Distinct Pathways for Stimulation of Cytochrome c Release by Etoposide*

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Running title: Etoposide-induced Cytochrome c Release

Abbreviations: z, benzyloxycarbonyl; VAD, Val-Ala-Asp; VDVAD, Val-Asp-Val-Ala-Asp; DEVD, Asp-Glu-Val-Asp; LEHD, Leu-Glu-His-Asp; fmk, fluoromethyl ketone; W, weight; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis; AMC, 7-amino-4-methylcoumarin; MPT, mitochondrial permeability transition; Δψ, mitochondrial membrane potential; CsA, cyclosporin A

*This work was supported by grants from the Swedish Medical Research Council (03X-2471) and the Swedish Cancer Society (Cancerfonden, 3829-B98-03XAC). J.D.R. is supported by a Visiting Scientist grant from The Swedish Foundation for International Cooperation in Research and Higher Education (STINT) and V.G. by a grant from the Royal Swedish Academy of Sciences.
SUMMARY

Induction of apoptosis by DNA-damaging agents, such as etoposide, is known to involve the release of mitochondrial cytochrome c, although the mechanism responsible for this event is unclear. In the present study, using Jurkat T-lymphocytes, a reconstituted cell-free system or isolated liver mitochondria, we demonstrate the ability of etoposide to induce cytochrome c release via two distinct pathways. Caspase inhibition by either z-VAD.fmk or z-VDVAD.fmk attenuates cytochrome c release triggered by a low dose of etoposide via an apparent inhibition of nuclear events involving the release of protein factor(s) that is (are) able to interact with mitochondria. In contrast, caspase inhibition has no effect on cytochrome c release induced by a higher dose of etoposide. Moreover, the higher dose of etoposide heightens the sensitivity of Ca\textsuperscript{2+}-loaded isolated mitochondria to mitochondrial permeability transition; an effect that is completely abolished by cyclosporin A. Interestingly, cyclosporin A is ineffective at preventing similar mitochondrial damage in Jurkat cells treated with etoposide. We propose that lower doses of etoposide predominantly target the nucleus and stimulate the release of caspase-sensitive protein factor(s) that interact with mitochondria to trigger cytochrome c release, whereas higher doses of the drug impart a more direct effect on mitochondria and thus are not mitigated by caspase inhibition.
INTRODUCTION

DNA damage brought about by the exposure of cells to any number of cytotoxic stimuli, including oxidants, ultraviolet radiation, x-rays, environmental toxicants and chemotherapeutic drugs, can stimulate the onset of a series of intracellular changes characteristic of a form of cell death known as apoptosis (1-5). Numerous studies have reported the ability of specific DNA-damaging agents to stimulate well-known changes at the mitochondrial level that are key initiative steps in the apoptotic process (6-9). However, the signaling mechanism responsible for linking DNA damage with downstream mitochondrial events is unknown. It is also unclear whether these reputedly specific DNA-damaging agents, such as etoposide, are capable of exerting their toxicity, at least in part, by directly damaging mitochondria.

Etoposide is a topoisomerase II poison that is routinely prescribed for the treatment of cancer (1). Topoisomerase II contains three primary domains and is involved in various aspects of DNA metabolism (10,11). Normally, this enzyme binds to DNA, and in the presence of a divalent cation (12), as well as ATP (13), generates a transient, double-stranded break through which an entire intact helix is passed in order to prevent intertwining of DNA. Etoposide does not altogether inhibit the activity of topoisomerase II. Instead, it selectively exploits the catalytic activity of this enzyme by increasing the frequency and duration of DNA cleavage sites, resulting ultimately in permanent double-stranded breaks that are lethal to the cell (14).

Recently, it was reported that etoposide-induced cytochrome $c$ release is a caspase-independent event and that caspase-9 is the most apical caspase in chemical-induced apoptosis (6,15,16). While the precise mechanism controlling cytochrome $c$ release from mitochondria remains obscure, several models have been proposed that largely focus on the role that mitochondrial permeability transition (MPT) and/or a loss of membrane potential may serve in the process (17-20). According to Sun et al. (15), the general caspase inhibitor z-VAD.fmK failed to inhibit decreases in both mitochondrial membrane potential and cell size in etoposide-treated (50 µM) Jurkat T-lymphocytes. Moreover, z-VAD.fmK did not abrogate cytochrome $c$ release, whereas it was able to inhibit caspase-9 and executioner caspases. In contrast, Tepper et al. (21) demonstrated a clear reduction in cytochrome $c$ release in response to etoposide (17 µM).
mediated by z-VAD.fmk, although the authors concluded that caspases are not involved in this process since cytochrome c release was not completely eliminated by this caspase inhibitor.

