Anticancer Drugs Induce Increased Mitochondrial Cytochrome c Expression That Precedes Cell Death

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ABSTRACT

Recent studies have demonstrated that cytochrome c plays an important role in cell death. In the present study, we report that teniposide and various other chemotherapeutic agents induced a dose-dependent increase in the expression of the mitochondrial respiratory chain proteins cytochrome c, subunits I and IV of cytochrome c oxidase, and the free radical scavenging enzyme manganese superoxide dismutase. The teniposide-induced increase of cytochrome c was inhibited by cycloheximide, indicating new protein synthesis. Elevated cytochrome c levels were associated with enhanced cytochrome c oxidase-dependent oxygen uptake using TMPD/ascorbate as the electron donor, suggesting that the newly synthesized proteins were functional. Cytochrome c was released into the cytoplasm only after maximal levels had been reached in the mitochondria. The release of mitochondrial cytochrome c in particular is a critical step in the apoptotic as well as the necrotic process (5–7). However, the mechanism(s) by which cytochrome c is released from mitochondria remains unclear. It has been proposed that PT would allow the passive release of cytochrome c and other caspase-activating factor(s) from the mitochondrial intermembrane space into the cytosol (8, 9). However, it has also been reported that in various cell types the release of cytochrome c occurs before or even in the absence of a change in mitochondrial permeability, suggesting that this release can occur by mechanisms other than the opening of the PT pore (10–13). For example, overexpression of the pro-apoptotic protein Bax has been shown to trigger cytochrome c efflux from mitochondria and cell death (14, 15), possibly via the formation of membrane channels, whereas the redistribution of cytochrome c during apoptosis can be prevented by overexpression of the anti-apoptotic protein Bcl-2 (10, 11). Thus, the exact sequence of events leading to the onset of cell death remains unclear and may differ in different systems.

INTRODUCTION

Recent studies have shown that alterations of mitochondrial functions such as PT1 play a major role in the apoptotic process induced by chemotherapeutic agents (1, 2). Mitochondria undergoing PT release apoptogenic proteins such as cytochrome c or the apoptosis-inducing factor from the mitochondrial intermembrane space into the cytosol, where they can activate caspases and endonucleases (3, 4). The release of mitochondrial cytochrome c in particular is a critical step in the apoptotic as well as the necrotic process (5–7). However, the mechanism(s) by which cytochrome c is released from mitochondria remains unclear. It has been proposed that PT would allow the passive release of cytochrome c and other caspase-activating factor(s) from the mitochondrial intermembrane space into the cytosol (8, 9). However, it has also been reported that in various cell types the release of cytochrome c occurs before or even in the absence of a change in mitochondrial permeability, suggesting that this release can occur by mechanisms other than the opening of the PT pore (10–13). For example, overexpression of the pro-apoptotic protein Bax has been shown to trigger cytochrome c efflux from mitochondria and cell death (14, 15), possibly via the formation of membrane channels, whereas the redistribution of cytochrome c during apoptosis can be prevented by overexpression of the anti-apoptotic protein Bcl-2 (10, 11). Thus, the exact sequence of events leading to the onset of cell death remains unclear and may differ in different systems.

MATERIALS AND METHODS

Reagents. All reagents and drugs were obtained from Sigma (St. Louis, MO), unless otherwise indicated. Antibodies against COX subunits I and IV were from Molecular Probes (Eugene, OR); against cytochrome c (clone 7H8.2C12) and caspases 3 and 9 from PharMingen (San Diego, CA); against PARP from Roche Biochemicals (Indianapolis, IN); against GAPDH (clone 6C5) from Research Diagnostics, Inc. (Flanders, NJ); and against citrate synthase from Chemicon International, Inc. (Temecula, CA). Anti-VDAC antibody was a gift from Dr. C.A. Mannella, (Wadsworth Center, Albany, NY) and anti-manganous superoxide dismutase antibody was a gift from Dr. J. A. Melendez (Albany Medical College, Albany, NY). A complete cocktail of protease inhibitors was purchased from Roche Biochemicals (Indianapolis, IN).

