Temporospatial Sequence of Cellular Events Associated With Etoposide-Induced Neuronal Cell Death: Role of Antiapoptotic Protein Bcl-Xl

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Etoposide-induced death comprises such nuclear events as the formation of topoisomerase II-DNA cleavable complex and cytosolic events including caspase activation. By first establishing the temporospatial death sequence triggered by etoposide in a neuronal cell line, MN9D overexpressing Bcl-Xl (MN9D/Bcl-Xl) or control vector (MN9D/Neo), we examined whether formation of this complex is primarily responsible for cell death and at which strategic points and how Bcl-Xl blocks etoposide-induced neuronal death. Etoposide induced death that was dependent on caspase, cycloheximide, and calpain in MN9D/Neo cells. Etoposide also induced death in enucleated MN9D/Neo cells, although this was less severe. The level of topoisomerase II-DNA cleavable complex reached at a maximum of 2 hr after etoposide treatment was identical in MN9D/Neo and MN9D/Bcl-Xl cells. In MN9D/Neo cells, cytochrome c release into the cytosol and caspase activation occurred as early as 2 hr and 3–6 hr after etoposide treatment, respectively. Etoposide-induced DNA laddering potentially via caspase appeared as early as 12 hr after drug treatment, followed by nuclear swelling in MN9D/Neo cells (>18–20 hr). Subsequently, nuclear condensation started by 24–28 hr and became apparent thereafter. All of these events except for nuclear swelling were substantially blocked in MN9D/Bcl-Xl. At the later stage of cell death (<32–36 hr), a specific cleavage of Bax and fodrin appeared that was completely blocked by calpain inhibitor or by Bcl-Xl. Taken together, our data suggest that Bcl-Xl prevents etoposide-induced neuronal death by exerting its anticaspase and anticalpain effect on cellular events after the formation of topoisomerase II-DNA cleavable complex that may not be a major contributor to cell death. J. Neurosci. Res. 66:1074–1082, 2001.

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pro- and antiapoptotic functions by modifying the flux of small molecules or proteins at strategic positions, such as in mitochondria (Kroemer, 1997). For example, release of cytochrome c into the cytosol and subsequent caspase activation are inhibited or accelerated by the presence of Bcl-2 family proteins on mitochondria (Kluck et al., 1997; Yang et al., 1997; Jurgensmeier et al., 1998; Rosse et al., 1998).

Etoposide (VP-16; an inhibitor of topoisomerase II) stabilizes topoisomerase II-DNA cleavable complex, thereby inducing a series of events in various cell types that can eventually lead to cell cycle arrest and apoptosis (Hande, 1998). As demonstrated almost exclusively in nonneuronal cells and to a lesser extent in neuronal cells, etoposide-induced apoptosis seems to comprise nuclear events such as formation of topoisomerase II-DNA cleavable complex and cytosolic events that include the activation of caspase (Kamesaki et al., 1993; Nakajima et al., 1994; Dubrez et al., 1996; Tanabe et al., 1997). It has been shown that the antiapoptotic Bcl-2 family protects etoposide-induced, caspase-mediated apoptosis both in nonneuronal and in neuronal cells (MiyaShita and Reed, 1993; Dole et al., 1995; Yang et al., 1997). However, it has not been examined the extent to which etoposide-induced formation of topoisomerase II-DNA cleavable complex in the nucleus contributes to cell death. Similarly, it remains to be determined at which strategic points the antiapoptotic Bcl-2 family is able to stall the apoptotic processes induced by etoposide. In the present study, MN9D dopaminergic neuronal cells (Choi et al., 1991, 1992) overexpressing Bcl-X₁ (MN9D/Bcl-X₁) or control vector (MN9D/Neo) were used to establish first the temporospatial sequence of the nuclear and the cytosolic death processes induced by etoposide and then to determine the protective points conferred by Bcl-X₁. Our results suggest that, 1) following formation of topoisomerase II-DNA cleavable complex, etoposide induces not only the early phase of caspase activation but also the later phase of calpain activation in MN9D neuronal cells; 2) however, formation of topoisomerase II-DNA cleavable complex by etoposide does not seem to be primarily responsible for subsequent neuronal cell death; and 3) Bcl-X₁ plays a protective role by exerting its anticaspase and anticalpain effect on most of the cellular events without affecting the rate of topoisomerase II-DNA cleavable complex formation in the nucleus following etoposide treatment.