The present study examined the ability of different doses of etoposide to stimulate the release of cytochrome c from mitochondria in Jurkat T-lymphocytes or a reconstituted cell-free system, as well as mitochondria isolated from rat liver. The results indicate that 10 µM etoposide stimulates cytochrome c release in Jurkat cells and in a cell-free system that is significantly reduced by z-VAD.fmk or z-VDVAD.fmk. However, caspase inhibition was not able to prevent the release of cytochrome c in response to a higher dose (50 µM) of etoposide. At the same time, 50 µM, and not 10 µM, etoposide significantly diminished mitochondrial Ca\(^{2+}\) buffering capacity in digitonin-permeabilized cells and stimulated the release of cytochrome c from liver mitochondria.

**EXPERIMENTAL PROCEDURES**

*Cell Culture*—Jurkat T-lymphocytes were cultured in RPMI 1640 complete medium supplemented with 10% (v/v) heat-inactivated fetal calf serum, 2% (w/v) glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin in a humidified air/CO\(_2\) (19:1) atmosphere at 37°C. Cells were maintained in a logarithmic growth phase for all experiments. Apoptosis was induced with etoposide (10-50 µM) (Bristol-Myers, Solna, Sweden) and ethanol (0.05% final concentration) was used as a vehicle control. In some cases, cells were first treated for 1 h with z-VAD.fmk (25 µM) (Enzyme Systems Products, Dublin, CA, USA) to inhibit caspase activity.

*Preparation of Cytosol for Cell-free System and Cytochrome c Measurement*—Cells were collected and washed twice in ice-cold phosphate-buffered saline (PBS), resuspended in S-100 buffer (20 mM Heps, pH 7.5, 10 mM KCl, 1.9 mM MgCl\(_2\), 1 mM EGTA, 1 mM EDTA, mixture of protease inhibitors) and incubated on ice for 20 min. Cells were centrifuged at 10,000 x g for 15 min at 4°C. Supernatants were further centrifuged at 100,000 x g for 1 h at 4°C and used for cell-free experiments or western-blot analysis.

*Isolation of Rat Liver Nuclei*—Nuclei were isolated using a slightly modified version of a method described previously (22). Male Sprague-Dawley rats (6 to 8 weeks old) were killed by CO\(_2\) inhalation in accordance with the European directive of protection of vertebrate animals for scientific research. Livers were quickly removed, blotted and placed in (2 x W) ml of ice-cold
Buffer A (15 mM Hepes, pH 7.4, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 250 mM sucrose, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM phenylmethylsulfonyl fluoride). Tissue was minced finely and homogenized with a glass Dounce homogenizer and Teflon pestle. Homogenates were filtered through four layers of cheesecloth, mixed with (2 x W) ml of Buffer B (15 mM Hepes, pH 7.4, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 2.3 M sucrose, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine, 1 mM phenylmethylsulfonyl fluoride) and transferred to centrifuge tubes. Prior to centrifugation, 5 ml of Buffer B were added as a cushion to the bottom of the tube. Samples were centrifuged at 118,000 x g for 1.5 h at 4°C and resulting nuclei were resuspended in Buffer A at a final concentration of 200,000 nuclei/µl. Aliquots were stored at -80°C until used.

Isolation of Rat Liver Mitochondria—The liver of a male Sprague-Dawley rat was minced on ice, resuspended in 50 ml of MSH buffer (210 mM mannitol, 70 mM sucrose, 5 mM Hepes, pH 7.5) supplemented with 1 mM EDTA, and homogenized with a glass Dounce homogenizer and Teflon pestle. Homogenates were centrifuged at 600 x g for 8 min at 4°C. The supernatant was decanted and recentrifuged at 5,500 x g for 15 min to form a mitochondrial pellet that was resuspended in MSH buffer without EDTA and centrifuged again at 5,500 x g for 15 min. The final mitochondrial pellet was resuspended in MSH buffer at a protein concentration of 80-100 mg/ml.

Measurement of Functional Activity of Isolated Mitochondria—Mitochondria (1 mg/ml) were incubated in a buffer containing 150 mM KCl, 1 mM KH2PO4, 5 mM succinate, and 5 mM Tris, pH 7.4 at 25°C. Rotenone (2 µM) was added to maintain pyridine nucleotides in a reduced form. Estimation of Δψ was performed using an electrode sensitive to the lipophilic cation tetraphenylphosphonium (TPP+). Energized mitochondria rapidly accumulate TPP+ from the incubation buffer and release this cation as Δψ decays. Ca2+ fluxes across the inner mitochondrial membrane were monitored using a Ca2+-sensitive electrode (model 97-20, Orion Research, Inc, Beverly, MA). Mitochondrial swelling was monitored continuously as changes in OD540. Oxygen consumption by isolated rat liver mitochondria was measured using a Clark-type oxygen electrode (Yellow Spring Instrument Co., OH, USA) at 25°C. Mitochondria with a respiratory control ratio (defined as the rate of respiration in the presence of ADP divided by the rate obtained following the
expenditure of ADP) above 4 were used for all experiments. Fresh mitochondria were prepared for each experiment and used within 4 h.

