Hypersensitivity of mtDNA-depleted cells to staurosporine-induced apoptosis: roles of Bcl-2 downregulation and cathepsin B

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Rommelaere G, Michel S, Mercy L, Fattaccioli A, Demazy C, Ninane N, Houbion A, Renard P, Arnould T. Hypersensitivity of mtDNA-depleted cells to staurosporine-induced apoptosis: roles of Bcl-2 downregulation and cathepsin B. Am J Physiol Cell Physiol 300:C1090–C1106, 2011. First published November 10, 2010; doi:10.1152/ajpcell.00037.2010. — We show that mitochondrial DNA (mtDNA)-depleted 143B cells are hypersensitive to staurosporine-induced cell death as evidenced by a more pronounced DNA fragmentation, a stronger activation of caspase-3, an enhanced poly-(ADP-ribose) polymerase-1 (PARP-1) cleavage, and a more dramatic cytosolic release of cytochrome c. We also show that B-cell CLL/lymphoma-2 (Bcl-2), B-cell lymphoma extra large (Bcl-Xl), and myeloid cell leukemia-1 (Mcl-1) are constitutively less abundant in mtDNA-depleted cells, that the inhibition of Bcl-2 and Bcl-Xl can sensitize the parental cell line to staurosporine-induced apoptosis, and that overexpression of Bcl-2 or Bcl-Xl can prevent the activation of caspase-3 in p0143B cells treated with staurosporine. Moreover, the inactivation of cathepsin B with CA074-Me significantly reduced cytochrome c release, caspase-3 activation, PARP-1 cleavage, and DNA fragmentation in mtDNA-depleted cells, whereas the pan-caspase inhibitor failed to completely prevent PARP-1 cleavage and DNA fragmentation in these cells, suggesting that caspase-independent mechanisms are responsible for cell death even if caspases are activated. Finally, we show that cathepsin B is released in the cytosol of p0 cells in response to staurosporine, suggesting that the absence of mitochondrial activity leads to a facilitated permeabilization of lysosomal membranes in response to staurosporine.

mitochondrial DNA depletion; lysosome

The number of identified pathologies linked to impaired mitochondrial activity has increased incredibly since the first neuropathies and myopathies associated with mitochondrial dysfunction were reported in the 1960s (67). Indeed, mitochondrial disorders are now also associated with type II diabetes, many cancers, and late-onset neurodegenerative disorders such as Alzheimer’s and Parkinson’s diseases (68). This large variety of pathological situations in which mitochondrial dysfunctions have been found could be linked to the major role of mitochondria in the integration and execution of the “intrinsic” and “extrinsic” apoptotic pathways (39).

Numerous links between mitochondrial dysfunction and apoptosis have been reported, and cell death seems to play a role in the pathogenesis of mitochondrial disorders associated with mitochondrial DNA (mtDNA) defects. For example, apoptosis in mitochondrial encephalomyopathies has been shown to result from mitochondrial DNA mutations (52). Thus apoptosis most probably contributes to mitochondrial pathology, and it is now accepted that an increase in apoptosis susceptibility is tightly linked with mitochondrial proliferation and a high mutation load in the genome (3). However, under these conditions, it seems that a putative apoptotic inducer is required to initiate an apoptotic response as cells that display mitochondrial dysfunction do not spontaneously die by apoptosis in vitro.

It was first reported that functional mitochondria were not a prerequisite for apoptosis to occur since mtDNA-depleted (p0) cells still undergo apoptosis (32). Later on, it was shown that mitochondria-like organelles from p0 cells are still able to release cytochrome c when apoptosis is induced (35), demonstrating that mtDNA-depleted mitochondria are still able to mediate apoptotic responses. However, several elements that lead to mtDNA-depleted cell hypersensitivity to apoptosis, triggered by mitochondrial dysfunction or lack of activity, are still poorly understood and some of the proposed mechanisms even remain controversial since cell death resistance or hypersensitivity to apoptosis have both been reported in cells exposed to mitochondrial dysfunction or in p0 cells (6, 43). Furthermore, it has recently been proposed that some p0 cell lines are resistant to apoptosis due to a higher expression of multidrug resistance complex proteins compared with the corresponding parental cell line (21).

The release of mitochondrial proteins in the cytosol is a key event in mitochondria-dependent cell death. This mechanism is thought to be regulated by B-cell CLL/lymphoma-2 (Bcl-2) protein family members, which control the outer mitochondrial membrane permeabilization and cytochrome c release in the cytosol, enabling the activation of caspases (14). Among these proteins, anti- and pro-apoptotic proteins can be distinguished based on their conserved α-helical domains that correspond to the Bcl-2 homology domains (BH 1–4) (30). Relative abundance, interactions between pro- and anti-apoptotic members, active conformation acquisition, posttranslational modifications, and modifications of localization are all key determinants in the onset and regulation of apoptosis by Bcl-2 family members (30).

To add to the complexity of relationships between the lack of activity and apoptosis in organelles, it is becoming more and more accepted that in addition to the “classical” apoptotic pathways that involve the activation of caspases downstream of “extrinsic” and/or “intrinsic” pathways, there are other mechanisms leading to cell death that involve noncaspase proteases and interactions between several organelles such as mitochondria, the endoplasmic reticulum, and lysosomes (9). Indeed, the contribution of proteases other than caspases, such as serine
proteases (65), calcium-dependent activated calpains (65), proteasome-associated proteases (71), and cathepsins (65) has been reported in caspase-independent apoptotic cell death. Lysosomal proteolytic enzymes or cathepsins can be cysteine (cathepsins B and L), aspartate (cathepsin D), or serine (cathepsin G) proteases. It has been shown that cathepsins B and D, activated by DNA damage, cross signal with mitochondria at the mitochondrial-lysosomal axis, directly or indirectly, and thus activate a caspase cascade leading to apoptosis (7). Rapidly, the mitochondrial-lysosome axis could refer to either mechanisms that would affect mitochondrial effectors involved in apoptosis (matrix metalloproteinase, cyclochrome c, procaspases activation, etc.) in response to lysosomal dysfunction accompanied by an increase in lysosomal membrane permeabilization allowing the release of cathepsins and/or mechanisms by which mitochondrial dysfunction could affect lysosomes to trigger and/or amplify apoptosis (8, 22).

It has also been reported that pro-apoptotic conditions can lead to an increase in lysosomal membrane permeability and the subsequent release of cathepsins (mainly cathepsins B, L, and D) in the cytosol where they might play an important role in the regulation of apoptosis (15). For instance, cathepsin B was shown to be able to cleave Bid in vitro (16) and to cause chromatin condensation, a major morphological feature of apoptosis. On the other hand, it is now accepted that another type of programmed cell death called autophagic cell death or type II programmed cell death can occur after cell exposure to various cell death inducers such as etoposide or staurosporine (STS) (48, 64). This revisited form of cell death in the apoptotic cell field is a lysosome-mediated, self-digestion process of the cell that needs cathepsins to achieve (27).

In conclusion, the issue of addressing whether or not cells with a lack of mitochondrial activity are hypersensitive or more resistant to apoptosis is still under debate in the literature (6, 43, 72), and the mechanisms behind these processes are still poorly understood. We therefore used \( \rho^{0} \)143B human osteosarcoma cells (40) were cultured as previously described (50). Depending on the experiment, both parental and \( \rho^{0} \)143B cells were grown on glass coverslips in 24-well or 6-well plates or 25- or 75-cm\(^2\) culture flasks (Corning), until confluency was reached, and they were then treated for the indicated times with 500 nM or 2 \( \mu \)M STS (Sigma) to induce apoptosis. When the inhibitors were used as indicated, the 143B and \( \rho^{0} \)143B cells were preincubated for 60 min with 10 \( \mu \)M \( \pi \)VAD-fmk (BD Pharmingen), 10 \( \mu \)M N-acetyl-L-leucyl-L-leucyl-L-norleucinal (ALLN, Sigma), 1 \( \mu \)M peptatin A (Sigma), 10 \( \mu \)M lactacystin (Calbiochem), 10 mM 3-methyladenine (Calbiochem), or 10 or 20 \( \mu \)M CA074-Me (Calbiochem) before STS addition. For the treatment with ABT-737, the inhibitor of Bcl2 and Bcl-X\(_{L}\), or its inactive enantiomere, the 143B and \( \rho^{0} \)143B cells were preincubated with STS and the inhibitor at a final concentration of 100 nM before treatment with STS for 4 h. At the end of the incubations, detached and attached/adherent cells were observed by confocal cell/surface microscopy for morphological changes, cell fractionation was performed by differential centrifugation, and clear cell lysates were prepared for Western blot analysis and a caspase-3 activity assay.