MATERIALS AND METHODS

Cell Culture and Drug Treatments

To examine the effects of etoposide in the presence or absence of other drugs, cells stably overexpressing human Bcl-X₁ (MN9D/Bcl-X₁; Kim et al., 1999) or its control vector (MN9D/Neo) were plated on 50 μg/ml poly-D-lysine-coated culture plates and dishes (Corning Glass Works, Corning, NY). Cultures were maintained in 90% DMEM supplemented with 10% fetal bovine serum and 250 μg/ml G-418 (Gibco, Grand Island, NY) for 3 days in an incubator with an atmosphere of 10% CO₂ at 37°C. Cells were subsequently switched to serum-free N2 medium containing the various experimental reagents. Reagents used included etoposide, ZnCl₂, aurintricarboxylic acid, cytochalasin B, and cycloheximide (all from Sigma, St. Louis, MO); N-benzoyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD; Enzyme Systems Products, Dublin, CA); and calpeptin (Calbiochem, San Diego, CA).

Preparation of Enucleated Cytoplasts

Cytoplasts were prepared as described previously, with some modifications (Wiegler and Weinstein, 1975). Briefly, MN9D/Neo (3 × 10⁵) cells were incubated for 5 min at 37°C in 12.5% Ficoll with 10 μg/ml cytochalasin B. Cells were then layered on a discontinuous gradient of 12.5%, 15%, 16%, 17%, and 25% Ficoll containing 10 μg/ml cytochalasin B. The gradients were ultracentrifuged for 1 hr at 33°C using an SW-41Ti rotor in an Optima LE-80K Ultracentrifuge (Beckman, Palo Alto, CA). Cytoplasts were collected from the 12.5% and 15% interface. Subsequently, cytoplasts were washed with DMEM, plated on culture dishes, and subjected to further experiments. Purity of the enucleated cells was determined by staining with 1 μg/ml bisbenzimide (Hoechst dye 33258; Molecular Probes, Leiden, The Netherlands). Only <2–3% of enucleated cells were positive for bisbenzimide.

MTT Reduction Assay

For the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) reduction assay, cells or cytoplasts from MN9D/Neo and MN9D/Bcl-X₁ plated onto culture dishes were treated with 100 μM etoposide in the presence or absence of various inhibitors. After incubation with various experimental reagents, cell survival was assessed by colorimetric measurement of MTT reduction, an indicator of the pyridine nucleotide dehydrogenase activity of the cell, as described previously (Shearman et al., 1994). Briefly, the MTT solution was added to a final concentration of 1 mg/ml. The cells were incubated for 2 hr at 37°C, and lysed in 20% sodium dodecyl sulfate (SDS) in 50% aqueous dimethylformamide for 24 hr. The optical density (O.D.) of the dissolved formazan grains within the cells was measured at 540 nm in a microplate reader (Molecular Devices, Palo Alto, CA). Values from each treatment were calculated as a percentage of the nontreated control (100% survival). The data were analyzed for statistical significance by Student’s t-test.

Assay for Rate of Topoisomerase II-DNA Cleavable Complex Formation

Cells from MN9D/Neo and MN9D/Bcl-X₁ plated on P-100 culture plates were treated with 100 μM etoposide for the indicated times. After lysing of the cells with 1% sarkosyl solution, the lysates were carefully layered onto a CsCl step gradient (1.82, 1.72, 1.5, 1.3 g/ml) and ultracentrifuged for 20 hr at 20°C using an SW-41Ti rotor in an Optima LE-80K Ultracentrifuge (Beckman). This gradient centrifugation resulted in 12 fractions collected with a micropipette from the top of the gradient. The three fractions containing the DNA peak were dialyzed overnight in 0.1M TE buffer at 4°C, and assayed for DNA content. Serial dilutions of the dialyzed samples were slot blotted and subjected to immunodetection for topoisomerase II with polyclonal antibodies raised against human topoisomerase II that were cross-reactive with murine topoisomerase (1:100; TopoGEN, Co-
lumbus, OH; Son et al., 1998). Reactivity was visualized using enhanced chemiluminescence (ECL; Amersham, Buckinghamshire, United Kingdom) and quantified using a Bio-Rad densitometer (Hercules, CA).