**Digitonin-permeabilized Cells and Estimation of Mitochondrial Ca\(^{2+}\) Accumulation**—Jurkat cells (2.5×10\(^6\)) were washed in PBS, resuspended in 500 µl of buffer (150 mM KCl, 5 mM KH\(_2\)PO\(_4\), 1 mM MgSO\(_4\), 5 mM succinate, 5 mM Tris, pH 7.4) and added to the incubation chamber. Following a 2 min stabilization period, cells were permeabilized with 0.005 % digitonin and 5 µM rotenone was added in order to maintain pyridine nucleotides in a reduced form. MPT was induced by sequential additions of Ca\(^{2+}\) and changes in the level of this cation were monitored using a Ca\(^{2+}\)-selective electrode.

**Reconstituted Cell-free System**—Standard reactions were carried out in a 30-µl reaction volume with reaction buffer (20 mM Hepes, pH 7.2, 10 mM KCl, 1.5 mM MgCl\(_2\), 1 mM EGTA, 1 mM DTT, 250 mM sucrose, 10 mM succinate, 2 mM ATP, 10 mM creatine phosphate, 50 µg/ml creatine kinase, mixture of protease inhibitors) in the absence or presence of liver nuclei (5 x 10\(^6\)), isolated liver mitochondria (15 µg of protein), and 25 µg of Jurkat cytosol protein. Nuclei and mitochondria were suspended separately in reaction buffer prior to their addition to the reaction mix. Samples were incubated at 37°C for up to 3 h. Nuclei and mitochondria were removed by centrifugation at 12,500 x g for 10 min at 4°C and the supernatants stored at -20°C until used for western-blot analysis.

**Western-blot analysis**—Samples were mixed with Laemmli’s loading dye, boiled for 5 min and subjected to 15% SDS-PAGE at 130 V followed by electroblotting to nitrocellulose for 2 h at 100 V. Membranes were blocked for 1 h with 5% nonfat milk in phosphate-buffered saline at room temperature and subsequently probed overnight with an anti-cytochrome c (1:2500) or anti-G3PDH (1:5000) antibody. The membranes were rinsed and incubated with a horseradish-peroxidase-conjugated secondary antibody (1:10,000). Following the secondary antibody incubation, the membranes were rinsed and bound antibodies were detected using enhanced chemiluminescence according the manufacturer’s instructions.

**Measurement of Caspase Activity**—The measurement of DEVD-AMC, VDVAD-AMC or LEHD-AMC (Peptide Institute, Osaka, Japan) cleavage was performed using a modified version of a fluorometric assay reported previously (23). One million cells were pelleted and washed once
with PBS. After centrifugation, cells were resuspended in 25 µl PBS, added to a microtiter plate and combined with the appropriate peptide substrate dissolved in a standard reaction buffer (100 mM HEPES, 10% sucrose, 5 mM dithiothreitol (DTT) and 0.1 % 3-[ (3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), pH 7.25). Cleavage of the fluorogenic peptide substrate was monitored by AMC liberation in a Fluoroscan II plate reader (Labsystems, Stockholm, Sweden) using 355 nm excitation and 460 nm emission wavelengths. Fluorescence units were converted to pmol of AMC using a standard curve generated with free AMC. Data from duplicate samples were then analyzed by linear regression.

RESULTS

Etoposide Induces Cytochrome c Release and DEVDase Activity in Jurkat cells—

Treatment of Jurkat T-lymphocytes with etoposide stimulates the release of cytochrome c (Fig. 1A). To investigate whether this effect is dose- and/or caspase-dependent, cells were treated with either 10 or 50 µM etoposide for 6 h in the absence or presence of the general caspase inhibitor z-VAD.fmk (25 µM). A similar amount of cytochrome c was detected in cytosolic extracts from cells treated with 10 or 50 µM etoposide at 3 and 6 h. Interestingly, z-VAD.fmk pre-treatment blocked cytochrome c release only in response to the lower dose of etoposide, and only at 3 h, indicating that there may be more than one pathway controlling etoposide-induced cytochrome c release (Fig. 1A). Fluorometric analysis of DEVDase activity indicated that 25 µM z-VAD.fmk completely blocked caspase activation following both 10 and 50 µM etoposide (Fig. 1B). No significant difference in DEVDase activity was detected between the high and low dose of etoposide until 3 h post-treatment, when levels of AMC released were ~3.5-fold higher in cells treated with 50 versus 10 µM etoposide (Fig. 1B). A smaller ~1.5-fold difference was observed between the two treatments at 6 h, which appeared to be due to a flattening out of DEVDase activity in response to the higher dose of etoposide.