Cell Culture. Human breast carcinoma MDA-MB-231 were cultured in IMEM medium supplemented with penicillin, streptomycin, glutamine (2 mM), and 10% fetal bovine serum at 37°C in a humidified atmosphere of 5% CO2 in air.

Separation of Attached and Floating Cells. During the process of cell death, MDA-MB-231 cells detached from the culture flask and floated in the medium. We exploited this phenomenon as an easy way to separate attached/living cells from floating/dead cells. Thus, floating cells were collected from the culture supernatant by centrifugation (5 min at 1200 rpm), whereas the attached cells were harvested by trypsinization. Viability of floating and attached cells was determined by PI staining and microscopic evaluation. Only samples of attached cells with >85% of viable cells were used for additional studies. The floating cell population was 100% PI-positive.

DNA Fragmentation Analysis. For DNA fragmentation analysis, 3 × 106 MDA-MB-231 cells were exposed to teniposide (10 μM) for 24, 48, or 72 h. Attached and floating cells were harvested and combined, washed with cold PBS, and resuspended in 400 μl of hypotonic lysis buffer A [10 mM Tris/HCl (pH 7.5), 1 mM EDTA, and 0.2% Triton X-100]. The cell lysates were centrifuged at 13,000 rpm for 15 min in a microcentrifuge. The supernatant (350 μl) was then incubated with 106 μl of lysis buffer B [150 mM NaCl, 10 mM Tris-HCl (pH 8.0), 40 mM EDTA, 1% SDS, and 0.2 mg/ml proteinase K, final concentrations] for 4 h at 37°C. The DNA was extracted with phenol/chloroform/isooamyl alcohol (25:25:1, v/v/v) and ethanol precipitated for 12–18 h. After centrifugation for 5 min at 13,000 rpm and 4°C, the DNA pellet was washed with 50 μl of 70% ethanol and resuspended in 15 μl of 10 mM Tris/HCl (pH 8.5), 1 mM EDTA containing 50 μg/ml RNase, and incubated for...
1 h at 37°C. Each DNA sample was then analyzed on a 1% agarose gel containing 0.1 µg/ml ethidium bromide. The same amount of DNA, as assessed by spectrophotometric measurement, was loaded in each lane. A mixture of 
Har III-digested 
Hind III-digested ADNA and Hind III-digested ADNA was run as size markers. DNA fragmentation was also measured by quantitation of hypoplo-
oid nuclei after DNA staining with PI. After teniposide treatment and harvesting as above, cells were fixed with 70% cold ethanol at 4°C overnight.

After centrifugation, the fixed cells were resuspended in 1 ml PI staining solution (5 µg/ml PI in 0.1% sodium citrate and 0.1% Triton X-100) and incubated for 30 min at 4°C. Stained nuclei were analyzed on a FACScan (Becton Dickinson Immunocytochemistry System, San Jose, CA). Hypoploid cells appear as a sub-G1 peak.

**COX Activity in Whole Cells.** COX activity was measured as described (16). MDA-MB-231 cells were treated with teniposide (10 µM) for 24, 48, or 72 h. After the indicated time, the floating cells were removed. The attached cells were harvested and resuspended in respiration buffer [0.25 M sucrose, 72 h. After the indicated time, the floating cells were removed. The attached cells were harvested and resuspended in respiration buffer [0.25 M sucrose, 0.1% BSA, 10 mM MgCl₂, 10 mM NaF, 5 mM KH₂PO₄ (pH 7.2)] at a final concentration of 4 × 10⁶ cells/ml. One-half ml of the cell suspension was injected into a chamber containing 3.5 ml of air-saturated respiration buffer and 1 mM ADP at 37°C. The cells were permeabilized with digitonin (final concentration, 0.005%), and substrates and inhibitors were added in the following order and final concentrations: (a) antimycin A, 50 nM; (b) ascorbate, 1 mM; and (c) TMPD, 0.4 mM. Antimycin A was used to inhibit autologous mitochondrial electron transport. TMPD is an electron donor that reduces cytochrome c nonenzymatically. Therefore, when TMPD is used as a substrate, changes in O₂ uptake rates reflect changes in COX activity. Ascorbate was used to reduce TMPD. The oxygen consumption was calibrated with air-saturated buffer, assuming 390 ng-atoms of oxygen/ml of buffer. Rates of potassium cyanide-sensitive oxygen consumption are expressed as ng-atoms of oxygen/min/2 × 1⁰⁷ cells.