**Cell detachment and cell death assays.** Cells were grown in six-well plates and treated with or without STS for 6 or 24 h. In some conditions, cells were incubated with STS in the presence or in the absence of 100 nM ABT-737 for the indicated incubation period. At the end of the incubations, cell detachment and cell death assays determined by the incorporation of acridine orange (AO)/ethidium bromide (EB) (Sigma) were performed. At the end of the incubations, detached cells were counted in a hemocytometer (Neubauer chamber) while attached cells were counted after cells were collected by a treatment with trypsin (GIBCO). The proportions of detached cells in each experimental condition were then compared with the total cell number in the different cell populations seeded. For the cell death determination, detached cells were harvested by centrifugation and incubated for 2 min in PBS containing AO (3 \( \mu \)g/ml) and EB (10 \( \mu \)g/ml). Attached cells were rinsed once with PBS and then incubated for 2 min in PBS containing AO and EB at the same concentrations. Detached and attached/adherent cells were observed by fluorescence microscopy, and the number of cells positive (orange fluorescence) for EB (that stains dead cells with permeabilized plasma membrane) was counted out of a total cell number counted by the green fluorescence conferred by AO (a membrane permeable dye that stains alive cells) (4). To quantify the proportion of dead cells, micrographs were taken and counting was performed on three representative micrographs for each condition (meaning 3 countings of about 100 cells).

Each experiment was done in triplicates and dead cells were counted in both attached and detached cell populations in each experimental condition.

**Protein extraction.** Protein samples were prepared as previously described (50). For the determination of the abundance of Bcl-2 family proteins, Western blot analysis was also performed on samples prepared from total cell lysates (see supplementary data S3 online at the *AJP-Cell Physiol* website). Cells were treated or not with 2 \( \mu \)M STS for 6 h. The cells were then directly scraped in 1 \( \times \) Western blot loading buffer (100 nM Tris-HCl, pH 6.8, SDS 1.2%, 2-mercaptoethanol 3%, glycerol 6%, and bromophenol blue 0.01%). Detached Cells were harvested by centrifugation and collected in the same buffer. Western blot analysis was then performed on 30 \( \mu \)g of proteins of the total cell lysates, and the equal protein loading was checked by the immunodetection of \( \alpha \)-tubulin.

**Western blotting analysis.** The proteins were resolved by electrophoresis on 12% Tricine gels or 4–12% bis-Tris gel (NuPage, Invitrogen). After semidry transfer onto polyvinylidene fluoride (PVDF) membranes, Western blotting analysis was performed with primary and secondary antibodies against human PARP-1 (dilution 1:5,000, Pharmingen), human caspase-9 (dilution 1:3,000, Cell Signaling), and a caspase-3 activity assay.
human caspase-3 (dilution 1:1,000, Cell Signaling), human Bcl-2 (dilution 1:2,000, Sigma), Mcl-1 and cytochrome c (dilution 1:1,000, Santa Cruz), Bcl-XL (dilution 1:1,000, Sigma), Bcl-W (dilution 1:2,000, Cell Signaling), and LC3 (dilution 1:2,500, Nanotools). The anti-cathepsin B antibody was purchased from Calbiochem (dilution 1:2,500).

Western blot analysis was performed either by chemoluminescence (ECL advanced, Amersham Biosciences) or by infrared fluorescence (Odysey scanner, Li-Cor). For ECL detection, a horseradish peroxidase-conjugated antibody (Amersham Biosciences) was used as a secondary antibody at a 1:100,000 or a 1:300,000 dilution. For the Western blot in fluorescence, a secondary antibody coupled to infrared dyes (LiCor) was used at a 1:10,000 dilution. Protein loading for samples prepared from either total or clear cell lysates and cytosolic fractions was controlled by the immunodetection of α-tubulin (dilution 1:20,000, Sigma) or β-actin (1:20,000). Protein loading for "mitochondrial fractions" was controlled by the immunodetection of TOM-20 (Transporter of Outer Membrane-20; dilution 1:20,000; BD Biosciences). Fluorescence Western blot analysis by infrared technology (Li-Cor) allows the quantification of the fluorescence intensity of the bands corresponding to the protein of interest. For the quantification of lysosomal contamination in the cytosolic fractions, the fluorescence intensity of the band corresponding to lysosomal associated membrane protein-2 (LAMP-2) was measured for all the fractions [N, MLP, and S] and for each condition by fluorescence Western blotting. Based on the volume of the different fractions, a total fluorescent value for LAMP-2 was calculated for each condition. The sum of the values for N, MLP, and S gives a 100% intensity value for each condition allowing calculation of the percentage of lysosomal contamination in the different cytosolic fractions.

Caspase activity assay. For the caspase-3 activity assay, 143B and p143B cells were grown to confluence in six-well plates and were then incubated with or without 2 μM STS for the indicated times. The cells were then processed as follows: adherent and floating cells were collected in 50 μl PBS and pelleted by centrifugation (5 min, 1,000 g). They were then resuspended in 50 μl of a lysis buffer [10 mM HEPES/KOH; pH 7.0, 10% sucrose, 2 mM EDTA, 0.1% 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonate (CHAPS), 5 mM DTT, and 10 μg/ml aprotinin]. After a 30-min incubation period at 4°C with gentle agitation, the cell lysates were centrifuged at 10,000 g for 10 min at 4°C. Aliquots of the supernatant corresponding to equal amounts of proteins between the different samples were then diluted in a reaction buffer [20 mM piperazine-N,N’-bis(2-ethanesulfonic acid) (PIPES); pH 7.2, 100 mM NaCl, 2 mM EDTA, 0.2% CHAPS, 10% sucrose, and 10 mM DTT], and the proteolytic reaction was determined by measuring the fluorescence at the end of incubation (37°C for 1 h) with 10 μg/ml of Ac-Asp-Glu-Val-Asp-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC), a caspase-3/7 fluorescent substrate (BD Pharmingen). The fluorescence of the cleaved substrate was measured with a spectrofluorometer (excitation: 400 nm; emission: 505 nm; Kontron instruments).

Subcellular fractionation by differential centrifugation. Subcellular fractionation was performed by differential centrifugation. Briefly, 143B and p143B cells were preincubated with or without 20 μM CA074- Me for 60 min and then incubated with 2 μM STS for the indicated times before cell fractionation. Cells were then scraped in the culture medium with a rubber policeman and centrifuged for 5 min at 1,000 rpm. Cells were then rinsed once with PBS and once with 0.25 M sucrose by centrifugation at 1,000 rpm at 4°C. Cells were then resuspended in 2 ml 0.25 M sucrose and homogenized by six strokes of a B-type pestle in a 7-ml Dounce homogenizer (Kontes Glass). Cell homogenate was submitted to a centrifugation at 2,700 rpm for 10 min at 4°C in an International Centrifuge PR-J (International Equipment). The pellet was suspended in 2 ml of ice-cold 0.25 M sucrose, disrupted by six other strokes in a Dounce homogenizer, and submitted to a centrifugation at 2,100 rpm for 10 min at 4°C. This second pellet corresponded to the nuclear fraction (N) and was discarded.

MLP and S fractions were obtained from the postnuclear supernatant by a centrifugation carried out in a Beckman Coulter Optima TLX ultracentrifuge equipped with a TLA 100.3 rotor. MLP fractions are referred in this study as "mitochondrial fraction" as they contain largely distributed mitochondria among M, L, and P fractions, and S fraction is referred as "nonmitochondrial fraction," mainly containing soluble molecules.

DNA fragmentation assays. The DNA fragmentation assay on agarose gel was previously described in detail (43). Quantitative DNA fragmentation was also assessed with the Cell Death Detection ELISA kit (Roche) following the manufacturer’s recommendations. Briefly, cells were first incubated for 60 min with or without 10 μM CA074-Me or 10 μM zVAD-fmk before incubation with 2 μM STS for 6 h. At the end of the incubations, cells were incubated for an extra 30 min in 200 μl of an incubation buffer and then centrifuged at 13,000 rpm for 7 min at 4°C. The supernatants were collected and ELISA was performed on 10 μl of supernatant diluted 10 times with an incubation buffer in a 96-well plate coated with a anti-histone antibody. Mono- and oligonucleosomes were then detected by a colorimetric reaction in the presence of a horseradish peroxidase-conjugated anti-DNA antibody. Finally, absorbance was measured at 405 nm using a spectrophotometer (Bio-Rad), and the data were normalized for the protein content determined by a Bio-Rad protein assay.