**Nuclear Staining and Detection of DNA Fragmentation**

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) assays were performed on MN9D/Neo and MN9D/Bcl-X<sub>L</sub> cells after treatment with 100 μM etoposide for 24 hr as described previously with some modifications (Gavrieli et al., 1992; Choi et al., 1999). To assess a pattern of DNA laddering, cells from MN9D/Neo and MN9D/Bcl-X<sub>L</sub> plated on P-100 plates were treated with 100 μM etoposide for the indicated time periods in the presence or absence of a caspase inhibitor (200–400 μM Z-VAD) or with inhibitors of Ca<sup>2+</sup>/Mg<sup>2+</sup>-dependent endonuclease (5–25 μM ZnCl<sub>2</sub> or 10–40 μM aurantricarboxylic acid). Cells were then lysed in a buffer containing 0.5% Triton X-100/5 mM Tris, pH 7.4, 20 mM EDTA for 30 min on ice. As described previously (Hockenbery et al., 1990), Triton X-100-soluble DNA was extracted and subjected to electrophoresis on 1.2% agarose gels. Gels were treated with 1 mg/ml DNase-free RNase, stained with ethidium bromide, and visualized under a UV transilluminator. To examine nuclear morphology, cells were plated on P-100 culture plates, treated with 100 μM etoposide in the presence or absence of 1 μg/ml cycloheximide for the indicated times, fixed with 4% paraformaldehyde, exposed to 1 μg/ml bisbenzimide for 15 min at room temperature, and examined under UV illumination on a Carl Zeiss Axiovert 100. Cells from randomly selected areas of two or three dishes from three independent cultures were photographed, and the numbers of TUNEL-positive cells and bisbenzimide-positive condensed or swollen nuclei were quantitated.

**In Vitro Fluorogenic Caspase Cleavage Assay**

Caspase activity was measured by a fluorometric assay as recommended by the manufacturer. Briefly, MN9D/Neo and MN9D/Bcl-X<sub>L</sub> cells plated on P-100 culture plates were treated with 100 μM etoposide for the indicated time periods and lysed in a buffer containing 50 mM Tris, pH 7.0, 2 mM EDTA/1.0% Triton X-100. Cell lysates (10 μg) recovered after centrifugation at 13,000g for 10 min were incubated with 25 μM acetyl-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC, a substrate for caspase-3 as well as caspases-6, -7, -8, and -10; Calbiochem) or acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethylcoumarin (Ac-LEHD-AFC, a substrate for caspase-9 as well as caspases-4, -9, and -5; Calbiochem). Incubation was for 1 hr at 37°C in the presence of a caspase reaction buffer containing 100 mM HEPES, pH 7.4/10% sucrose/5 mM dithiothreitol/0.1% CHAPS. Proteolysis of the fluorescent peptides was measured using a fluorescence spectrophotometer (Hitachi).

**Immunoblot Analysis**

For immunoblot analysis, cells from MN9D/Neo and MN9D/Bcl-X<sub>L</sub> were plated on P-100 plates and treated with 100 μM etoposide for the indicated times in the presence or absence of a calpain inhibitor, calpeptin. Cells were then washed with ice-cold PBS and lysed on ice in a buffer containing 50 mM Tris, pH 7.0, 2 mM EDTA/1.0% Triton X-100/2 mM phenylmethylsulfonyl fluoride (PMSF)/10 μg/ml aprotinin and leupeptin for 10 min. Lysates were centrifuged at 13,000g for 15 min at 4°C. Protein content was measured using the Bio-Rad protein assay kit. For detecting cytochrome c released into the cytosolic fraction, cells were treated with 100 μM etoposide, lysed, and subsequently fractionated as previously described (Holinger et al., 1999). Equal amounts of soluble proteins (50 μg) were separated on 10% SDS-PAGE gels. Detection of specific bands was by ECL. Primary antibodies used were rabbit polyclonal anti-murine Bax (1:3,000; Krajewski et al., 1994) and rabbit polyclonal anti-human and murine Bcl-X (1:3,000; Krajewska et al., 1996), mouse monoclonal anticytochrome c (1:3,000; Pharmingen, San Diego, CA), mouse monoclonal anti-PARP (1:5,000; Enzyme Systems Products; Tewari et al., 1995), and rabbit polyclonal anti-150 kDa calpain-proteolyzed fodrin fragment (1:500; Shields et al., 1999). Exact duplicate gel from the same batch of samples was stained with Coomassie brilliant blue to demonstrate that overall protein profiles and amounts of all lanes were similar.