**Western Blot Analysis of Cytosolic and Mitochondrial Fractions.** Cytosolic and mitochondrial fractions were prepared as described (17). Floating and attached MDA-MB-231 cells were collected separately, washed twice with ice-cold PBS (pH 7.4), and resuspended in 600 µl of extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM PIPES-KOH (pH 7.4), 50 mM KCl, 5 mM EGTA, 1 mM DTT, and protease inhibitors. After 30 min incubation on ice, cells were homogenized with a Teflon homogenizer for 15 min at 300 rpm. Cell homogenates were centrifuged at 14,000 × g for 15 min, and the cytosolic supernatants removed and stored at −70°C. The quality of the cytosolic fraction was routinely monitored by Western blotting for cytochrome oxidase subunit I as a marker of mitochondrial contamination, whereas the mitochondrial fraction was routinely monitored for dihydrofolate reductase as a marker of cytoplasmic contamination. The pellet containing the mitochondria was resuspended in extraction buffer and stored at −70°C. Twenty-five µg of cytosolic or mitochondrial proteins were separated on a

**Measurement of Mitochondrial Membrane Potential by Flow Cytometry.** Changes in the mitochondrial membrane potential ΔΨₘ were analyzed using JC-1 (Molecular Probes, Inc., Eugene, OR). This cyanine dye accumulates in the mitochondrial matrix under the influence of the ΔΨₘ and forms J-aggregates that have characteristic absorption and emission spectra (18). The JC-1 staining method is reported to provide more accurate estimates of ΔΨₘ than DIOC6 (Refs. 3 and 19). Untreated controls and cells treated with teniposide (10 µM) for 24, 48, or 72 h were incubated in 0.4 ml of IMEM with 0.5 µM JC-1 for 10 min. As a positive control for reduction of ΔΨₘ control cells were treated with the uncoupling agent CCCP (1 µM) before labeling with JC-1. Cell suspensions were prepared for flow cytometry, and the 488-nm line of an argon ion laser was used for excitation. Red and green emitted fluores-
cence was collected through 585/42 (FL2) and 530/30-nm (FL1) bandpass filters, respectively. Flow cytometry was performed on a Coulter Elite flow cytometer. After gating out small-sized debris, 10,000 events were collected for each analysis. The ratio of FL2 versus FL1 was used to analyze ΔΨₘ. Forward scatter was used to differentiate live from dead cells.

**Immunostaining and Microscopy.** For immunostaining, MDA-MB-231 cells were seeded onto 18 × 18 mm no. 1 glass coverslips and grown for 24–48 h in IMEM supplemented with 10% FBS. In some experiments, cells were treated with teniposide for 48 h. To differentiate between live and dead cells, the “Dead Red” reagent (Molecular Probes, Eugene, OR) was added to the cells 10 min before fixation. For immunostaining, cells were fixed in 3.8% paraformaldehyde for 5 min at room temperature, permeabilized in 0.1% saponin for 5 min, and stained with anti-cytochrome c antibody dilute at 1:100. FITC-conjugated goat antimouse secondary antibody (Sigma, St. Louis, MO) was used at 1:100. DNA was stained using Hoechst 33342 at 0.4 µg/ml. Preparations were mounted in FluoroGard antifade reagent (Bio-Rad, Hercules, CA) and analyzed using a Nikon Diaphot microscope, equipped with a QuadFluor epi-fluorescence attachment. Images were recorded with a Photometrics PXL cooled charge-coupled device camera.

