Apoptosis-associated Derangement of Mitochondrial Function in Cells Lacking Mitochondrial DNA

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ABSTRACT

U937 cells lacking mitochondrial DNA (p° cells) are auxotrophic for uridine and pyruvate, hypertensive to hypoglycemic conditions, and resistant to antimycin A-induced apoptosis. In spite of their obvious metabolic defects, p° cells possess a normal mitochondrial transmembrane potential, as well as a near-normal capacity to generate superoxide anion after menadione treatment. Similarly to p° controls, p° cells undergo apoptosis in response to tumor necrosis factor-α plus cycloheximide. Detailed comparison of the apoptotic process in p° and p+ cells reveals essentially the same sequence of events. In response to tumor necrosis factor/cycloheximide, cells first lose their mitochondrial transmembrane potential (Δψm) and then manifest late apoptotic alterations, such as generation of reactive oxygen species and DNA fragmentation. Experiments involving isolated mitochondria from p° and p+ cells confirm that p° mitochondria can be induced to undergo permeability transition, a process thought to account for the pre-apoptotic Δψm disruption in cells. Like p° mitochondria, p° mitochondria contain a pre-formed soluble factor that is capable of inducing chromatid condensation in isolated nuclei in vitro. This factor is released from mitochondria upon induction of permeability transition by calcium or the specific ligand of the adenine nucleotide translocator atractyloside. In conclusion, it appears that all of these p° sublines conserved TNF receptor expression and demonstrated a comparable TNF sensitivity. The P subline morphologically resembles most closely the parental wild-type cell line, and results are shown for this cell line. In all experiments, both p° and p+ control cells were maintained in the same culture medium supplemented with glucose, pyruvate, and uridine. Forty-eight h before experiments were performed, ethidium bromide was routinely removed from the medium of p° cells.

INTRODUCTION

For many years, it has been believed that the nucleus would constitute the prime target of the apoptotic process. Since 1994, however, it has become clear that even anucleate cells can undergo programmed cell death and that nonnuclear cytoplasmic structures participate in the control of apoptosis (1, 2). Recently, it has been found that cells undergo alterations in mitochondrial structure and function early during the apoptotic process, i.e., well before nuclear or chromatin structures are affected (3–11). This applies to rather different cell types: neurons (3), fibroblasts (4), thymocytes (6, 10), peripheral T lymphocytes (5, 9, 10), B cells (5), and myelomonocytic cells (7, 8). Before cells undergo nuclear apoptosis, they manifest a reduction in the mitochondrial transmembrane potential (Δψm), suggesting the involvement of mitochondria in the apoptotic process (3–11). This idea has been corroborated by the observation that cytoplasmic extracts enriched in mitochondria can induce apoptosis in isolated nuclei in vitro (12, 13). Contrasting with this hypothesis, however, it has been shown that a human fibroblast cell line lacking mtDNA3 dies from apoptosis in a Bcl-2-inhibitable fashion when stimulated with the phosphorytase kinase inhibitor staurosporine or cultured in the absence of FCS (14). Based on this observation, it has been widely assumed that mtDNA, and by extension mitochondria, would not be involved in the regulation of apoptosis.

Based on these premises, we decided to characterize cell lines lacking mtDNA and to determine the putative contribution of defective mitochondria to the apoptotic process. Our data indicate that mitochondria from cells lacking mtDNA (p° cells) undergo the same early apoptosis-associated changes as mitochondria from control (p+) cells. In normal conditions, both p° and p+ cells possess an intact Δψm. This Δψm is lost at an early pre-apoptotic stage when cells are induced to die from apoptosis. In addition, both p° and p+ mitochondria contain a pre-formed factor capable of inducing signs of nuclear apoptosis (chromatid condensation and DNA fragmentation) in isolated nuclei in vitro. These data underline the participation of mitochondria in the apoptotic process and indicate that all apoptotic functions of mitochondria are products of the nuclear rather than mitochondrial genes. Thus, our data confirm that mtDNA is not important for the regulation of apoptosis yet underscore the probable involvement of mitochondria in the apoptotic process.