Immunofluorescence confocal laser scanner microscopy. The 143B and p143B cells were seeded onto glass coverslips in 24-well plates. The next day, or 48 h posttransfection in the case of the transfection experiments, the cells were incubated with or without 2 μM STS and then processed for confocal microscopy as previously described (50). The antibodies used were the following: an anti-Bax6A7 antibody (dilution 1:100, Sigma Aldrich) that specifically recognizes Bax conformational changes, an anti-active caspase-3 antibody (dilution 1:100, Promega), or an anti-cathepsin B antibody (dilution 1:50, Calbiochem). For nuclear staining, the cells were incubated with TOPRO-3 (Molecular Probes) for 30 min and then processed for confocal microscopy. The cell number exhibiting caspase-3 activation was counted in three independent experiments performed on a minimum of 200 cells per experimental condition.

Cell transfection method. For Bcl-2-green fluorescent protein (GFP) and Bcl-XL-GFP overexpression, p143B cells were transiently transfected with plasmids encoding either GFP-tagged Bcl-2 or Bcl-XL using FuGENE 6 (Roche) according to the manufacturer’s instructions. Briefly, cells were grown on coverslips in 24-well plates and transiently transfected when 30% confluence was reached with FuGENE 6 for 48 h (DNA: FuGENE 6 transfection reagent ratio of 1:10).

Cathepsin B activity assay. Cathepsin B activity was assessed in N, MLP, and S fractions prepared as described above. Samples from N, MLP, and S fractions were mixed with activating buffer (340 sodium acetate, 60 acetic acid, 4 EDTA, and 4 aminoethane-thiol, pH 6) at 37°C for 10 min before the addition of the enzyme substrate (Z-Arg-Arg-AMC, 100 μM, Sigma). MLP and S samples were then incubated along with the fluorogenic substrate for 10 and 60 min, respectively. Reaction was stopped by the addition of fixation buffer (50 mM glycine, 5 mM EDTA, 0.05% Triton X-100, pH 10.5), and fluorescence was measured in a spectrofluorometer (Exλ: 360 nm and Emλ: 460 nm). Results are expressed as percentages of cathepsin B activity in MLP and S fractions compared with the total cathepsin B activity (sum of the activity measured in N + activity in MLP + activity in S).

Statistical analysis. Data from at least three independent experiments are presented as means ± SD and were analyzed with the appropriate statistical test as determined by the software SigmaStat 3.1. Differences between means were only considered as statistically significant when P < 0.05.
RESULTS

ρ0 143B cells are more sensitive to STS than parental 143B cells. Cells depleted in mtDNA have been extensively used as valuable tools for studying cellular responses to the mitochondrial impairment involved in various neuropathies and encephalomyopathies (67). As mitochondria play a major role in the commitment to cell death, we first tested the sensitivity of ρ0 143B cells to STS, a general inhibitor of protein kinases (PKCs and CDKs, among others) and a potent inducer of apoptosis (56).

To characterize cell death and cell detachment of 143B and ρ0 143B exposed to STS, several assays were performed. While both cell lines displayed cell shrinkage suggestive of modifications in the cytoskeleton after 6 h of treatment in the presence of STS, the cell detachment was stronger in ρ0 143B cells (Fig. 1, A and B). Indeed, as seen in the micrographs (Fig. 1A), fewer mtDNA-depleted cells remained attached to the culture plate after STS exposure. This effect was quantified by measuring the proportion of cells that are detached during the STS treatment in both cell lines by counting the number of cells in the media collected after STS treatments (Fig. 1B). To determine the proportion of cells that are detached after a 6- and a 24-h treatment with 2 μM STS, 143B and ρ0 143B cells were counted in the conditioned culture media with a Neubauer chamber, and attached cells were counted after a treatment with trypsin to collect cells in suspension. Cell detachment increases in ρ0 143B cells treated with STS for 6 h (12%) but not in 143B cells. However, after a 24-h treatment, cell detachment represents 54% of the total cell population in ρ0 143B cells, whereas only 35% of the 143B cells are detached in these conditions. In each cell population (attached or detached cell population), we also counted the number of dead cells using AO and EB assay, a test allowing discrimination of dead cells based on the detection of increased plasma membrane permeability (4). After a 6-h treatment with 2 μM of STS, only 1.3% of the attached cells from the ρ0 143B cell population were positive for EB while 23.5% of the detached cells of the ρ0 143B cell population were dead (Fig. 1C). In these conditions, there is no detectable cell death in 143B cells. After 24 h, the percentages of cell death reaches 3.9% and 52.7% in the attached and detached ρ0 143B cell populations, respectively, whereas we calculated that only 0.55% and 19.3% of the attached and detached 143B cell populations were positive for ethidium bromide, respectively (Fig. 1C). If these results confirm the hypersensitivity of ρ0 143B to STS, the percentages of cell death might appear relatively low, especially for the attached cell populations.

To assess the sensitivity of ρ0 143B and parental 143B cells to STS at the molecular level, both 143B and ρ0 143B cells were incubated with 2 μM STS for 0, 4, or 6 h before PARP-1

Fig. 1. The effect of staurosporine (STS) on cell death in ρ0 143B and 143B osteosarcoma cells. A: micrographs taken with a phase contrast microscope (Leitz Labover FS equipped with a Leica DC 100 digital camera) showing the morphological modifications of both 143B and ρ0 143B cells incubated for 6 h with or without (CTL) 2 μM STS. B: effect of STS on cell detachment. Parental 143B cells (open bars) or ρ0 143B cells (solid bars) were treated with or without (CTL) 2 μM STS for 6 or 24 h before cell detachment was evaluated. At the end of the incubations, cellular media were harvested and cells in suspension were counted (Neubauer chamber). Cells still attached were trypsinized and counted. Results are expressed as percentages calculated from the ratio between cells in suspension and total number of cells present in each condition (detached + adherent). Cell counting has been performed in triplicates for each condition and results represent means ± SD for n = 3. C: effect of STS on cell death evaluated by acridine orange (AO) and ethidium bromide (EB) incorporation. Parental 143B cells (open bars) or ρ0 143B cells (solid bars) were treated with or without (CTL) 2 μM STS for 6 or 24 h before cell death was evaluated by an AO and EB incorporation assay. At the end of the incubations, attached cells were rinsed once with PBS and incubated in a solution containing AO + EB. Detached cells were sedimented by centrifugation and resuspended in the same solution. Cells were next observed by fluorescence microscopy. Living cells appear as green while dead cells appear as red. Cell counting has been performed on 3 representative micrographs for each condition (3 countings of about 50 to 100 cells), and each condition was repeated three times. Results are expressed as percentages of attached dead cells compared with the total number of attached cells (left) or as percentages of detached dead cells compared with the total number of detached cells (right) and represent means ± SD for n = 3. **Significantly different from untreated control cells as determined by t-test with P = 0.001. + + + + + + Significantly different from corresponding treated parental cells as determined by t-test with P < 0.01 and P < 0.001 respectively.
cleavage was analyzed by Western blot (Fig. 2A). The fragment resulting from PARP-1 cleavage by caspase-3 or caspase-7 in vivo (p89) was clearly detectable in p143B cells incubated for either 4 or 6 h with STS, whereas it was barely detectable in 143B cells. In addition, it was found that the abundance of the Bcl-2 protein was dramatically reduced in p143B cells compared with 143B cells, whereas STS treatment did not change the global cellular abundance of this protein in these cell lines (Fig. 2A). We next checked for caspase-3 cleavage by Western blot analysis. As shown in Fig. 2B and as observed for the PARP-1 fragments, the caspase-3 active fragments (p19 and p17) were only detected in p143B after 4 and 6 h of treatment. To confirm the activation of caspase-3 in STS-treated cells, we then assessed the activity of caspases-3/7 by quantifying the fluorescence associated with the release of 7-amino-4-trifluoromethylcoumarin (AFC) from Ac-DEVD-AFC, a caspase-3/7 fluorogenic substrate. Caspase-3/7 activation induced by STS was clearly stronger in p143B.
cells after up to 12 h of treatment compared with the parental cells (Fig. 2C). Similar results were obtained with a lower STS concentration (500 nM) and with another ρ0 cell line derived from HeLa cells (see supplementary data Figs. S1 and S2A, respectively). These results suggest that the hypersensitivity found for ρ0143B cells is not specific to a particular cell background or a clonal effect but that it reflects a differential response to STS due to a lack of mitochondrial activity. As caspase-3 can be activated by the cytosolic release of cytochrome c, a protein sequestered in the mitochondrial intermembrane space (24), we thus assessed the abundance of cytochrome c released in the cytosolic fractions of 143B and ρ0143B cells incubated with or without STS (Fig. 2D). The data clearly show that cytochrome c was released in abundance from the mitochondria of ρ0143B cells in response to STS, whereas under these same conditions it was undetectable in the cytosol of 143B cells.