**Protein Determinations.** Protein concentrations were determined by the Bradford assay (20).
Statistical Analysis. All results are expressed as means ± SD unless stated otherwise. The unpaired Student’s t test was used to evaluate the significance of differences between groups, accepting \( P < 0.05 \) as the level of significance.

RESULTS

Characterization of Teniposide-induced Cell Death in MDA-MB-231 Cells. In agreement with previous reports demonstrating that teniposide and other anticancer drugs can induce apoptosis in various cell lines, flow cytometric analysis of MDA-MB-231 cells treated with teniposide demonstrated that cell death was accompanied by a significant increase in the percentage of sub-G₁ cells with a subdiploid DNA content (Fig. 1A). Furthermore, internucleosomal DNA degradation was also demonstrated by the appearance of DNA ladders (Fig. 1B). Both of these features are considered typical of apoptosis, suggesting that teniposide-treated MDA-MB-231 cells underwent a form of cell death characterized by features common to the apoptotic pathway.

Teniposide Induces Increased Mitochondrial Cytochrome c Expression. Recent work on apoptosis and necrosis revealed that cytochrome c is released from the mitochondria into the cytoplasm during cell death (4–7). To examine whether cytochrome c release...
The Teniposide-induced Increase in Cytochrome c Is Functional. To test if the teniposide-induced up-regulation of mitochondrial respiratory chain protein expression was accompanied by a change in the function of these proteins, we measured the COX-dependent oxygen uptake in MDA-MB-231 cells treated with teniposide for 24, 48, or 72 h. Attached cells were harvested and placed into an oxygen electrode cuvette. Oxygen consumption was measured using ascorbate/TMPD as the electron donor. Values are given as means ± SD of three independent experiments. * P < 0.001 between control and treated cells.

Fig. 6. Effect of teniposide treatment on COX-dependent oxygen uptake in MDA-MB-231. MDA-MB-231 cells were treated with 10 μM teniposide for 24, 48, or 72 h. Attached cells were harvested and placed into an oxygen electrode cuvette. Oxygen consumption was measured using ascorbate/TMPD as the electron donor. Values are given as means ± SD of three independent experiments. * P < 0.001 between control and treated cells.

Taken together, these results indicate that teniposide induced a dose- and protein synthesis-dependent increase in respiratory chain proteins before cells died. Essentially the same results were obtained with HeLa cells treated with teniposide (data not shown) and Jurkat cells treated with camptothecin (21), suggesting that this is a more general phenomenon.

Immunofluorescence Microscopy. To confirm the finding that cytochrome c is released into the cytosol in living cells, the cellular localization of cytochrome c protein was examined by immunofluorescence microscopy in MDA-MB-231 cells treated with teniposide for 48 h. At this stage we found live and dead cells, which allowed us to compare the different cytochrome c expression patterns in both populations. To distinguish between live and dead cells, we used the Dead Red dye, which is a cell-impermeant red fluorescent nucleic acid stain that labels only dead cells (22). By using this stain, viability staining can take place before para-formaldehyde treatment (fixation) without disrupting the distinctive immunofluorescence-staining pattern. Untreated cells demonstrated a punctate pattern for cytochrome c consistent with its mitochondrial localization (Fig. 5A). Upon teniposide treatment, a brighter punctate, as well as a somewhat diffuse, staining was seen throughout the live cells (Fig. 5B). Together with the Western blot data, these results support the conclusion that there was an increased amount of cytochrome c in the mitochondria and some release of cytochrome c from the mitochondria to the cytosol before cell death. In contrast, the staining in dead cells was far more diffuse and mostly without a punctate pattern (see short arrow in Fig. 5B), which is in agreement with our observation by Western blot that mitochondria are depleted of cytochrome c after cell death. Similar results were also obtained in teniposide-treated HeLa cells (data not shown).