**RESULTS**

**Etoposide-Induced Death in Neuronal Cells**

We first attempted to characterize etoposide-induced cell death in a dopaminergic neuronal cell line, MN9D. As shown in Figure 1A, MN9D/Neo control cells had no detectable levels of Bcl-X<sub>L</sub>, whereas MN9D/Bcl-X<sub>L</sub> expressed substantial levels of Bcl-X<sub>L</sub>. In MN9D/Neo cells, 100 μM etoposide caused a time-dependent cell death. Approximately half of the MN9D/Neo cells died 18–20 hr after drug treatment as determined by the MTT reduction assay (not shown). By 48 hr, only 18.4% of the MN9D/Neo cells were alive (Fig. 1B). This was attenuated by cotreatment with 300 μM Z-VAD (a broad-spectrum caspase inhibitor; 61.7% ± 7.5%), 1 μg/ml cycloheximide (a protein synthesis inhibitor; 68.8% ± 4.5%), or 25 μM calpeptin (a calpain inhibitor; 45.5% ± 3.7%). Effective concentrations of all the inhibitors used here were chosen based on our previous studies (Choi et al., 1999, 2001). By contrast, MN9D/Bcl-X<sub>L</sub> cells displayed 79.4% survival following treatment with 100 μM etoposide for 48 hr. In this paradigm of cell death, many TUNEL-positive nuclei were observed in MN9D/Neo cells were alive (Fig. 1B). This was attenuated by cotreatment with 300 μM Z-VAD (a broad-spectrum caspase inhibitor; 61.7% ± 7.5%), 1 μg/ml cycloheximide (a protein synthesis inhibitor; 68.8% ± 4.5%), or 25 μM calpeptin (a calpain inhibitor; 45.5% ± 3.7%). Effective concentrations of all the inhibitors used here were chosen based on our previous studies (Choi et al., 1999, 2001). By contrast, MN9D/Bcl-X<sub>L</sub> cells displayed 79.4% survival following treatment with 100 μM etoposide for 48 hr. In this paradigm of cell death, many TUNEL-positive nuclei were observed in MN9D/Neo cells following etoposide treatment (22.4% for 24 hr group, 56.6% for 48 hr group). By contrast, TUNEL reactivity was very minimal in MN9D/Bcl-X<sub>L</sub> cells (Fig. 1C).

**Formation of Topoisomerase II-DNA Cleavable Complex Following Etoposide Treatment and Its Contribution to Cell Death**

Etoposide is known first to stabilize topoisomerase II-DNA cleavable complex, thereby inducing formation of double-stranded breaks in a variety of apoptotic cell deaths (Hanne, 1998). To examine first whether formation of topoisomerase II-DNA cleavable complex in the nucleus is critical and necessary for etoposide-induced cell death and how Bcl-X<sub>L</sub> affects rate of the complex formation, we prepared enucleated cells from MN9D/Neo. As
shown in Figure 2A, etoposide significantly induced cell death in MN9D/Neo enucleated cytoplasts, although the extent of death in cytoplasts was less severe than that in MN9D/Neo cells (Fig. 2A). Then, we compared the relative level of topoisomerase II-DNA cleavable complex in etoposide-treated MN9D/Neo and MN9D/Bcl-XL cells by fractionating the topoisomerase II-DNA adducts from other cellular components in a CsCl density gradient. As determined by DNA analysis, topoisomerase II-DNA cleavable complex appeared as early as 1 hr after treatment with 100 μM etoposide, and its level reached a maximum by 2 hr of treatment in both MN9D/Neo and MN9D/Bcl-XL cells, whereas background level of topoisomerase II-DNA cleavable complex in untreated cell was not detectable in DNA-containing fractions 4–6 (not shown). As shown in Figure 2B, the level of topoisomerase II-DNA cleavable complex was found to be the same for both MN9D/Neo and MN9D/Bcl-XL cells 2 hr after etoposide treatment following serial dilutions (76.5 for...
12μg DNA from MN9D/Neo cells vs. 75.6 for 12μg DNA from MN9D/Bcl-XL cells by arbitrary units from three independent cultures), suggesting that formation of topoisomerase II-DNA cleavable complex that may not be a primary contributor to etoposide-induced cell death was not affected by overexpression of Bcl-XL.