The release of cytochrome c in the cytosol is a common feature of the intrinsic apoptotic pathway usually associated with caspase-9 activation in the “apoptosome” (24). We therefore wanted to determine whether or not the release of cytochrome c and the activation of caspase-3 were correlated with caspase-9 activation; we checked for the processing of caspase-9 by Western blot. We found a cleaved fragment of caspase-9 (p35) in ρ0143B cells treated for either 4 or 6 h with STS, whereas caspase-9 processing was undetectable in 143B cells (Fig. 2E). Note that the abundance of procaspase-9 decreased with incubation time in the presence of STS in both cell lines, which could be correlated with the activating cleavage of the protein even though the p35 fragment was not detectable in the wild-type cell line. Taken together, these results show that a challenge with STS leads to the activation of the “intrinsic pathway”-related caspasas in mtDNA-depleted cells. Nuclear morphological alterations such as DNA fragmentation, chromatin condensation, and karyorrhexis (fragmentation of the nucleus) are considered as definitive hallmarks of apoptosis (74). Figure 2F clearly shows that the nuclear DNA of ρ0143B cells displays typical oligonucleosomal fragmentation after a 6-h treatment with STS, whereas the DNA in 143B cells is less affected. Similar results were obtained with another ρ0 cell line derived from HeLa cells (see supplementary data Fig. S2B). As already mentioned, we also found that the number of nuclei exhibiting karyorrhexis, a feature that can be highlighted by fluorescent DNA-specific probes such as TOPRO-3, increased in a time-dependent manner upon the addition of STS to ρ0143B cells (data not shown). In conclusion, these results not only show that STS induces the main features of apoptosis (i.e., PARP-1 cleavage, caspase-3, and caspase-9 activation, cytochrome c release, DNA fragmentation, and karyorrhexis) in mtDNA-depleted osteosarcoma 143B cells, but they also clearly demonstrate that these cells are more sensitive to STS than the 143B parental cells.

Anti-apoptotic proteins are constitutively downregulated in ρ0143B cells. To better delineate the mechanisms responsible for the hypersensitivity of ρ0143B cells to STS, and since it is known that Bcl-2 is constitutively downregulated in these cells, we next monitored the abundance of Bcl-XL in cytosolic fractions and the abundance of Bcl-w in clear cell lysates of 143B and ρ0143B cells treated with or without STS (Fig. 3, A and B). In addition, since Mcl-1 can localize to mitochondria as well as in the cytosol (25), the mitochondrial and nonmitochondrial abundances of Mcl-1 were determined in the subcellular fractions (Fig. 3C). Interestingly, we found that both Bcl-XL and Mcl-1 are less abundant in ρ0143B cells than in 143B cells, whereas the abundance of Bcl-w is comparable in both cell lines. The results related to the abundance of these proteins in both lines exposed or not to STS were also verified in samples prepared from total cell lysates. As seen in supplemental Fig. S3, the pattern of abundance for Mcl-1, Bcl-XL, Bcl-2, and Bcl-w was comparable when assessed on total cell lysates. However, while Western blot quantification revealed a slight decrease in the constitutive abundance for Mcl-1 and Bcl-XL in ρ0143B, difference in the abundance of these proteins between both cell lines was less impresssive for Bcl-XL and Mcl-1 when compared with the difference found for the abundance in mitochondrial fractions. This result means that a small difference in total protein abundance of the Bcl-2 family members could be accompanied by a different localization of these proteins between the two cell lines, a condition promoting a higher sensitivity to inducers of apoptosis in ρ0143B cells.

In addition, similar results were obtained for ρ0HeLa cells, where we found that the abundances of Bcl-2 and Bcl-XL were also lower in these cells when compared with the parental HeLa cells (supplementary data Fig. S4). Furthermore, it is interesting to note that Bcl-2, but not Bcl-XL, was also downregulated at the transcriptional level in ρ0143B cells, as shown by the mRNA abundance analysis via real-time RT-PCR (supplementary data Fig. S5). In addition, even though both 143B and ρ0143B cells responded to STS by an upregulation of Bcl-XL (Fig. 3A) while no modifications were observed for Bcl-2 or Bcl-w abundances (Figs. 2A and 3B, respectively), the abundance of Bcl-XL always remained lower in STS-treated mtDNA-depleted 143B cells compared with the 143B cells. We then looked for Bax activation (a pro-apoptotic member of the Bcl-2 family) using a specific antibody enabling to detect the structurally active form of the protein. We found that Bax was activated in both parental and mtDNA-depleted cell lines in response to STS treatment. In addition, counting cells that were positive for active Bax in both lines exposed to STS revealed that the number of cells that display positive fluorescence signals for active Bax is significantly higher in ρ0143B cells (Fig. 3E). It is thus most likely that the hypersensitivity of mtDNA-depleted cells to STS could be caused, at least partly, by both a higher activation of Bax and a global downregulation of anti-apoptotic proteins such as Bcl-2, Bcl-XL, and Mcl-1.

The constitutive lower abundance of Bcl-2 and Bcl-XL is responsible for the hypersensitivity of ρ0143B cells to STS. To support the assumption that the basal downregulation of anti-apoptotic members of the Bcl-2 protein family could sensitize mtDNA-depleted cells to STS, we first checked whether or not the inhibition of Bcl-2 and Bcl-XL could sensitize the cells to an apoptosis-inducing agent. We preincubated 143B and ρ0143B cells with ABT-737, a molecule known to be a potent inhibitor of Bcl-2, Bcl-XL, and Bcl-w proteins (55), or its inactive enantiomer (Abbott, Chicago, IL) before the addition of STS. The cleavage of PARP-1 (p89) was then monitored by Western blot analysis (Fig. 4A). Interestingly, the inhibition of Bcl-2-related proteins was able to sensitize 143B cells to STS as PARP-1 cleavage was clearly enhanced in the cells incubated with ABT-737, whereas its inactive enantiomer had no effect. Surprisingly, while Bcl-2 was barely detectable in
Fig. 3. Expression of Bcl-2 family members in 143B and ρ0143B cells treated or not with STS. A: Western blot analysis of B-cell lymphoma extra large (Bcl-XL) protein abundance performed on cytosolic fractions (30 μg proteins) prepared from 143B and ρ0143B cells incubated with (STS) or without (CTL) 500 nM STS for 4 h. B: Western blot analysis of Bcl-w protein abundance performed on 20 μg of clear cell lysates prepared from 143B and ρ0143B cells treated with (CTL) or without 2 μM STS for 6 h. C: Western blot analysis of Mcl-1 protein abundance performed on mitochondrial and nonmitochondrial fractions (30 μg proteins) prepared from 143B and ρ0143B cells incubated with or without (CTL) 500 nM STS for 4 h. For all Western blot analyses, protein loading was controlled by the immunodetection of α-tubulin (clear cell lysates and nonmitochondrial fractions) and transporter of outer membrane-20 (TOM-20, mitochondrial fractions). D: Immunostaining for active Bax (green) in 143B and ρ0143B cells incubated with or without 2 μM STS for 6 h. Representative pictures are shown. Scale bars: 40 μm.