Etoposide Induces Cytochrome c Release in a Cell-free System—

To investigate the potential relationship between caspases and cytochrome c release in the presence of 10 µM etoposide, experiments were performed using a reconstituted cell-free system. Isolated rat liver nuclei (5 x 10⁹) were incubated with mitochondria (15 µg of protein) and/or Jurkat cytosol (25 µg
of protein) in the presence or absence of etoposide for 3 h at 37°C (Fig. 2A). Western-blot analysis for cytochrome c was first performed on isolated nuclei and Jurkat cytosol in order to verify that these fractions were free from mitochondrial contamination (data not shown). Results indicated that 10 µM etoposide was sufficient to stimulate cytochrome c release from mitochondria incubated in the presence of nuclei and Jurkat cytosol (Fig. 2A, lanes 3 versus 4). Furthermore, this effect was not dependent on the presence of cytosol as evidenced by the similar results that were obtained when cytosol was absent (Fig. 2A, lanes 1 versus 2). To ensure that 10 µM etoposide was not exerting a direct effect on mitochondria, these organelles were incubated under the same conditions as described for Fig. 2A except nuclei were excluded (Fig. 2B). However, when mitochondria were incubated with a higher dose (25 µM) of the drug, cytochrome c release was observed as compared to vehicle alone (Fig. 2C).

To further characterize this effect, nuclei alone were incubated with 10 µM etoposide for 1 h at 37°C. Following the 1 h incubation, nuclei were removed by centrifugation and the resulting supernatant was collected and used to treat isolated mitochondria for an additional 2 h. As shown in Fig. 2D (lanes 1 and 2), the supernatant from etoposide-treated nuclei stimulated significant cytochrome c release as compared to control supernatants, suggesting that etoposide stimulated the release of some factor(s) from nuclei which was (were) capable of targeting mitochondria and triggering the release of cytochrome c. Next, to determine if the factor(s) released from nuclei in the presence of etoposide was (were) a protein(s), supernatants were heat-inactivated at 70°C for 30 min prior to being added to mitochondria. Heat-inactivation successfully mitigated the ability of the supernatant to stimulate cytochrome c release (Fig. 2D, lane 3 versus 2), indicating that the factor(s) released from etoposide-treated nuclei is (are) likely to be a protein(s). Moreover, it would appear that this factor is present constitutively since pre-treatment of intact Jurkat cells with the protein synthesis inhibitor cycloheximide (1 µg/ml) for 1 h failed to block etoposide-mediated cytochrome c release (data not shown).

The potential inhibitory effect of z-VAD.fmk was tested to see whether caspase activity was associated with cytochrome c release in this system. Nuclei and mitochondria were co-treated with 25 µM z-VAD.fmk and 10 µM etoposide for 3 hours. This was sufficient to block cytochrome c release induced by etoposide (Fig. 2E, lane 3 versus 2), suggesting that caspase activity is involved
in this response. When the supernatants of isolated nuclei that had been treated with etoposide and z-VAD.fmK for 1 h were subsequently added to mitochondria, cytochrome c release was also inhibited (Fig. 2F, lane 3 versus 2), whereas treatment of nuclear supernatants with z-VAD.fmK at the end of etoposide treatment, but before addition to mitochondria, was unable to retard release of cytochrome c (Fig. 2F, lane 4 versus 2). Taken together, this suggests that z-VAD.fmK most likely inhibits pro-apoptotic nuclear, and not mitochondrial, events in this system.

Experiments with more specific caspase inhibitors indicated that only z-VDVAD.fmK, which primarily inhibits caspase-2, and to a lesser extent –3 and -7, mimicked the effect observed with z-VAD.fmK (Fig. 2G, lanes 6 versus 3). Neither z-DEVD.fmK (caspase-3 and –7) nor z-LEHD.fmK (caspase-9) was able to inhibit cytochrome c release when nuclei and mitochondria were treated with 10 µM etoposide for 3 h (Fig. 2G, lanes 4 and 5). Moreover, enzymatic studies revealed that caspase-2 activity was detected in advance of caspase-9 in Jurkat cells treated with 10 µM etoposide (Table I), suggesting that the nuclear signal responsible for stimulating cytochrome c release in response to etoposide involves caspase-2.