Role of Bcl-XL in Other Temporally Occurring Cellular Events Induced by Etoposide

To assess activation of caspase following etoposide treatment, assays for cytochrome c release and proteolytic activity of caspase using a fluorogenic substrate were carried out. As shown in Figure 3A, cytochrome c released into the cytosolic fraction was first detected in MN9D/Neo cells 2 hr after etoposide treatment, whereas it was less apparent in MN9D/Bcl-XL cells. Consequently, caspase-9-like activity in MN9D/Neo cells was elevated 3 hr after treatment with 100μM etoposide and continued to increase up to 3.5-fold at 12 hr over the level in untreated control cells (Fig. 3B). Caspase-3-like activity was also slightly increased at 6 hr and continued to increase up to 5.3-fold at 18 hr after etoposide treatment. These caspase activities remained largely unaltered in MN9D/Bcl-XL cells.

Fig. 3. Time course of cytochrome c release and caspase activation following etoposide (ETP) treatment. Both MN9D/Neo and MN9D/Bcl-XL cells were treated with 100μM ETP for the indicated times and lysed. A: Fifty micrograms of the cytosolic fraction were processed for immunoblot analysis of cytochrome c (Cyt c) released into the cytosol as described in Materials and Methods. Primary antibody used was mouse monoclonal anticytochrome c (1:3,000; PharMingen). A specific band was detected by ECL. B: For fluorogenic caspase assay, 10μg of lysates harvested from MN9D/Neo (solid circles and squares) or MN9D/Bcl-XL (open circles and squares) cells following ETP treatment for the indicated times were incubated with the fluorogenic peptide substrate Ac-LEHD-AFC (25μM for caspase-9-like activity; circles) or Ac-DEVD-AMC (25μM for caspase-3-like activity; squares) for 1 hr at 37°C. Data are expressed as-fold increase relative to untreated control. Each point represents a mean from triplicate wells from one experiment representative of two similar repetitions. C: Fifty micrograms of lysates were processed for immunoblot analysis for PARP. Primary antibody used was mouse monoclonal anti-PARP (1:5,000; Enzyme Systems Products). Immunoreactivity was visualized using ECL.

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and MN9D/Bcl-X<sub>L</sub> were stained with bisbenzimide dye. Nuclear staining revealed that abnormally enlarged nuclei both in MN9D/Neo and in MN9D/Bcl-X<sub>L</sub> cells appeared as early as 18–20 hr following etoposide treatment. By 24 hr of treatment, swollen nuclei were very apparent in the majority of cells (70.4% for MN9D/Neo vs. 78.1% for MN9D/Bcl-X<sub>L</sub>; Fig. 5B,F). However, visible nuclear condensation and fragmentation did not appear until 24–28 hr after treatment in MN9D/Neo cells. By 48 hr of treatment, most nuclei of MN9D/Neo cells were either condensed or fragmented (61.7% of cells; Fig. 5C). Despite the fact that nuclear enlargement was still apparent in MN9D/Bcl-X<sub>L</sub> cells, nuclear condensation and fragmentation did not appear in Bcl-X<sub>L</sub>-overexpressing cells under the same conditions (Fig. 5G). Interestingly, cotreatment of MN9D/Neo cells with 1 μg/ml cycloheximide not only prevented etoposide-induced nuclear swelling but also largely abolished nuclear swelling in both MN9D/Neo and MN9D/Bcl-X<sub>L</sub> cells (Fig. 5D,H).

