were observed in LHON cells upon treatment with anti-Fas, consistent with apoptotic cell death.

The increased sensitivity of LHON cells to Fas-dependent cell death is consistent with previous studies that have shown increased Fas receptor expression in other cell types. This suggests that the LHON mutation may be promoting increased Fas receptor expression, which could result in increased apoptosis. Further investigations are needed to determine if this is the case and to identify the mechanisms by which the LHON mutation promotes increased Fas receptor expression.

**Fig. 3.** A, DEVDase-specific activity. Control and 11778 LHON cells were treated with 1 μg/ml anti-Fas for 6 h. Cytosolic extracts were prepared from cells and caspase-3-like activity was determined by measuring the cleavage of the substrate DEVD-pNA. $p = 0.07$ for untreated control cells versus treated control cells. A single asterisk indicates $p < 0.05$ for treated control cells versus treated LHON cells. $p < 0.005$ for untreated LHON cells versus treated LHON cells. $p < 0.005$ for untreated LHON cells versus treated LHON cells. B, increased caspase-3 cleavage in LHON extracts versus controls. LHON and control cells were treated with anti-Fas (1 μg/ml) for 6 h, extracts from cell lysates were prepared, and then 25 μg of protein were electrophoresed, blotted, and probed with anti-caspase-3 antibody. Lanes 1–3, 11778 extracts; lanes 4–5, 3460 extracts; lanes 6–10, control extracts.

**Fig. 4.** DNA laddering. Chromosomal DNA was isolated by phenol chloroform extraction, and 5 μg of DNA was loaded onto a 2% agarose gel, electrophoresed, and stained with ethidium bromide. Starting from left to right, lane 1 contains a DNA molecular weight marker, lanes 2 and 3 contain DNA isolated from control cells (untreated), lane 4 contains DNA from control cells (+anti-Fas), and lanes 5–7 contain DNA from LHON 11778 cells + anti-Fas.
tions may predispose cells to apoptosis. Using Fas as the stimulus, we observed a consistent and significant increase in sensitivity to cell death of the LHON cybrids treated with anti-Fas compared with control cells, which shared the identical nucleus. This sensitivity was both time- and dose-dependent and was not related to mtDNA haplogroup variation, in particular to the presence of the J haplogroup. The J haplogroup has been found associated with the 11778 and 14484 mutations in European populations, and one explanation of this association was that it could enhance the penetrance of the disease (20). However, we observed no haplogroup-specific differences in Fas-mediated apoptosis in either the presence or absence of the LHON pathogenic mutations.

**The Increased LHON Cell Death Is Apoptotic**—The increased cell death observed in LHON cybrids was consistent with apoptotic cell death. In fact, it was dependent on Fas, abrogated by the caspase inhibitor zVAD-fmk, resulted in an increase of DEVDase activity and specific cleavage of caspase-3, caused eversion of phosphatidyserine residues in the plasma membrane, and resulted in increased DNA fragmentation. The most straightforward interpretation of these results is that the 11778 and 3460 LHON pathogenic mutations increase the sensitivity of osteosarcoma cells to apoptotic death mediated through the Fas pathway.
Although we observed no difference in expression of Fas receptor, we did observe a difference in DEVDase activity and, specifically, caspase-3 cleavage between LHON mutants and control cells. Thus, the site of activation of apoptosis presumably lies downstream of Fas-receptor binding by ligand, but upstream of the activation of caspase-3.