MATERIALS AND METHODS

Generation of p° Cells and Culture Conditions. U937 cells were cultured at 37°C in a humidified atmosphere containing 5% CO2 in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 4.5 mg/ml glucose, 100 μg/ml sodium pyruvate, and 50 μg/ml uridine. To deplete mtDNA, cells were cultured during 6 months in the presence of ethidium bromide (50 ng/ml). Four different sublines were derived and controlled for mtDNA depletion using a specific PCR reaction (15). Three of these p° sublines survived TNF receptor expression and demonstrated a comparable TNF sensitivity. The P subline morphologically resembles most closely the parental wild-type cell line, and results are shown for this cell line. In all experiments, both p° and p+ control cells were maintained in the same culture medium supplemented with glucose, pyruvate, and uridine. Forty-eight h before experiments were performed, ethidium bromide was routinely removed from the medium of p° cells.

Induction of Apoptosis. Cells were cultured during the indicated interval in the presence of recombinant TNF-α (4 ng/ml; Research Diagnostics, Flanders, NJ) plus CHX (0.5 μg/ml; Sigma Chemical Co., St. Louis, MO), antimycin A (100 μM; Sigma), or glucose-free medium (Life Technologies, Inc.) plus 2-deoxy-glucose (2.5 mM; Sigma; Ref. 16), followed by the determination of apoptotic features. DNA fragmentation was assessed as described by agarose gel electrophoresis of nuclear DNA (5 × 106 cells/lane; Ref. 17).

Cytofluorometric Analysis and Confocal Microscopy. To measure the Δψm, cells were incubated at 37°C during 15 min in the presence of DiOC6(3) (40 mM; Molecular Probes; Ref. 4). To determine superoxide anion generation, cells were kept at 37°C during 15 min in the presence of HE (2 μM; Molecular

3 The abbreviations used are: mtDNA, mitochondrial DNA; TNF, tumor necrosis factor; Δψm, mitochondrial transmembrane potential; CHX, cycloheximide; DiOC6(3), 3,3'-diethyloxacarbocyanine iodide; HE, hydroethidine; mCCCP, carbonyl cyanide m-chlorophenylhydrazone; ROS, reactive oxygen species; Atr, atractyloside; BA, bongkrekic acid; RR, ruthenium red; p° cells, cells lacking mitochondrial DNA; p+ cells, cells bearing an intact mitochondrial genome.
Probes; Ref. 7). After incubation with DiOC6(3) plus HE, cells were immediately analyzed in an Epics Profile II cytofluorometer (Coulter, Miami, FL). In control experiments, cells were labeled after incubation in the presence of the protonophore mCICCP (50 µM; 30 min; Sigma) or the ROS-generating agent menadione (1 mM; 1 h; Sigma). DiOC6(3) fluorescence was recorded in FL1, and HE was recorded in FL3. Confocal microscopy was performed on cells labeled with either DiOC6(3)/HE or with ethidium bromide (50 ng/ml; 1 h) using a Meridian Acas 570 confocal microscope (Meridian Instrument Co., Okemos, MI). The frequency of cells having lost part of their chromosomal DNA (hypoploid cells) was determined by the propidium iodine staining of ethanol permeabilized cells, as described (18).