E: Quantification of the percentage of positive cells for active Bax in 143B and ρ0143B cells incubated with or without 2 μM STS for 6 h. Counting has been performed on 6 representative micrographs for each condition (6 counting of about 50 to 100 cells). Open and solid bars represent 143B and ρ0143B cell lines, respectively. Results are expressed as percentages of cells stained for active Bax compared with the total number of cells present in the microscopic field as visualized by TOPRO-3 nuclear staining and represents means ± SD for n = 6. **, ***Significantly different from untreated control cells as determined by t-test with P < 0.01 and P < 0.001, respectively. ∗Significantly different from corresponding treated parental cells as determined by t-test with P < 0.05.

ρ0143B cells, we observed that ρ0143B cells could still be sensitized to STS in the presence of ABT-737. This effect could probably be explained by the inhibition of Bcl-2-related anti-apoptotic proteins still expressed in ρ0143B cells, such as Bcl-XL and Bcl-w (Fig. 3, A and B) (55).

Since 143B cells can be sensitized by the inhibition of Bcl-2-related anti-apoptotic proteins, the next step was to test the putative effect of an ectopic overexpression of either Bcl-2 or Bcl-XL in ρ0143B cells on the sensitivity to apoptosis induced by STS. For this purpose, the cells were transiently transfected with expression plasmids encoding either Bcl-2-GFP or Bcl-XL-GFP, treated with 2 μM STS for 6 h and then processed for confocal microscopy to assess the activation of caspase-3 (Fig. 4, B and C). As shown in Fig. 4D, the proportion of cells that display a positive signal for active caspase-3 after incubation with STS for 6 h is almost 3.5 times higher in ρ0143B cells than in 143B parental cells (21% vs. 6%; Fig. 4, B and D). However, we found a strong decrease in the number of stained cells for active caspase-3 in ρ0143B cells that overexpressed either Bcl-2-GFP or Bcl-XL-GFP (Fig. 4, C and D). The proportion of cells that displayed active caspase-3 (red) among the cells positive for GFP was reduced to the proportion found for cells showing active caspase-3 in parental cells (about 5% of the cell population, Fig. 4D). Thus the differential activation of caspase-3 in STS-treated ρ0143B cells seems to be directly linked to the constitutive downregulation of anti-apoptotic proteins. Under the same conditions, similar results were found for nuclear fragmentation (karyorrhexis) in ρ0143B cells that overexpressed Bcl-2-GFP or Bcl-XL-GFP when compared with the 143B cells (data not shown).

While ρ0143B cells have already been reported to be more prone than 143B cells to undergoing apoptosis under various conditions (STS, UV irradiation, or cisplatin) (43, 72), the underlying mechanisms are still poorly documented. Since the ABT-737 was able to sensitize both cell lines to STS as
evidenced by the increase in PARP-1 cleavage observed in these conditions (Fig. 4A), cell detachment and cell death assessed by the AO/EB incorporation was analyzed in 143B and rho143B cells treated with STS for 6 h in the presence or in the absence of ABT-737 to inhibit Bcl-2 family members. As seen in Fig. 4E, Bcl-2 inhibition by ABT-737 induces an increase in STS-induced cell detachment in 143B (from 0.9% to 7.7%) and in rho143B cells (from 12.1% to 43.3%). Percentages of cell death were also evaluated by AO/EB staining in both attached and detached cell populations (Fig. 4F). ABT-737 also induces a slight increase in the percentage of cell death in the attached cell population of rho143B cells (from 1.3% to 3%) but not in 143B cells. In detached cell populations, the proportion of dead cells slightly (but not significantly) decreases in rho143B cells (from 23.5% to 16.2%) while it significantly increases in 143B cells (from 0 to 4.3%). In conclusion, the overall cell death proportion (attached dead cells + detached dead cells) induced by STS is increased in both cell lines in the presence of ABT-737 confirming the involvement of Bcl-2 family members in apoptotic cell death induced by STS. Taken together, our results show that the basal downregulation of important anti-apoptotic proteins such as Bcl-2 and Bcl-XL can alone explain the hypersensitivity of mtDNA-depleted cells to apoptotic inducers.

However, while increasing evidence can be found to illustrate that caspase activation is usually found in cells responding to pro-apoptotic molecules (19), it might not always be a necessary key determinant in the onset of apoptotic features and lethal late events (9, 75).

**Alternative cell death in STS-treated rho143B cells.** It has been well reported that in addition to caspases, several other proteases such as lysosomal proteases, calcium-dependent proteases, or serine proteases can mediate cell death either independently or in a complementary manner to the activation of caspases (9, 37, 65). Therefore, to identify the class of proteases that can potentially contribute to rho143B cell death induced by STS, we next tested the effects of various inhibitors of pan-caspases (zVAD-fmk), calpains, cathepsin B/L (ALLN), and cathepsin D (pepstatin A) on PARP-1 cleavage in both cell lines incubated in the presence of STS (Fig. 5A). For these experiments, cells were preincubated with the inhibitors for 1 h before the addition of STS. We found that while zVAD-fmk completely inhibited PARP-1 cleavage in parental 143B cells, it was tremendously less efficient in rho143B cells (Fig. 5A, lanes 3 and 8). On the other hand, ALLN efficiently inhibited the cleavage of PARP-1 in rho143B cells (compare lane 9 with lane 7). Pepstatin A also had a differential effect as it reduced PARP-1 cleavage in rho143B cells but enhanced its cleavage in 143B cells (lanes 5 and 10). Therefore, while STS leads to PARP-1 cleavage in both cell lines, caspases might only play a critical role in the cleavage of this protein in the parental cells, while their contribution to PARP-1 cleavage in rho143B cells seems minor. Next, we monitored DNA fragmentation in rho143B cells under the same conditions (Fig. 5B). Whereas zVAD-fmk weakly inhibited DNA fragmentation in these cells, pepstatin A and, even more efficiently, ALLN prevented DNA fragmentation. In conclusion, it seems that caspase activation weakly contributes to DNA fragmentation in mtDNA-depleted 143B cells. This surprising data suggests that other proteases could be implicated in the DNA fragmentation observed in mtDNA-depleted 143B cells in response to STS, and we hypothesized that lysosomal proteases could be involved as STS has already been described as inducing apoptosis mediated by cathepsins (5).

**The role of autophagy in STS-induced cell death.** There is a complex and not fully understood relationship between autophagy and apoptosis, depending on the biological context (27). Intriguingly, regulators of apoptosis, such as members of the anti-apoptotic Bcl-2 family, can also directly “disarm” autophagy execution proteins such as Beclin-1, a protein that contains a structural domain considered to be a hallmark of pro-apoptotic proteins (42). We next wondered whether or not autophagy could be differentially stimulated in 143B and rho143B cells incubated with STS. To assess autophagy in both cell lines, we monitored LC3II protein abundance in cells treated with 2 μM STS. Indeed, during the autophagy process, a phosphatidylinositol degra-molamine group becomes attached to the LC3I soluble form and converts it into LC3II, which becomes inserted in the autophagosomal membranes (47). As shown in supplemental Fig. S6A, we found that STS induced an increase in LC3II abundance in both cell lines. However, the intensity of LC3 conversion in response to STS did not seem to be different between the 143B and rho143B cells. Furthermore, when both cell lines were incubated in the presence of STS with 3-methyladenine (3-MA), a well-known inhibitor of autophagy that acts by inhibiting class III PI3K activity (63), we observed an enhanced oligonucleosomal DNA fragmentation in both cell lines compared with the DNA fragmentation measured for STS-treated cells (supplemental Fig. S6B). Whereas it seems that STS induces autophagy in both cell lines, this process slightly protects cells against STS-induced apoptosis rather than contributes to it.