**Retinal Ganglion Cells Are Sensitive to Apoptotic Death—** There are several observations that suggest RGCs are sensitive to apoptotic cell death, both in LHON and other optic neuropathies. In LHON, a distinguishing feature of the acute phase of the disease, in which the progress of massive RGC death may take approximately 6 months, is a complete lack of both pain and inflammation, hallmarks of apoptosis (1, 2). The lack of inflammation is supported both by the absence of leakage on fluorescein angiography (suggesting that the blood-brain barrier is not damaged) and by the lack of any inflammatory signs in histopathological investigations of the optic nerve (1, 2, 15). This “silent” massive death of RGCs observed in LHON has previously been suggested to be the result of an apoptotic process (2, 16). In other optic neuropathies apoptosis of RGCs is also evident. Within 14 days after cutting or crush-axotomy of the optic nerve, more than 90% of the RGCs die by apoptosis (34). Apoptosis of RGCs has also been documented in human pathological conditions such as glaucoma (17) and anterior ischemic optic neuropathy (35). Thus it is clear that RGCs are capable of apoptosis, and that the 11778 and 3460 mutations sensitize osteosarcoma cells to Fas-dependent apoptosis.

**The Stimulus for the Fas Pathway, Fas Ligand, Is Expressed in Retina—** Our data demonstrate that the most common and severe LHON mutations, the 11778 and the 3460, sensitize cells to Fas-dependent apoptotic death. One requirement for this observation to be significant in LHON pathophysiology is the presence of Fas ligand, which triggers the Fas pathway, and in fact Fas ligand is expressed endogenously in the retina (36). Thus if the Fas ligand stimulus is present in retinas of control and LHON individuals, but the LHON mutations specify an increased sensitivity to the stimulus, this could explain increased apoptotic death in LHON individuals.

**Potential Mechanisms for Increased Fas-dependent Apoptosis in LHON Cells—** Given that the only relevant difference between control cybrids and LHON is the presence of the 11778 and 3460 pathogenic mutations, both potentially affecting some function of Complex I, we suggest that alterations in Complex I structure or function may be involved in the increased cell sensitivity the Fas-induced apoptotic death. There are multiple potential mechanisms by which this could occur. For example, recent studies pointed to a regulatory role of Complex I, and ubiquinone analogs in the opening of the mitochondrial permeability transition pore (37, 38). And in fact the most consistent biochemical finding common to all the LHON pathogenic mutations is an alteration of Complex I interaction with ubiquinone substrates and inhibitors at the quinone/quinol binding site (10–13). A direct link between complex I and ubiquinone analogs with the permeability transition pore opening and, consequently, with the activation of the apoptotic cascade downstream mitochondria seems to fit with our findings in LHON cybrids, where the increased sensitivity to apoptosis is postulated to be mediated by the presence of Complex I mutations.

Another mechanistic possibility for an interaction between Complex I and apoptosis has been put forward by Barrientos and Moraes (39), who have modeled a partial Complex I defect in a trans-species cybrid system and have demonstrated increased apoptotic death in that system which may be the result of increased reactive oxygen species (ROS).

Our study provides the first evidence that cells bearing LHON pathogenic mutations are sensitized to Fas-dependent apoptosis. We believe this observation may be relevant to the pathophysiologival mechanism of cell death in LHON. These findings could also have profound implications for LHON therapy by using anti-apoptotic drugs, by contrast to most current therapy for LHON, which is focused on the use of antioxidant vitamins and bioenergetic-supporting drugs, which have demonstrated little success (1, 2). Thus the above observations suggest not only a novel pathophysiological mechanism, but also perhaps a novel strategy for therapy of LHON neurodegeneration.

**Acknowledgments—** We thank Drs. Giuseppe Attardi and Michael King for the kind gifts of cells.

**Note Added in Proof—** We have also observed increased mitochondrial reactive oxygen species in a neuronal cell model of LHON (Wong et al. (2002) Hum. Mol. Genet., in press).
Cells Bearing Mutations Causing Leber's Hereditary Optic Neuropathy Are Sensitized to Fas-induced Apoptosis
Steven R. Danielson, Alice Wong, Valerio Carelli, Andrea Martinuzzi, Anthony H. V. Schapira and Gino A. Cortopassi

J. Biol. Chem. 2002, 277:5810-5815.
doi: 10.1074/jbc.M110119200 originally published online December 11, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M110119200

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 39 references, 13 of which can be accessed free at http://www.jbc.org/content/277/8/5810.full.html#ref-list-1