**In Vitro Tests of Mitochondrial Permeability, Transition, and Generation of Mitochondrial Supernatants.** Mitochondria were purified on a Percoll gradient (19) and were stored on ice in buffer B (400 mM mannitol, 10 mM PO4H,K, 5 mg/ml BSA, and 50 mM Tris-HCl, pH 7.2) for up to 4 h at a concentration of 100 µg protein/10 µl buffer. For determination of swelling, mitochondria (10 µl) were resuspended in 90 µl CFS buffer (220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM PO4H,K, 0.5 mM EGTA, 2 mM MgCl2, 5 mM pyruvate, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM DTT, and 10 mM HEPES-NaOH (pH 7.4); reagents from Sigma), and adsorption was recorded at 540 nm in a Beckman DU 7400 spectrophotometer, as described (20). Atr (final concentration, 5 mM; Sigma), BA (50 µM; kindly provided by Dr. J. A. Duine, Delft University, Delft, the Netherlands), CaCl2 (500 µM), and/or RR (100 µM; Sigma) were added to mitochondria. The loss of absorption induced by 5 mM Atr within 5 min was considered as the 100% value of large amplitude swelling. Supernatants (150,000 × g for 1 h at 4°C) of mitochondria treated with Atr, BA, CaCl2, and/or RR during 5 min at room temperature were recovered and used in a cell-free system of apoptosis.

**Cell-free System of Apoptosis.** Nuclei from HeLa cells were purified on a sucrose gradient, as described (21), and were resuspended in CFS buffer. Nuclei were preserved at −20°C in 50% glycerol for up to 8 days, as described (21, 22). Nuclei (1 × 10⁷/µl final concentration) were mixed with the supernatants from mitochondria and were incubated for 90 min at 37°C, followed by staining with 4′,6-diamidino-2-phenylindole dihydrochloride (10 µM) and examination by fluorescence microscopy (23).

**Statistical Analysis.** Results were calculated as arithmetic means ± SEM, and significance values were calculated by means of the Student t test. 

**RESULTS AND DISCUSSION**

Mitochondrial Inner Transmembrane Potential (Δψm) and Mitochondrial Superoxide Anion Generation in U937 ρ° Cells. The cationic lipophilic fluorochrome DiOC6(3) allows for the assessment of the mitochondrial transmembrane potential (Δψm; Refs. 5 and 24). Normal control U937 cells incorporate 1 to 1.5 log more DiOC6(3) than cells treated with the protonophore mCICCP, indicating that most of the dye incorporation is indeed driven by the inner mitochondrial transmembrane proton gradient (Fig. 1A). A detailed comparison of normal U937 cells (ρ° cells) and U937 sublines lacking mtDNA (ρ0 cells) revealed no significant difference in the Δψm; ρ° U937 cells possess a normal Δψm (Fig. 1A). In addition, the determination of superoxide anion in ρ° and ρ° cells does not reveal any major differences. The superoxide-driven conversion of nonfluorescent lipophilic HE into fluorescent hydrophilic ethidium is increased in the presence of menadione, a substance that undergoes redox cycles in mitochondria (Ref. 25; Fig. 1B). Menadione enhances the rate of HE—ethidium conversion both in ρ° and ρ° cells. However, as expected for partially respiration-deficient cells, the maximum HE—ethidium conversion is lower in ρ° than in ρ° cells (Fig. 1B). Thus, ρ° cells retain at least part of their capacity to generate superoxide anion after menadione treatment. In conclusion, it appears that ρ° cells possess a normal Δψm as well as a near-normal capacity of generating ROS species in response to the mitochondrion-targeted drug menadione.

**Early Δψm Loss and ROS Generation in ρ° Cells Undergoing Apoptosis.** In contrast to some ρ° cell lines that become resistant to the induction of necrosis in response to TNF (26, 27), U937 ρ° cells stimulated with a combination of TNF and CHX exhibit hallmarks of apoptosis such as oligonucleosomal DNA fragmentation (Fig. 2A) and loss of chromosomal DNA (hypoploidy; Fig. 2B). As described (7, 8), ρ° U937 cells treated with the apoptosis-inducing combination of TNF plus CHX exhibit a reduction in the Δψm, as well as an increase in superoxide anion generation (Fig. 3A, upper panels). As described (28), TNF and CHX exhibit a clear synergistic effect on