Etoposide Stimulates Cytochrome c Release via MPT in Isolated Mitochondria—Since 25 µM etoposide stimulated the release of cytochrome c from mitochondria alone in our cell-free system, the next step was to test whether this effect might be due to an induction of MPT. It is well-known that mitochondrial Ca\(^{2+}\) accumulation is obligatory for MPT induction, although the sensitivity of MPT to Ca\(^{2+}\) can be significantly enhanced by different factors. Among these factors are the depletion of adenine nucleotides, an elevated level of inorganic phosphate and oxidative stress.

The addition of etoposide to mitochondria induced a concentration-dependent sub-maximal shift of Δψ that was not preventable by CsA, which was added to mitochondria prior to etoposide (Fig. 3A, trace 5 versus 3). The addition of Ca\(^{2+}\) to mitochondria in the presence of different concentrations of etoposide stimulated an additional and more prominent drop in Δψ and a release of accumulated Ca\(^{2+}\) (Fig. 3A and B, traces 1 and 2); effects that were completely abolished by CsA (Fig. 3A and B, trace 3), indicating that the second phase of the drop of Δψ was due to MPT induction. Importantly, Ca\(^{2+}\) alone was not sufficient to induce a drop in Δψ or a release of Ca\(^{2+}\) (Fig. 3A and B, trace 4). Similarly, etoposide treatment alone did not induce a complete collapse of Δψ (Fig. 3A, trace 5).
Etoposide-induced drops in Δψ and the release of Ca\(^{2+}\) from mitochondria were accompanied by mitochondrial swelling, an indicator of mitochondrial permeability transition (Fig. 3C, traces 1 and 2 versus 4). Swelling only occurred in Ca\(^{2+}\)-loaded mitochondria and led to the rupture of the outer membrane and the release of cytochrome c (Fig. 3D). CsA (1 µM) or EGTA (1 mM) completely prevented swelling of Ca\(^{2+}\)-loaded mitochondria (Fig. 3C, trace 3) and the release of cytochrome c (Fig. 3D).

**Effect of Etoposide on Mitochondrial Ca\(^{2+}\) Accumulation in Permeabilized Cells**—As mentioned previously, earlier reports have demonstrated the inability of z-VAD.fmk to inhibit etoposide-induced cytochrome c release, while other investigators indicate that z-VAD.fmk does mitigate this response to etoposide. Here, we provide evidence (Fig. 1) indicating that z-VAD.fmk is able to attenuate cytochrome c release in response to 10 µM, but not 50 µM, etoposide. To determine whether this difference was due to a direct targeting of mitochondria by 50 µM etoposide, mitochondrial Ca\(^{2+}\) accumulation was evaluated in permeabilized Jurkat cells that had been treated with either 10 or 50 µM etoposide for up to 6 h. The addition of Ca\(^{2+}\) to permeabilized cell suspensions led to a rapid increase in the level of this cation in the reaction buffer followed by a return to the initial level (Fig. 4A) as mitochondria accumulated the excess Ca\(^{2+}\); an effect that was completely abrogated by antimycin, an inhibitor of mitochondrial respiratory chain (data not shown). Mitochondria accumulated sequential additions of Ca\(^{2+}\) until MPT was induced and the accumulated Ca\(^{2+}\) released (Fig. 4A and B). Treatment with 10 µM etoposide for 3 h did not significantly alter either the rate of Ca\(^{2+}\) accumulation or the threshold level of Ca\(^{2+}\), which is necessary for MPT induction (Fig. 4C and D). In contrast, 50 µM etoposide markedly suppressed both parameters by 3 h (Fig. 4B-D). At 6 h, the Ca\(^{2+}\) buffering capacity of mitochondria was significantly impaired at both concentrations of etoposide. CsA, which was added to the cells at the same time as etoposide, did not offer protection, indicating that MPT was not involved in etoposide-mediated damage to these organelles (data not shown).

**DISCUSSION**

Currently, there is some confusion and uncertainty in the literature as to the mechanism controlling cytochrome c release in response to specific DNA-damaging agents. In particular, what
is the signal linking DNA damage to downstream mitochondrial events? Are these reputedly specific DNA-damaging agents, in fact, “specific” or can some of them exert their toxicity by directly targeting mitochondria? Which, if any, of these effects is/are caspase-dependent?