**Lysosomal proteases account for the hypersensitivity of rho143B cells to STS-induced apoptosis.** As apoptosis does not contribute to STS-induced cell death, we wondered how cathepsins could be involved in this process. Indeed, the contribution of lysosomal cathepsins (mainly cathepsins B, L, and D) to apoptosis has been recently reported, and it seems that they could act as major regulators once released into the cytosol (15). Although it was found that ALLN could inhibit typical apoptotic DNA fragmentation (Fig. 5B), it is a nonspecific inhibitor; therefore, we next tested the effect of CA074-Me, a specific cell-permeable cathepsin B inhibitor (33), on rho143B cell death triggered by STS. As illustrated in Fig. 6, we found that the inhibition of cathepsin B by CA074-Me strongly prevented both the cleavage of PARP-1 (Fig. 6A) and the activation of caspase-3/7 (Fig. 6B) in rho143B cells. Furthermore, the addition of a nonestereified form of the inhibitor at the same concentration directly in the caspase-3/7 assay mixture had no effect on the activity of the cysteinyl-aspartate proteases (data not shown). Therefore, a direct inhibition of caspase-3/7 activity by the inhibitor could be ruled out. Moreover, we also showed that, in the presence of the cathepsin B inhibitor, less cytochrome c was released into the cytosol of STS-treated rho143B cells (Fig. 6C), suggesting that cathepsin B acts upstream of cytochrome c release. We next compared the efficiency of both pan-caspase and cathepsin B inhibitors (zVAD-fmk and CA074-Me, respectively) on the DNA fragmentation triggered by STS in both cell lines (Fig. 6D). Very interestingly, we found that zVAD-fmk was the most effective inhibitor of DNA fragmentation in STS-treated 143B cells, but it had no significant effect on rho cells, whereas CA074-Me had a
significant inhibitory effect on DNA fragmentation only in STS-treated \( \rho^0 \)143B cells. Therefore, it seems that cathepsin B could be a major mediator of STS-induced cell death in cells that display a lack of mitochondrial activity. Cathepsins have been associated with cell death when acting outside the lysosome (15). We thus wondered whether or not a modification in the distribution of cathepsin B could be observed in \( \rho^0 \)143B cells incubated with STS. In Fig. 7A, the immunostaining for cathepsin B in both cell lines clearly shows that while the control cells display a punctuated fluorescence consistent with lysosomal localization, the STS-treated cells present a more diffuse fluorescence consistent with a redistribution of the protease from lysosomes to the cytosol. As seen in the fluorescence intensity plots that quantify and monitor the fluorescence distribution associated with cathepsin B and TOPRO-3 (Fig. 7A, bottom), in the control cells the stains of cathepsin B and TOPRO-3 are separated in space with a good resolution, whereas in STS-treated cells there is an overlap between the two stains, particularly in the \( \rho^0 \)143B cells. Indeed, based on quantification of the fluorescence intensity of the diffuse signal, the signal associated with cathepsin B outside the expected lysosomal localization was definitely stronger in \( \rho^0 \)143B cells responding to the apoptotic inducer than in 143B cells. To establish a causal link between the role of anti-apoptotic Bcl-2 family members and the activation of distinct proteolytic cascades by STS in these cell lines, 143B and \( \rho^0 \)143B cells were next treated for 6 h with 2 \( \mu \)M STS in the presence or in the absence of 100 nM ABT-737, an inhibitor of anti-apoptotic Bcl-2 family members. Then, a subcellular fractionation was performed and the abundance of cathepsin B protein was analyzed in the cytosolic fractions by fluorescence Western blotting using Li-Cor odyssey technology. This sensitive Western blot technique allows quantification of the abundance of both native (inactive form of 43 kDa) and cleaved cathepsin B.
Fig. 4. Bcl-2 and Bcl-XL control the sensitivity of 143B and \( \rho^0 \)143B cells to STS. A: effect of Bcl-2, Bcl-XL, and Bcl-w inhibition on PARP-1 cleavage. The 143B and \( \rho^0 \)143B cells were preincubated with or without 100 nM ABT-737 or with its inactive enantiomer for 60 min before STS addition (2 \( \mu \)M) for 4 h. At the end of the incubations, clear cell lysates were prepared and PARP-1 cleavage was analyzed by Western blotting performed on 20 \( \mu \)g of proteins. Protein loading was controlled by the immunodetection of \( \alpha \)-tubulin. B: immunostaining for active caspase-3 (red) in 143B and \( \rho^0 \)143B cells exposed to STS for 6 h. At the end of the incubations, cell lysates were prepared and the abundance of cleaved PARP-1 was monitored by Western blot analysis performed on 25 \( \mu \)g of proteins. Protein loading was controlled by the immunodetection of \( \alpha \)-tubulin. For a clearer presentation, four irrelevant conditions related to other inhibitors tested have been sliced from the original blot. Band cut is indicated by the black lines. C: \( \rho^0 \)143B cells were preincubated with or without 10 \( \mu \)M zVAD-fmk, 10 \( \mu \)M ALLN, or 1 \( \mu \)M pepstatin A for 1 h before the addition of 2 \( \mu \)M STS for an extra incubation of 6 h. The end of the incubations, the cells were harvested and the DNA was extracted and resolved on 2% agarose gel.

Fig. 5. STS-induced DNA fragmentation and PARP-1 cleavage in \( \rho^0 \)143B cells is mediated by noncaspase proteases. A: parental 143B and \( \rho^0 \)143B cells were preincubated with or without 10 \( \mu \)M zVAD-fmk (pan-caspase inhibitor), 10 \( \mu \)M ALLN (proteasome, cathepsin B/L, and calpain inhibitor), or 1 \( \mu \)M pepstatin A (cathepsin D inhibitor) for 1 h before the addition of 2 \( \mu \)M STS for an extra incubation of 6 h. At the end of the incubations, clear cell lysates were prepared and the abundance of cleaved PARP-1 was monitored by Western blot analysis performed on 25 \( \mu \)g of proteins. Protein loading was controlled by the immunodetection of \( \alpha \)-tubulin. For a clearer presentation, four irrelevant conditions related to other inhibitors tested have been sliced from the original blot. Band cut is indicated by the black lines. B: \( \rho^0 \)143B cells were preincubated with or without 10 \( \mu \)M zVAD-fmk, 10 \( \mu \)M ALLN, or 1 \( \mu \)M pepstatin A for 1 h before the addition of 2 \( \mu \)M STS for an extra incubation of 6 h. At the end of the incubations, the cells were harvested and the DNA was extracted and resolved on 2% agarose gel.
Fig. 6. Cathepsin B participates in the hypersensitivity of \( \rho^143B \) cells to STS. 143B and \( \rho^143B \) cells were preincubated with or without 10 or 20 \( \mu \)M CA074-Me for 1 h before incubation with 2 \( \mu \)M STS for an extra 6 h. At the end of the incubations, A: clear cell lysates were prepared and PARP-1 cleavage was analyzed by Western blotting performed on 20 \( \mu \)g of proteins. B: clear cell lysates were prepared and caspase-3 activity was determined in 143B cells (white) and \( \rho^143B \) cells (black) on 8 \( \mu \)g of proteins incubated with the fluorescent substrate (Ac-DEVD-AFC) for 1 h. The fluorescence of the cleaved substrate was subsequently determined with a spectrofluorometer (Ex: 400 nm; Em: 505 nm) and normalized for protein content. The results are expressed as fluorescence arbitrary units (FAU) normalized for protein content (FAU/\( g \) protein) and represent means \( \pm \) SD for \( n = 3 \). **, ***Significantly different from untreated control cells as determined by a \( t \)-test with \( P < 0.01 \) and \( P < 0.001 \), respectively. + + +Significantly different from STS-treated cells as determined by a \( t \)-test with \( P < 0.01 \). C: subcellular fractions were prepared by differential centrifugation and the release of cytochrome c was evaluated by fluorescence Western blot analysis (presented in black and white) performed on 4 \( \mu \)g of proteins of soluble fractions. D: parental 143B (left) and \( \rho^143B \) cells (right) were preincubated with or without 20 \( \mu \)M CA074-Me or 10 \( \mu \)M zVAD-fmk for 1 h before an additional incubation in the presence of 2 \( \mu \)M STS for 6 h. At the end of the incubations, cells were processed for the colorimetric DNA fragmentation assay as described in the MATERIAL AND METHODS section. The results are expressed as optical density (O.D.) normalized for protein content (O.D./\( \mu \)g protein) and represent means \( \pm \) SD for \( n = 3 \). **, ***Significantly different from corresponding untreated control cells as determined by a \( t \)-test with \( P < 0.05 \) and \( P < 0.001 \), respectively. + + +Significantly different from STS-treated cells as determined by a \( t \)-test with \( P < 0.05 \) and \( P < 0.01 \), respectively, NS, not significant. For all Western blot analyses, protein loading was controlled by the immunodetection of \( \alpha \)-tubulin.
These results suggest that mitochondrial DNA depletion can also lead to an increase in lysosomal membrane permeabilization in cells incubated with STS, leading to a stronger release of active cathepsin B in the cytosol of mtDNA-depleted cells when challenged with the pro-apoptotic molecule.