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**Fig. 1.** Mitochondrial transmembrane potential (Δψm) and superoxide anion generation in ρ° and ρ° U937 cells. Cells were treated with the uncoupling agent mCICCP (A) or with the superoxide anion-generating agent menadione (B), followed by labeling with the Δψm-sensitive dye DiOC6(3) or the superoxide anion oxidizable probe hydroethidine, respectively. Results are representative for three different experiments. Similar results were obtained with two additional U937 ρ° sublines (data not shown).
panels). Confocal microscopy confirmed the similarity between p° cells maintained in standard culture conditions exhibited <5% hypoploidy. The addition of TNF and/or CHX, followed by the determination of apoptotic parameters, i.e., Subdiploidy was determined by ethidium bromide staining of ethanol-fixed cells. Control cede nuclear signs of apoptosis. Analysis of several time points accelerate HE—»ethidium conversion, giving rise to a diffuse fraction of either p° or p+ cells lose DiOC6(3) incorporation and ethidium bromide, exhibit a normal distribution of intra-mitochondrial apoptosis induction, suggesting that they trigger different death path p° cells tend to exhibit a slower kinetics of apoptosis induction than p° p° controls (Fig. 4B). Moreover, p° cells are resistant to apoptosis induction and hypoploidy, irrespective of the presence of mtDNA (Fig. 4/4). Control experiments confirmed that p° cells, which rely on enhanced glycolysis, are only fully capable of undergoing permeability transition but also contain normal quantities of a protein that induces apoptotic nuclear changes in a cell-free system.

Concluding Remarks. As mentioned in the Introduction to this report, a human fibroblast cell line lacking mtDNA was shown to die from apoptosis in a Bcl-2-inhibitable fashion (14). Based on this observation, it has been postulated that apoptosis itself and the anti-apoptotic activity of Bcl-2 would not be related to mitochondria. The data contained in this report disagree with this interpretation. As expected, U937 rapidly underwent apoptosis in response to a combination of TNF and CHX, irrespective of their mtDNA status. p° U937 thus behave similar to p° L929 cells, which die in response to TNF plus actinomycin D (26), exactly as control p+ cells do. However, in contrast to L929 fibrosarcoma cells, which undergo necrosis in response to TNF (27, 34), U937 pro-monocytes undergo apoptosis (8, 16, 28), and this type of cell death can be induced in p° sublines, even in the absence of inhibitors of macromolecule synthesis (15). When induced to undergo apoptosis, p° U937 cells behave much as wild-type p+ cells in the sense that they exhibit an early reduction in ΔΨm that precedes all other manifestations of apoptosis, such as superoxide anion hypergeneration, cell shrinkage, hypoploidy, DNA fragmentation, and loss of plasma membrane integrity. It appears that the molecular mechanisms accounting for the pre-apoptotic ΔΨm disruption, i.e., mitochondrial permeability transition (7, 11), are operative in p° mitochondria and that p° mitochondria preserve their capacity to release a factor causing isolated nuclei to undergo apoptotic chromatin condensation. Therefore, in line with the published data (14), mtDNA does not encode any of the mitochondrial struct-

Fig. 2. Apoptosis sensitivity of p° and p+ U937 cells. Cells were cultured in the presence of TNF and/or CHX, followed by the determination of apoptotic parameters, i.e., DNA fragmentation (A; 12 h), or subdiploidy (B; 6 h). Oligonucleosomal DNA fragmentation was assessed by horizontal agarose gel electrophoresis (5 × 10^5 cells/ lane). Subdiploidy was determined by ethidium bromide staining of ethanol-fixed cells. Control cells maintained in standard culture conditions exhibited <5% hypoploidy. The addition of the relevant substance.

Normal Permeability Transition and Induction of Nuclear Apoptosis by p° Mitochondria. Our previous studies indicate that the opening of so-called mitochondrial permeability transition pores (30, 31) accounts for the pre-apoptotic ΔΨm disruption (7, 11). We, therefore, tested whether purified p° and p+ mitochondria would respond in a similar fashion to standard protocols for the induction of permeability transition. As shown in Fig. 5A, no difference could be detected in the permeability transition-dependent colloidiosmotic swelling of isolated p° and p+ mitochondria induced by calcium or by atracuridione, a specific ligand of the adenine nucleotide translocator (32). As a control, calcium-induced permeability transition was inhibited in the presence of RR, an inhibitor of the inner mitochondrial membrane calcium uniport (33). Similarly, the Atr-induced permeability transition was inhibited in the presence of its antagonist BA, both in p° and p+ mitochondria (Fig. 5A).