Numerous reports have described the ability of various specific and non-specific DNA-damaging agents to stimulate the release of mitochondrial cytochrome c (6,15,16,24). In contrast to death receptor-mediated apoptosis, during which caspase-8 activity is often responsible for the cleavage of a cytosolic substrate, e.g., Bid, which targets mitochondria triggering the release of cytochrome c, this event is traditionally accepted as caspase-independent in chemical- and/or DNA-damage-induced apoptosis (16,18,25). Moreover, recent results from our laboratory indicate that neither is caspase-8 activated nor is Bid cleaved in response to etoposide up to 3 h (J.D. Robertson, B. Zhivotovsky, and S. Orrenius, unpublished data). In this respect, the general caspase inhibitor z-VAD.fmk is believed to protect against chemical-induced apoptosis downstream of mitochondria by preventing the activation of caspase-9 (15), which is activated within the apoptosome complex (26,27). However, a more recent report proposes that z-VAD.fmk is able to inhibit the second of a two-stage cytochrome c release process that involves caspase activation, a reduction in ATP levels and decreases in Δψ (28). In this study, we examined the nature of cytochrome c release in response to two different, and frequently cited, doses of the topoisomerase II inhibitor etoposide. We demonstrate that z-VAD.fmk is effective at mitigating early cytochrome c release in response to a low (10 µM), and not a high (50 µM), dose of etoposide in Jurkat T-lymphocytes. These results were subsequently extended using a cell-free system where it was determined that etoposide-mediated (10 µM) release of cytochrome c was dependent upon the presence of nuclei, but not Jurkat cytosol. Moreover, studies performed with isolated liver mitochondria revealed the inability of this same dose of etoposide to exert a direct effect on mitochondria, whereas 25 µM etoposide prominently diminished the ability of mitochondria to accumulate Ca^{2+}, suggesting that higher doses of the drug are able to directly target and damage these organelles. Taken together, the current data clearly separate two different pathways for etoposide-induced cytochrome c release that are largely dose-dependent (Fig. 5).

Low doses of etoposide primarily exert their effect at the nuclear level, stimulating changes that ultimately lead to the release of protein factor(s) that target, and interact with, mitochondria to
stimulate the release of cytochrome c. Since caspase inhibition by either z-VAD.fmk or z-VDVAD.fmk blocked this effect, and given that caspase-2 was the most apical caspase activated in intact Jurkat cells in response to etoposide, it is tempting to conclude that cytochrome c release induced by a low dose of etoposide requires active caspase-2. This conclusion is further supported by other work reporting the early activation of this caspase during apoptosis (29,30) and its nuclear localization (30,31). Additional studies designed to characterize caspase involvement in this process are currently ongoing in our laboratory.

The second pathway accounting for etoposide’s toxic effect involves a direct targeting of mitochondria (Fig. 5). In this case, higher doses (≥ 25 µM) of the drug elicit a caspase-independent release of cytochrome c, as well as earlier and more robust DEVDase activity, as compared to low doses, which signal through the nucleus and hence require more time to impart an effect at the mitochondrial level. Results with isolated liver mitochondria demonstrate the ability of etoposide to facilitate Ca\(^{2+}\)-dependent MPT and cytochrome c release, which were preventable by CsA or EGTA. Because EGTA was able to block cytochrome c release, these results might seem to be in conflict with our original cell-free observation that a higher dose of etoposide directly targets mitochondria and stimulates cytochrome c release since the buffer used for those experiments contained EGTA. However, mitochondria were incubated in the presence of etoposide for 3 h in our cell-free system, whereas etoposide facilitated Ca\(^{2+}\)-dependent MPT within several minutes in our isolated mitochondria model. Nonetheless, given that cytochrome c release was demonstrated in the presence of EGTA, a known inhibitor of MPT, suggests that there is more than one mechanism by which etoposide directly targets and damages mitochondria, resulting in cytochrome c release. It should be mentioned that the addition of etoposide to energized isolated mitochondria resulted in a sub-maximal decrease in \(\Delta \psi\), which was not CsA- or EGTA-preventable. Therefore, this effect was not due to MPT but more likely due to a dissipation of \(\Delta \psi\) induced by futile cycling of either protons (indicative of mitochondrial uncoupling) or potassium ions (indicative of potassium entry into mitochondria).

A high dose of etoposide induced a decrease in the Ca\(^{2+}\)-buffering capacity of mitochondria in permeabilized cells. This might be due to both an induction of MPT, at least in a sub-population of mitochondria, and a decrease in mitochondrial membrane potential, the main driving force for
Ca\textsuperscript{2+} accumulation. However, CsA failed to prevent mitochondrial deterioration in response to etoposide, indicating that MPT is not likely to be a dominating factor. Nonetheless, since Jurkat, and other, cells in culture normally maintain a lower rate of mitochondrial respiration (Crabtree effect) (32), and hence a lower $\Delta \psi$, etoposide-induced dissipation of $\Delta \psi$ observed in isolated mitochondria may be more severe in these cells as even a slight disruption in $\Delta \psi$ could significantly increase the likelihood of MPT.