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Functional increase in the transport of electrons through the respiratory chain, we examined COX-dependent oxygen uptake in teniposide-treated MDA-MB-231 cells (Fig. 6). The results showed a significant increase in COX activity, consistent with the observed up-regulation of components of the respiratory chain, and suggested that the newly synthesized proteins were functional.

**Various Chemotherapeutic Drugs Induced Increased Levels of Cytochrome c and Cytochrome Oxidase Subunits I and IV.** To assess if the teniposide-mediated increase in mitochondrial respiratory chain protein levels was specific to this drug, we also treated MDA-MB-231 cells with various other anticancer drugs for 24 h, and determined levels of cytochrome c and subunits I and IV of COX proteins in the mitochondrial fraction. As seen in Fig. 7, all of the drugs tested had a similar effect on the levels of these proteins, suggesting that it might be a more general response to drug treatment.

**Teniposide Did Not Induce ΔΨ\textsubscript{m} Reduction in Attached Cells.** Previous studies have suggested that a decline of the mitochondrial membrane potential ΔΨ\textsubscript{m} may be an early event in the process of cell death. Therefore, we determined ΔΨ\textsubscript{m} at various times after teniposide treatment in MDA-MB-231 cells. For this purpose we used the membrane potential-sensitive probe JC-1, which forms monomers (green fluorescence) at low membrane potential and J-aggregates (red fluorescence) at higher membrane potential. The ratio between the red and the green signals is indicative of the ΔΨ\textsubscript{m}. JC-1 fluorescence ratios remained essentially unchanged during the entire incubation time in the attached cell population (Fig. 8). In contrast, there was a dramatic drop of the red fluorescence in the floating cells, indicating a loss of ΔΨ\textsubscript{m}. To confirm that the JC-1 dye was sensitive to mitochondrial transmembrane depolarization, control MDA-MB-231 cells were treated with the mitochondrial uncoupling agent CCCP (1 μM). Within 10 min, essentially the entire population exhibited a decline of red fluorescence, indicative of a loss of ΔΨ\textsubscript{m}. These results indicate that the mitochondrial membrane potential remained intact in the attached population.

**Caspase Activation and PARP Cleavage Occurred in Floating, but not in Attached, Cells.** Recent studies have demonstrated that the release of cytochrome c from mitochondria leads to activation of the caspase cascade in the cytosol (3, 10, 11, 23). To assess whether the appearance of cytochrome c in the cytosol of teniposide-treated MDA-MB-231 cells led to the activation of caspases 9 and 3, cytosolic fractions from both attached and floating cells were analyzed by immunoblotting with anti-caspases 9 and 3 antibodies (Fig. 9). Significant amounts of both caspases were detected in the cytoplasm, but there was no apparent activation of either caspase in the attached cell population. In contrast, however, clear activation of both caspases was detected in the cytosol of the floating cells. Furthermore, PARP cleavage was observed in the cytosol of the floating, but not the attached, cells as indicated by the appearance of the M\textsubscript{r} 85,000 fragment and consistent with the activation pattern of the caspases. Thus, it appeared that caspase activation was associated with cell death but did not correlate with the release of cytochrome c into the cytoplasm of attached cells.