DISCUSSION

Mitochondrial dysfunction and a lack of mitochondrial activity have been directly and indirectly associated with various diseases, including numerous neuropathies and myopathies (67), type 2 diabetes (44), many cancers, and late-onset neurodegenerative disorders (68). In all these pathological situations, the question of whether or not cells with mitochondrial energetic dysfunction are more or less prone to undergoing apoptosis (spontaneously or in response to apoptotic inducers) is of prime interest. Indeed, it is now well recognized that apoptosis plays an important role in the clinical features of human mitochondrial pathologies (3). Furthermore, cancer treatments would also benefit from a better understanding of how mitochondrial activity can affect cell death in response to pro-apoptotic chemotherapeutic molecules since the aggressiveness of transformed cells with and without impaired mitochondrial activity seems to be different (1). Whereas mitochondria are recognized as major integrator organelles of apoptotic pathways, the requirement of their activity for integrating the apoptotic response is still a matter of debate (11, 43, 59). Indeed, the mechanisms by which mtDNA-depleted cells respond to apoptotic conditions either by a hypersensitivity or a higher resistance is still controversial and might be dependent on cell types and apoptosis-inducing stimuli. Interestingly, while conflicting results exist regarding the in vitro sensitivity of mtDNA-depleted cells to apoptosis-inducing molecules, in vivo experiments performed in Tfam knockout animals leading to mtDNA depletion revealed an increased embryonic apoptosis (69).

In this study, using a human osteosarcoma cell line totally depleted of mtDNA (ρ⁰143B), we present evidence that a lack of mitochondrial activity leads to a hypersensitivity of the cells to STS induced-apoptosis. Indeed, when parental and ρ⁰143B cells were incubated with STS, ρ⁰143B cells displayed enhanced cell detachment, cell death, as well as apoptotic features such as caspase-3 and caspase-9 activation, an increase in PARP-1 cleavage, a stronger cytochrome c release in the cytosol, and a more severe DNA fragmentation (Figs. 1 and 2). Furthermore, using PARP cleavage as an apoptotic marker, we also found a higher sensitivity of ρ⁰143B cells when cells were exposed to tumore necrosis factor-α (TNF-α) in the presence of cycloheximide (data not shown).

This hypersensitivity of ρ⁰143B cells to STS can be at least partly explained by an imbalance between the pro-apoptotic
Fig. 8. Effect of ABT-737 on STS-induced cathepsin B release in the cytosol. A: cytosolic fractions were prepared by differential centrifugation from 143B and r0143B cells treated with or without (CTL) 2 μM STS for 6 h (STS) in the presence or in the absence of 100 nM ABT-737 (STS + ABT). Cathepsin B abundance was monitored in cytosolic fractions by fluorescent Western blot analysis using secondary fluorescent antibodies. Protein loading and lysosomal contamination were controlled by the immunodetection of α-tubulin and LAMP-2, respectively. B and C: quantification of native (B) or cleaved (C) cathepsin B fluorescence by measuring intensities of appropriate signals as revealed by Western blot analysis. Results are expressed as fluorescence intensity normalized for α-tubulin for 143B (white columns) and r0143B cells (black columns). D: quantification of lysosomal contamination in cytosolic fractions based on quantification of LAMP-2 fluorescence intensity in the different cytosolic fractions.

and anti-apoptotic members of the Bcl-2 family. Indeed, in this study, we show that the main anti-apoptotic members of the family (Bcl-2, Bcl-XL, and Mcl-1) are constitutively down-regulated in r0143B cells while the abundance of active Bax is significantly higher in cells depleted in mtDNA exposed to STS (Fig. 3). The hypersensitivity of mtDNA-depleted cells to STS is thus, at least partly, the result of a decrease in the protection efficiency conferred by the anti-apoptotic proteins. To test this hypothesis, we analyzed the effect of inhibiting Bcl-2 and Bcl-XL on the onset of several apoptotic and cell death markers and found that these treatments sensitized 143B cells to STS, whereas overexpression of these proteins in r0143B cells rendered them more resistant to STS-induced apoptosis (Fig. 3). Based on this data, we can conclude that the constitutive imbalance between pro-apoptotic and anti-apoptotic proteins in r0143B cells explains their hypersensitivity to apoptotic stimuli. This set of data is in agreement with previous reports since it was shown that, in melanoma cells, the Bax/Bcl-2 ratio was correlated with cell susceptibility to Fas-mediated apoptosis (61). A downregulation of Bcl-2 and Bcl-XL was also reported to increase cell sensitivity to anticancer drugs (46). Interestingly, Bcl-2 mRNA, but not Bcl-XL mRNA abundance, was constitutively lower in r0143B cells compared with 143B cells. It has been reported that Bcl-2 expression can be suppressed by both c-myc and E2F-1 transcription factors (20). It is interesting to note our observation that c-myc nuclear abundance was constitutively higher in mtDNA-depleted cells (data not shown), suggesting a negative correlation between the low abundance of Bcl-2 and the higher content of nuclear c-myc in these cells.

With the growing number of studies dedicated to programmed cell death, it becomes evident that the “classical” apoptotic pathway involving caspases as the main executioner proteases might only represent a particular mode of a more sophisticated process (13). Moreover, it is also known that the same inducer can trigger both caspase-dependent and caspase-independent pathways (75). Thus we wondered whether or not STS-induced apoptosis could also be mediated by caspase-independent mechanisms. Indeed, while zVAD-fmk, a pan-caspase inhibitor, could prevent PARP-1 cleavage and DNA fragmentation in parental cells, this molecule was found to be quite ineffective in cells without mtDNA, thus minimizing the potential role of caspases in mtDNA-depleted cell death induced by STS. In addition, noncaspase inhibitors such as ALLN and pepstatin A were able to block DNA fragmentation and PARP-1 cleavage in r0143B cells (Fig. 5), suggesting the participation of other effectors in these cells. Thus it seems that mitochondrial dysfunction does not only sensitize the cell to an apoptotic stimulus such as STS by enhancing the activation of key effectors of the apoptotic canonical pathway but also by activating alternative pathways that might involve other proteases.

A growing number of recent studies report that lysosomal proteases could potentially be involved in programmed cell death. For instance, cathepsin D is known to mediate the release of cytochrome c and the activation of caspase during apoptosis of human fibroblasts exposed to STS (37), while cathepsin B has been shown to be involved as a dominant executioner protease in TNF-α-induced apoptosis of WEHI-S fibrosarcoma cells (23) and in bile salt-induced apoptosis of rat hepatocytes (62). Furthermore, lysosomal proteases and, more generally, lysosomal proteolytic enzymes are essential for autophagy to occur. Deregulated autophagy represents a different form of programmed cell death called autophagic cell death or Type II programmed cell death (10). This cell death pathway also involves Bcl-2 family members, such as Bcl-2 or Bcl-XL, which are common and shared by the apoptotic cell death pathway (47). Here, in analyzing the abundance of
LC3II, an autophagic marker, we also showed that STS induces autophagy in both 143B and ρ0143B cells, as evidenced by the accumulation of LC3II under the conditions used. It is important to note that LC3II abundance could be related to an increase in the autophagic process as well as to an accumulation of autophagosomes due to an inhibition of degradation by the lysosomes (53). Nevertheless, it is unlikely that autophagy would contribute to cell death under our experimental conditions since STS-induced DNA fragmentation was enhanced in the presence of 3-MA, an autophagy inhibitor (supplemental Fig. S6).

Lysosomal proteases are also involved in lysosomal membrane permeabilization (LMP), which is now widely accepted as an event occurring during programmed cell death (for a recent review see Ref. 8). Indeed, among cathepsins, cathepsin B, D, and L, more stable at neutral pH, are the three major cathepsin isoforms involved in programmed cell death (15) and their release from the lysosome can be induced by several apoptotic inducers. For example, Castino and colleagues (12) have shown that cathepsin D is released in the cytosol of SH-SY5Y neuroblastoma cells in response to hydrogen peroxide, allowing Bax translocation to mitochondria (12). TNF-α-induced cell death in human macrophages has been shown to be mediated by cathepsin B release and subsequent mitochondrial outer membrane permeabilization and caspase-dependent apoptosis. On the other hand, docetaxel induces cathepsin B-dependent and caspase independent cell death in prostate cancer cells (49). Interestingly, tunicamycin, which is an endoplasmic reticulum stressor, is also able to induce caspase-2 activation, which in turn induces cathepsin B release and subsequent caspase-8 and caspase-3 activation independently of mitochondria, which shows that the endoplasmic reticulum stress pathway can also be linked to the permeabilization of lysosomal membrane (31). More specifically, in the case of STS-induced cell death, LMP has already been reported in different studies (5, 37, 38). Bidère and colleagues (5) have shown that STS induces cathepsin B, D, and L release in the cytosol of activated T lymphocytes, which allows Bax activation by cathepsin D and mitochondrial outer membrane permeabilization. On the other hand, Kagedal and collaborators (38) have postulated that, in STS-treated fibroblasts, Bax is activated, translocates to the lysosome, and mediates LMP. This shows that in STS-induced programmed cell death, Bax could be involved either upstream or downstream of LMP.