**Role of Bcl-X<sub>L</sub> in Calpain-Mediated Bax Cleavage**

Calpain has been demonstrated to be responsible for cleaving a proapoptotic protein, Bax (Wood et al., 1998; Wood and Newcomb, 1999; Gao and Dou, 2000; Choi et al., 2001). Because cotreatment with the calpain inhibitor calpeptin partially rescued MN9D/Neo cells from etoposide-induced cell death, we examined the cleavage pattern of Bax by immunoblot analysis. As shown in Figure 6A, endogenous Bax abundantly expressed in MN9D/Neo cells displayed a cleavage pattern at the later stages of cell death. A specific cleavage of Bax into 18 kDa fragment first appeared after 32–36 hr of etoposide treatment in MN9D/Neo cells and continued thereafter. In contrast, Bcl-X<sub>L</sub> overexpression in MN9D cells was able to block Bax cleavage (Fig. 6B). Cotreatment of MN9D/Neo cells with the calpain inhibitor calpeptin (25 μM) largely prevented an appearance of the 18 kDa Bax fragment (Fig. 6C). Consistently, 150 kDa fodrin fragments became apparent at 36 hr of etoposide treatment, whereas cotreatment with calpeptin or overexpression of Bcl-X<sub>L</sub> inhibited generation of 150 kDa fragments as determined by immunoblot analysis using a specific antibody against

![Fig. 5. Changes in the nuclear morphology following etoposide (ETP) treatment. Both MN9D/Neo (A–D) and MN9D/Bcl-X<sub>L</sub> (E–H) cells were treated with 100 μM ETP in the presence or absence of 1 μg/ml CHX for the indicated time periods. Subsequently, cells were fixed with 4% paraformaldehyde, exposed to 1 μg/ml bisbenzimide, and examined under UV illumination on a Carl Zeiss Axiovert 100. Note the condensed (single arrowheads), fragmented (arrows), and swollen (double arrowheads) nuclei. Scale bar = 20 μm.](image-url)
calpain-proteolyzed form of fodrin (Fig. 6D,E). This sug-
gests that Bcl-X<sub>L</sub> in MN9D cells inhibits etoposide-
derived calpain activity in general.

**DISCUSSION**

The apoptotic process induced by etoposide can be separated into a phase of double-stranded breaks in the DNA and later cell death (Han, 1998). Several events associated with cell death induced by etoposide have been identified, but no attempts to delineate the cellular death sequences in neuronal or nonneuronal cell types have been made so far. The apoptotic process in dopaminergic MN9D neuronal cells following etoposide treatment occurs over a prolonged period (>48 hr), so the temporal and spatial sequences of cellular events can be resolved more easily, allowing for detailed analysis of the apoptotic process. In the present study, etoposide-induced formation of topoisomerase II-DNA cleavable complex (<1 hr) seems to precede early cytoplasmic signals, including release of mitochondrial cytochrome c into the cytosol and subsequent activation of caspase(s) (>2–6 hr). This, in turn, seems to activate caspase-dependent endonucleases responsible for the degradation of DNA into the so-called oligonucleosomal ladder (between 12 and 24 hr). Intrigu-
ingly, chromatin condensation/fragmentation has not oc-
curred even after DNA laddering is maximal at about 24 hr of treatment. Thus, it may be inferred that chroma-
tin condensation and fragmentation during etoposide-
duced apoptosis in MN9D cells are controlled, at least in part, independently of degradation of DNA into nucleo-
somal unit. Finally, we have demonstrated that Bax and fodrin cleavage by calpain follows at the later stage of etoposide-induced MN9D neuronal cell death. We have demonstrated, as demonstrated in other cell types (see, e.g., Yang et al., 1997), that activation of caspase following cytochrome c release from the mitochondrial into the cytosol appears to be the key event in etoposide-induced apoptosis of MN9D neuronal cells. Furthermore, etoposide-induced DNA laddering is prevented in MN9D/Neo cells cotreated with a caspase in-
hibitor (Z-VA D) but not with inhibitors of Ca<sup>2+</sup>/Mg<sup>2+</sup>
dependent endonuclease (ZnCl<sub>2</sub>, aurantricarboxylic acid). Therefore, our finding that caspase activation in MN9D cells may be required for DNA laddering is in agreement with the discovery of a caspase-activated DNase (Enari et al., 1998). We also observe here that the cleavage of the prototypical caspase substrate PARP by activated caspase does not occur even at the later stage of MN9D cell death. This is not in agreement with the finding that caspase activation is accompanied by a cleavage of PARP in etoposide-treated neuroblastoma as well as leukemic cells (Dubrez et al., 1996; Ibrado et al., 1996; Shah et al., 1996; Bursztajn et al., 2000). By contrast, previous studies by others have demonstrated that PARP is resistant to cleavage by caspase-3 in some cases of apoptosis (Germain et al., 1999; Jones et al., 1999). Therefore, it seems plausible that the appearance of an 85 kDa PARP band produced by activated caspase is not a common event associated with etoposide-induced apoptosis and may well be cell type-dep