Further experiments involving a cell-free system of apoptosis (22) revealed that mitochondria induced to undergo permeability transition release a factor capable of inducing chromatin condensation in isolated nuclei. Mitochondria were treated with different inducers of permeability transitions and centrifuged. After ultracentrifugation, supernatants were recovered and added to isolated nuclei to determine their effect on chromatin distribution. An apoptosis-inducing factor was detectable in the supernatant of mitochondria treated with Atr or Ca^2+ but not in control supernatants of mitochondria treated with Atr/BA or Ca^2+ /RR (Fig. 5B), indicating a strict correlation between permeability transition (Fig. 5A) and release of the pro-apoptotic activity (Fig. 5B). This apoptosis-inducing activity is destroyed by proteinase K treatment, indicating that it is a protein. The addition of antioxidants did not abolish the apoptosis-inducing activity of mitochondrial supernatants, indicating that ROS are dispensable for nuclear apoptosis induction (data not shown). Both the supernatant of Atr- or calcium-treated p° and p+ mitochondria contain a similar amount of pro-apoptotic activity. No qualitative or quantitative differences could be appreciated between supernatants from p° and p+ mitochondria (Fig. 5, B and C). In conclusion, p° mitochondria are not only fully capable of undergoing permeability transition but also contain normal quantities of a protein that induces apoptotic nuclear changes in a cell-free system.

Moreover, p° cells lose DiOC6(3) incorporation and accelerate HE—»ethidium conversion, giving rise to a diffuse fraction of either p° or p+ cells lose DiOC6(3) incorporation and ethidium bromide, exhibit a normal distribution of intra-mitochondrial apoptosis induction, suggesting that they trigger different death pathways. This pattern was also obtained for p° cells (Fig. 3A, lower panels). Confocal microscopy confirmed the similarity between p° and p+ cells. p° cells, the mitochondria of which fail to stain with ethidium bromide, exhibit a normal distribution of intra-mitochondrial DiOC6(3) uptake (Fig. 3B). After stimulation with TNF/CHX, a fraction of either p° or p+ cells lose DiOC6(3) incorporation and thereby indicate a higher permeability to ethidium bromide, giving rise to a diffuse nonnuclear cytoplasmic staining (Fig. 3B). In view of these results, we investigated whether alterations in mitochondrial function would preclude nuclear signs of apoptosis. Analysis of several time points revealed that the loss of DiOC6(3) uptake preceedes DNA fragmentation and hypoploidy, irrespective of the presence of mtDNA (Fig. 4A). p° cells tend to exhibit a slower kinetics of apoptosis induction than p+ cells. The reasons for this difference are not understood. It is possible that a reduced metabolic rate and/or generation of superoxide anion (which mediates some of the TNF effects; Ref. 27) by p° cells could account for this discrepancy. The sequence of events (first ΔΨm disruption, then superoxide anion generation and DNA fragmentation) is essentially the same in p° and p+ cells (Fig. 4A). Control experiments confirmed that p° cells, which rely on enhanced glycolysis, undergo apoptosis in the absence of glucose much more rapidly than p+ controls (Fig. 4B). Moreover, p° cells are resistant to apoptosis induction by antitumycin A in conditions in which most p+ cells are killed (Fig. 4B). This is in accord with the fact that p° cells have a reduced complex III activity and thus become resistant to antitumycin A, an inhibitor of respiratory chain complex III (29).

These data indicate that p° cells induced to undergo apoptosis exhibit an early ΔΨm reduction that precedes DNA fragmentation, exactly as this is the case in p° control cells. Once the cells have lost their ΔΨm, they overproduce HE-detectable ROS.