Altogether these data show that LMP can occur in many cell death models, but the mechanisms leading to cell demise may be different according to the cell line and/or the stimulus. Mechanistically, the release of cathepsins from the lysosome to the cytosol can be mediated by several effectors such as radical oxygen species (ROS), Bcl-2 family proteins, p53, caspases, calpains, or cathepsins themselves (for reviews see Refs. 8 and 36) that can, at least in part explain the heterogeneity and the complexity of the LMP contributing to apoptosis.

To determine whether cathepsin B could be involved in STS-induced apoptosis, we tested the effect of CA074-Me, a specific cathepsin B inhibitor, on several apoptotic markers. We clearly demonstrated that cathepsin B is a major protease responsible for the higher sensitivity of ρ0143B cells to STS-induced apoptosis. Indeed, the cathepsin B inhibitor strongly decreased caspase-3 activation, cytochrome c release, and PARP-1 cleavage in these cells. Moreover, we showed that CA074-Me is only effective in preventing DNA fragmentation in cells that display a lack of mitochondrial activity, whereas it had no significant effects on parental cells (Fig. 6). The action of lysosomal proteases in cell death is mainly related to their cytosolic activity that can trigger the caspase cascade and cleave pro-apoptotic proteins such as Bid (16). Here, using Western blot analysis, biochemical assay, and in situ immunostaining, we showed that STS induces the lysosomal release of active cathepsin B mainly in ρ0143B cells, suggesting that lysosomal membrane integrity might be more vulnerable in these cells exposed to STS than in the parental 143B cells.

However, the differential effect observed for the full-length enzyme and the cleaved and active enzyme (Fig. 8) suggests a regulated control for the cathepsin B release. STS is a molecule that was previously reported to trigger the translocation of cathepsins D and B during oxidative stress in cells treated with the kinase inhibitor (23, 28, 37). The apoptotic function of cathepsin B in vivo was clearly evidenced in liver cells. Indeed, it was shown that TNF-α failed to induce the release of cytochrome c or to trigger caspase activation in the hepatocytes of cathepsin B knockout mice (29). In addition, pharmacological inhibition of cathepsin B by CA074-Me also prevented apoptotic liver damage induced by TNF-α treatment (29).

Very interestingly, a lysosomal/mitochondrial pathway was also recently described in mifepristone-induced apoptosis in U937 cells (58). In this study, the authors found that a cathepsin B/L inhibitor partially prevented caspase activation and apoptosis induction. Under the conditions of this study, they also showed that the release of cathepsin B was inhibited by Bcl-2 overexpression (58). As the expression of both Bcl-2 and Bcl-XL is decreased in ρ0143B cells and as ABT-737 increases the release of cathepsin B in the cytosol in 143B and in ρ0143B cells (Fig. 8), it is tempting to speculate that LMP could be facilitated in mtDNA-depleted cells compared with 143B cells, promoting mitochondrial permeabilization and cell death.

While many studies have been dedicated to better understanding how the alteration of mitochondrial activity could trigger nuclear responses, the molecular pathways linking the mitochondrial dysfunction to the stability and/or activity of other organelles, such as the lysosome and endoplasmic reticulum, are still poorly investigated. As mitochondria and lysosomes could be closely related in some forms of programmed cell death, notably by sharing common regulatory proteins such as Bcl-2 (60), it would be interesting, in the future, to understand how both organelles directly or indirectly interact with each other, and how the mitochondrial/lysosomal and lysosomal/mitochondrial axes cross-talks are set up when their activity is impaired. Recently, several works showed that the chaperone heat shock protein (Hsp) 70 can also exert a prosurvival effect by inhibiting lysosomal membrane permeabilization and cathepsin release in the cytosol (17, 54). In fact, it seems that Hsp70 binds to bis-monoacylglycerophosphate (BMP), a phospholipid involved in lysosomal sphingomyelin metabolism. This interaction between BMP and Hsp70 would facilitate the conversion of sphingomyelin into ceramide, increasing the lysosomal membrane stability (41). Previously, we established a gene expression profile in 143B and ρ0143B cell lines using a low-density DNA microarray. This array allows gene expression analysis for 202 genes involved in various cellular pathways such as cell cycle, apoptosis, and...
signal transduction (“DualChip human general microarray,” Eppendorf; 18). We found that Hsp 70 was constitutively and significantly downregulated in $\rho^0$143B when compared with 143B cells (ratio: 0.58 ± 0.11, $n = 3$; $P < 0.001$) (L. Mercy, unpublished data). We thus hypothesized that this downregulation could facilitate the lysosomal membrane permeabilization and cathepsin B release in $\rho^0$143B cells exposed to STS.

In addition to Bcl-2 family members expression and to lysosomal permeabilization, the metabolism alteration and the glycolytic shift displayed by mtDNA-depleted cells might also contribute to modulate cell death sensitivity to apoptotic inducers. Indeed, we know that $\rho^0$143B cells and, more generally, mtDNA-depleted cells are characterized by major changes in metabolic activities. Indeed, the respiratory chain in these cells is not functional anymore, $O_2$ consumption is decreased when compared with parental cells (57), mitochondrial membrane potential is lower (2), and ATP is mainly generated by the glycolysis pathway (66). These features could directly influence cell sensitivity to apoptosis. For example, Jeong et al. (34) have shown that glycolysis inhibition in Chinese hamster ovary cells induces resistance to oxidative-stress-induced cell death (34). Moreover, it is now well accepted that cancer cells produce ATP mainly by the glycolytic pathway, which allows them to resist to cell death induced by many agents (26). In the cell lines used in our study, the glycolytic shift of $\rho^0$143B cells does not induce resistance to cell death compared with 143B. It is even the opposite as $\rho^0$143B cells are more sensitive to STS and a cocktail containing TNF-α + cycloheximide. However, here we are comparing two cancerous/transformed cell lines already known to mainly produce ATP by glycolysis when compared with untransformed cells (66). So, in these conditions, it is difficult to assess whether or not glycolysis modulates “resistance or increased sensitivity to apoptosis” phenotype as while 143B cells still produce, at least in part, ATP by the respiratory chain complexes, they also display a high glycolytic activity. Another characteristic of $\rho^0$ cells is the mitochondrial membrane potential that is lower than in the corresponding parental cell line (2). During the apoptotic process, the inner mitochondrial membrane is depolarized either before or after cytochrome c release (45). Furthermore, some studies indicate that a lower mitochondrial membrane potential could sensitize cells to apoptosis inducers (51). This could also be the case for $\rho^0$143B cells because these cells display a lower mitochondrial membrane potential than 143B cells. However, we do not have any data regarding the implication of the putative lower mitochondrial membrane potential in STS-induced cell death in $\rho^0$143B cells.

In summary, we demonstrated that both caspase-dependent and caspase independent mechanisms, induced by treatment with STS, participate in apoptotic cell death in mtDNA-depleted ($\rho^0$) 143B cells (Fig. 9). LMP and cathepsin B release in the cytosol are the major mechanisms induced by the kinase inhibitor in $\rho^0$143B cells while in parental 143B cells, cell death is mainly mediated by cytochrome c release and caspases activation. The LMP could be linked to Bcl-2, a protein constitutively downregulated in cells completely depleted of mtDNA that represents a major effector in apoptosis (73). In turn, this process might lead to the release of lysosomal proteases such as cathepsin B that could also play a role in the mitochondria by controlling the release of cytochrome c. These results will bring a new understanding to the sensitivity/resistance of cancer cells to chemotherapeutic drugs through their mitochondrial activity status.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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