In addition to caspases, excessive or uncontrolled activity of other proteases has been demonstrated to be intimately associated with apoptotic death. Moreover, ac-
tivation of another cysteine protease, calpain, has been suggested in neuronal cell death of the central nervous system (Chan and Mattson, 1999; Wang, 2000). In our study, cotreatment with calpain inhibitor (calpeptin) par-
tially attenuated etoposide-induced cell death, suggesting that calpain activity may be involved. In addition, we demonstrate that not only 150 kDa calpain-proteolyzed fodrin but also the 18 kDa Bax band appears at the later stage of etoposide-induced cell death. Generation of these bands is completely blocked by overexpression of Bcl-X<sub>L</sub> or cotreatment with the calpain inhibitor calpeptin in MN9D/Neo cells. Based on previous studies, including those from our laboratory (Wood et al., 1998; Kim et al., 1999; Wood and Newcomb, 1999; Gao and Dou, 2000; Choi et al., 2001), it is likely that calpain is responsible for cleaving Bax into an 18 kDa form after treatment of MN9D/Neo cells with etoposide. Therefore, calpain may play a role downstream of and distinct from caspases in etoposide-induced MN9D neuronal cell death. As we have proposed previously for the protective role of Bcl-2 in drug-induced Bax cleavage (Kim et al., 1999; Choi et al., 2001), it may be that Bcl-X<sub>L</sub> can block Bax cleavage either by inhibiting caspase activation upstream of calpain or directly by inhibiting calpain activity. Although it has been recently suggested that cleavage of Bax enhances its cell death function and promotes Bcl-2-independent cytochrome c release from the mitochondria into the cytosol (Gao and Dou, 2000; Wood and Newcomb, 2000), the
underlying significance of the 18 kDa Bax product in etoposide-induced apoptotic death of MN9D neuronal cells remains to be determined.

In this cell death paradigm, overexpression of Bcl-X\textsubscript{L} appears to have little effect on the rate of topoisoasemerase II-DNA cleavable complex as detected by the in vivo cross-link assay. Although it still remains to be unambiguously determined the extent to which formation of topoisoasemerase II-DNA cleavable complex is somehow linked to subsequent cell death signals, data from MN9D/Neo enucleated cytoplast favor that formation of topoisoasemerase II-DNA cleavable complex may not be an initiator of and a critical contributor to etoposide-induced death. Therefore, it may be that the protective role of Bcl-X\textsubscript{L} is primarily restricted to its anticaspase and anti-calpain activity in MN9D cell death induced by etoposide. Inhibition of caspase by Bcl-X\textsubscript{L}, in turn, seems to block nuclear events such as DNA fragmentation. Therefore, it is likely that Bcl-X\textsubscript{L} in MN9D cells exerts its antiapoptotic effects on cytosolic and nuclear events after the formation of topoisoasemerase II-DNA cleavable complex in the nucleus during etoposide-induced death. The findings that cycloheximide blocks nuclear swelling but that Bcl-X\textsubscript{L} does not, however, imply some functional selectivity or limitation of Bcl-X\textsubscript{L}.

Studies on the biochemical mechanisms of apoptosis induced by etoposide largely center on the nucleus and its associated events. Our data from enucleated MN9D/Neo cytoplast and our data demonstrating that Bcl-X\textsubscript{L} exerts its antiapoptotic effect without affecting the rate of topoisoasemerase II-DNA cleavable complex formation raise the possibility that etoposide may directly activate the cytosolic death machinery independently of this complex formation in the nucleus. Further studies unequivocally delineating this hypothesis and potential cytosolic trigger(s) induced by etoposide will expand our understanding of the cellular mechanisms underlying etoposide-induced apoptosis.

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