It is important to add that the two pathways are unlikely to be mutually exclusive. In other words, it does not seem to be the case that higher doses of etoposide only target mitochondria, whereas lower doses of the drug only damage DNA. This could account for the apparent inability of z-VAD.fmk to block the release of cytochrome $c$ induced by 10 $\mu$M etoposide in Jurkat cells at 6 h. Here, the lower dose of the drug ultimately targets mitochondria, exerting a similar effect as a higher dose at 3 h, and hence its ability to elicit cytochrome $c$ release at this time-point is not caspase-dependent. This was observed not only for cytochrome $c$ release in Jurkat cells but also in the decreased, and similar, abilities of Jurkat mitochondria treated with either a high or low dose of etoposide to accumulate Ca\textsuperscript{2+} in a permeabilized model (Fig. 5C and D).

In summary, we have demonstrated the ability of etoposide to stimulate cytochrome $c$ release via two distinct pathways. On the one hand, low doses of the drug predominantly target the nucleus where damage to DNA triggers subsequent caspase-dependent events that converge on the mitochondria and elicit cytochrome $c$ release. In contrast, higher doses of etoposide are directly toxic to mitochondria, altering their ability to accumulate Ca\textsuperscript{2+}, increasing their sensitivity to MPT and stimulating the release of cytochrome $c$; an event that is not diminished by caspase inhibition.

ACKNOWLEDGMENTS

We thank Dr. Ronald Jemmerson (University of Minnesota Medical School, Minneapolis, MN, USA) for the cytochrome $c$ antibody and Emma Mejhert for laboratory assistance.

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Table I

Caspase-2 is activated upstream of caspase-9 in etoposide-treated Jurkat T-lymphocytes

Jurkat cells (10^6/ml) were treated with either 10 or 50 μM etoposide for up to 6 h as described under “Experimental Procedures.” Enzyme activity was monitored by the release of AMC from either VDVAD-AMC or LEHD-AMC and the results of duplicate samples are shown.

| AMC released (pmol/min/10^6 cells) | 10 μM etoposide | 50 μM etoposide |
|-----------------------------------|-----------------|----------------|
|                                   | VDVADase        | LEHDase        | VDVADase        | LEHDase        |
| Time (h)                          |                 |                 |                 |                 |
| 0                                 | 0.8 ± 0.1       | 0.75 ± 0.07     | 0.8 ± 0.1       | 0.75 ± 0.07     |
| 1                                 | 1.5 ± 0.2       | 0.94 ± 0.05     | 1.35 ± 0.02     | 1.15 ± 0.07     |
| 2                                 | 2.5 ± 0.1       | 0.94 ± 0.05     | 3.15 ± 0.07     | 1.05 ± 0.05     |
| 3                                 | 4.7 ± 0.2       | 1.15 ± 0.07     | 7.4 ± 0.3       | 2.05 ± 0.05     |
| 4                                 | 7.8 ± 1.8       | 2 ± 0.2         | 11.8 ± 1.2      | 2.9 ± 0.4       |
| 5                                 | 13.3 ± 4        | 3.15 ± 0.07     | 21.3 ± 1        | 5.0 ± 0.4       |
| 6                                 | 12.1 ± 0.4      | 1.6 ± 0.3       | 14 ± 0.8        | 2.5 ± 0.1       |
FIGURE LEGENDS

Fig. 1. z-VAD.fmK inhibits both cytochrome c release and DEVDase activity in response to a low dose of etoposide. A, Jurkat T-lymphocytes were treated with two different doses of etoposide in the presence or absence of z-VAD.fmK for the indicated times, and cytosolic extracts were prepared. Samples were separated by SDS-PAGE and western-blotted as described in “Experimental Procedures.” G3PDH was used as a loading control. B, Jurkat cells (10^6/ml) were treated with either 50 µM (■) or 10 µM (▲) etoposide for the indicated times and harvested for DEVD-specific cleavage as described under “Experimental Procedures.” In some instances, cells were first pre-treated with z-VAD.fmK (25 µM) for 1 h at 37°C prior to the addition of 50 µM (□) or 10 µM (△) etoposide.