Unpublished data.
Fig. 3. Mitochondrial alterations occurring in $\rho^0$ and $\rho^+$ U937 cells stimulated with TNF/CHX. Cells were cultured in the presence or absence of TNF/CHX for 3 or 6 h ($\rho^+$ cells and $\rho^0$ cells, respectively), followed by simultaneous staining with DiOC$_6$(3) and HE. A, cytofluorometric analysis. Results are representative for five independent experiments. TNF plus CHX cause a significant ($P < 0.001$) reduction of the DiOC$_6$(3) incorporation, as well as a significant increase in the HE—ethidium conversion, both in $\rho^+$ and $\rho^0$ cells. B, confocal microscopy of $\rho^+$ and $\rho^0$. Untreated cells were labeled with ethidium bromide (which reveals mtDNA plus the nucleus) or DiOC$_6$(3) plus HE. In addition, TNF/CHX-treated cells bearing a DiOC$_6$(3)$^{\text{high}}$HE$^{\text{low}}$ phenotype are shown.
Fig. 4. Kinetics of mitochondrial and nuclear alterations induced by TNF/CHX. A, chronology of mitochondrial and nuclear alterations. p° and p* U937 cells were cultured during the indicated interval in the presence of TNF/CHX, followed by assessment of the frequency of DiOC6(3)low HE- and DiOC6(3)low HE+ (as in Fig. 3A), and hypoploid cells (as in Fig. 2B). Results are the means (bars, SEM) of three independent experiments. For the loss of DiOC6(3) incorporation, the difference between p° and p* is significant (P < 0.01) at 1.5 h of culture. For hypoploidy, the difference is significant at 3 h. B, control experiments revealing the functional impact of the p° genotype. p° and p* U937 cells were cultured during 12 h in the presence of 2-deoxy-D-glucose or in the presence of antimycin A, followed by assessment of the frequency of hypoploid cells. Results are the means (bars, SEM) of three independent experiments. The apoptotic response to both 2-deoxy-D-glucose and antimycin A is significantly different between p° and p* cells.

Fig. 5. Permeability transition and elaboration of an apoptotic factor by p° and p* mitochondria. A, permeability transition of isolated mitochondria. Mitochondria derived from p° and p* cells were monitored for changes in the absorbance (OD540) indicative of permeability transition-dependent swelling. As indicated by arrows, BA (50 μM), RR (100 μM), Atr (5 μM), and calcium (500 μM) were added to isolated mitochondria. Results are plotted as the percentage of mitochondrial swelling, considering the Atr-induced drop in OD540 obtained after 5 min as 100% maximum value. B, apoptosis-inducing activity released from p° and p* mitochondria. Mitochondria were treated with BA, RR, Atr, and/or calcium (same doses as in A) for 5 min. Mitochondria were spun down, and ultracentrifuged supernatants were tested for apoptosis-inducing activity on isolated HeLa nuclei. After a 60-min incubation period at 37°C, 50 μl were stained with 4′,6-diamidino-2-phenylindole dihydrochloride and examined in a fluorescence microscope. A minimum of 200 nuclei was counted for each point by two independent investigators. Results are the means (bars, SEM) of three experiments. C, representative samples of nuclei treated with supernatants of isolated mitochondria. Examples of the dominant phenotype of nuclei (as in B, >70% of nuclei) are shown.
mainly to so-called contact sites with the inner membrane (36) in which permeability transition pores are expected to form (30, 31, 38, 39). Indeed, at least in some experimental systems, Bcl-2 is only present in the outer mitochondrial membrane where the permeability transition pore is located (30).

In synthesis, our results clearly implicate mitochondria in the apoptotic process. Because it is not possible to generate higher eukaryotic cells lacking mitochondria, indirect approaches, including cell-free systems of (pre-)apoptosis, will be required to determine whether mitochondria play a facilitative or an obligatory role in the apoptotic process.

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