**DISCUSSION**

In the present study we demonstrated that anticancer drug treatment resulted in an early increase in mitochondrial respiratory chain protein levels, in particular cytochrome c, that preceded cytochrome c release.
into the cytosol. In mitochondria, cytochrome c is required as an electron carrier during oxidative phosphorylation, where cytochrome c shuttles electrons from complex III to complex IV of the respiratory chain (24). The electron transport between these complexes generates a proton gradient across the inner mitochondrial membrane, which is required to maintain $\Delta \Psi_m$. Thus, the release of cytochrome c from the electron transport chain is expected to result in impairment of the electron flow and a decrease in $\Delta \Psi_m$. The data in the present study indicate, however, that even when cytochrome c enters the cytosol, $\Delta \Psi_m$ is maintained for a substantial time afterward. In this context, our observation that teniposide treatment also induced an increase in several other mitochondrial respiratory proteins, in particular subunits I and IV of COX, may help us to understand how cells can maintain a high $\Delta \Psi_m$ despite the release of cytochrome c from the mitochondria. New synthesis of mitochondrial cytochrome c may serve to prevent its levels from falling below a critical threshold required to maintain $\Delta \Psi_m$. Although the exact signal that leads to cytochrome c protein synthesis is not known, one possible hypothesis is that the initial release of cytochrome c itself functions as the feedback signal that triggers new synthesis of mitochondrial proteins in a kind of compensatory reaction. Alternatively, it is possible that mitochondrial cytochrome c synthesis and enrichment is the primary event and cytochrome c release (or leakage) is simply a consequence of the higher levels of this protein. The fact that only a small amount of cytochrome c was detected in the cytosol at 24 h, a time when mitochondria were already maximally enriched with cytochrome c (Fig. 2B), suggests that up-regulation of cytochrome c expression is the primary event. The physiological and/or pathological significance of this effect in anticancer drug-treated cells is currently unknown. However, this effect does not seem to be unique to the present study’s model system, because increased cytochrome c levels were also observed within 1–2 h of staurosporine treatment of HeLa cells (data not shown) or camptothecin treatment of Jurkat cells (21). Recently, Skulachev (25) has proposed that the role of cytochrome c in apoptosis might represent one of the anti-oxidant functions inherent in this protein. First, cytochrome c can operate as an enzyme, oxidizing free oxygen radicals ($O_2^-$) back to $O_2$. The reduced cytochrome c is then reoxidized by COX. Second, the intermembrane cytochrome c can activate the electron transport chain in the outer mitochondrial membrane. This bypasses the initial and middle part of the main respiratory chain, which produces, as a rule, the major portion of reactive oxygen species in the cell. The fact that teniposide also up-regulated the mitochondrial expression of manganese superoxide dismutase suggested that free radicals are involved in the pathological changes observed. In this context, therefore, up-regulation of cytochrome c levels in mitochondria may play a protective role during the initial response of cells to drug treatment.

Different models have been proposed to explain the mechanism of cytochrome c release from the intermembrane space of mitochondria during apoptosis or necrosis. Swelling and subsequent rupture of the outer mitochondrial membrane have been proposed as a mechanism for the release of cytochrome c into the cytosol (26), events which are usually associated with the mitochondrial PT (27, 28) and with a loss of $\Delta \Psi_m$ (28). However, cytochrome c appeared in the cytoplasm of teniposide-treated cells with a normal $\Delta \Psi_m$. Furthermore, using electron microscopy, predominantly hyperdense and condensed mitochondria were observed after teniposide treatment of MDA-MB231 cells (data not shown), suggesting that the swelling of mitochondria is unlikely to be the primary mechanism of cytochrome c release. Together, these data suggest that mitochondrial depolarization was not required for cytochrome c release, a conclusion that is also consistent with previous results reported by us and by others (17, 29).

In conclusion, the present work demonstrated that teniposide and other chemotherapeutic drugs can induce an increase in cytochrome c and other mitochondrial respiratory chain proteins. We have also shown that cytochrome c release from mitochondria to the cytosol is an early event preceding the drop of $\Delta \Psi_m$ and caspase activation. We propose that mitochondrial cytochrome c enrichment may play a critical role in the initial defense response of a cell and precedes the final events leading to extensive cytochrome c release, a drop of $\Delta \Psi_m$, caspase activation, plasma membrane disruption, and eventually to cell death.

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