Fig. 2. Etoposide induces cytochrome c release in a cell-free system. A, Nuclei (5 x 10^6) and mitochondria (15 µg of protein) were incubated in the absence (lanes 1 and 2) or presence of Jurkat cytosol (25 µg of protein) (lanes 3 and 4) for 3 h at 37°C. In certain instances, the reaction mixture was treated with either vehicle (0.05% ethanol) (lanes 1 and 3) or 10 µM etoposide (lanes 2 and 4). B, Same conditions as (A) except nuclei were not added to these samples. C, Mitochondria were vehicle-treated (lane 1) or treated with 25 µM etoposide (lane 2) for 3 h. D, Mitochondria were incubated for 2 h with the supernatants of nuclei that had been treated for 1 h with vehicle alone (lane 1) or 10 µM etoposide (lane 2). Lane 3 is the same as lane 2 except the supernatant was heat-inactivated (70°C for 30 min) prior to its addition to mitochondria. E, Nuclei and mitochondria were incubated with vehicle (lane 1), etoposide alone (lane 2) or etoposide + z-VAD.fmK (lane 3) for 3 h. F, Supernatants of nuclei that had been treated for 1 h with either vehicle (lane 1) or etoposide (lane 2) were used to treat mitochondria for 2 h. Lanes 3 and 4 are under the same conditions as lanes 1 and 2 except 25 µM z-VAD.fmK was added to nuclei either before (lane 3) or after (lane 4) etoposide treatment, but before supernatants were added to mitochondria. G, Nuclei and mitochondria were treated with 10 µM etoposide (lanes 2-6) in the absence (lane 2) or presence (lanes 3-6) of different caspases inhibitors (25 µM final concentration). Lane 3, z-VAD.fmK; lane 4, z-DEVD.fmK; lane 5, z-LEHD.fmK; lane 6, z-VDVAD.fmK.
Fig. 3. **Etoposide induces cytochrome c release in isolated mitochondria by triggering MPT.** A, Isolated liver mitochondria (1 mg/ml of protein) were suspended in an incubation buffer containing 2 µM TPP⁺. After a 2 min stabilization period, mitochondria were treated with etoposide (10 or 25 µM), followed by 20 nmol of Ca²⁺ as described under “Experimental Procedures.” Trace 1, 25 µM etoposide and 20 nmol Ca²⁺; trace 2, 10 µM etoposide and 20 nmol Ca²⁺; trace 3, 25 µM etoposide, 20 nmol Ca²⁺ and 1 µM CsA; trace 4, 20 nmol Ca²⁺; trace 5, 25 µM etoposide. B and C, Same conditions as A except TPP⁺ was excluded. Trace 1, 25 µM etoposide and 20 nmol Ca²⁺; trace 2, 10 µM etoposide and Ca²⁺; trace 3, 25 µM etoposide, 20 nmol Ca²⁺, and 1 µM CsA (or 1 mM EGTA); trace 4, 20 nmol Ca²⁺. D, Resulting supernatants from B were separated by SDS-PAGE and western-blotted as described under “Experimental Procedures.”

Fig. 4. **Etoposide decreases Ca²⁺ buffering capacity of mitochondria in permeabilized Jurkat cells.** A and B, Cells (2.5 x 10⁶) were treated either with (A) vehicle (0.01% ethanol) or (B) 50 µM etoposide for 3 h, followed by digitonin-permeabilization and induction of MPT by the sequential addition of Ca²⁺ as described under “Experimental Procedures.” C and D, Cells (2.5 x 10⁶) were treated for either 3 or 6 h in the presence of vehicle (☐), 10 (■) or 50 µM (□) etoposide. Cells were washed in PBS and subsequently permeabilized and evaluated for (C) maximal Ca²⁺ capacity or (D) rate of Ca²⁺ accumulation.

Fig. 5. **Scheme for etoposide-mediated cytochrome c release.**
Figure 1

A

| Treatment                | 3 h  | 6 h |
|--------------------------|------|------|
| Etoposide (10 μM)        | -    | +    |
| Etoposide (50 μM)        | -    | +    |
| z-VAD.fmk (25 μM)        | -    | +    |
| Cyt c                    | -    | +    |
| G3PDH                    | -    | +    |

B

Graph showing DEVD-amc cleavage over time (h) with error bars.
Figure 2
Figure 3
Figure 4

A

B

C

D

Cells

Ca^{2+}

dig

Ca^{2+}

Ca^{2+}

Ca^{2+}

3 min

5 nmol Ca^{2+}

Cells

Ca^{2+}

dig

Ca^{2+}

3 min

5 nmol Ca^{2+}

C

Ca^{2+} capacity

nmol Ca^{2+}/10^6 cells

Time

0h

3h

6h

0

2

4

6

8

10

12

D

Rate of Ca^{2+} accumulation

nmol Ca^{2+}/min/10^6 cells

Time

0h

3h

6h

0

0.2

0.4

0.6

0.8

1.0

1.2

1.4
Distinct pathways for stimulation of cytochrome c release by etoposide
John D. Robertson, Vladimir Gogvadze, Boris Zhivotovsky and Šten Örrenius

J. Biol. Chem. published online August 28, 2000

Access the most updated version of this article at doi: 10.1074/jbc.C000518200

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