bcl-2 Protein Inhibits Etoposide-induced Apoptosis through Its Effects on Events Subsequent to Topoisomerase II-induced DNA Strand Breaks and Their Repair

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ABSTRACT

Previous studies have shown that bcl-2 overexpression can inhibit apoptosis induced by DNA-damaging agents widely used in cancer chemotherapy, including X-irradiation, alkylating agents (hydroperoxycyclophosphamide, etc.), and topoisomerase II inhibitors (etoposide, etc.). However, little is known about the mechanism by which bcl-2 overexpression inhibits apoptosis triggered by these agents. In this study, we examined whether bcl-2 overexpression could have effects on etoposide-induced DNA damage and its repair. For these experiments, we developed CH3I clones (mouse B-cells) stably transfected with human bcl-2 sense plasmids and compared these clones with a parental CH3I clone or CH3I clones with antisense plasmids. Overexpression of bcl-2 protein inhibited etoposide-induced apoptosis and cytotoxicity. However, there was no or little difference in the production and repair of DNA-protein cross-links, DNA single-strand breaks, and double-strand breaks among a parental CH3I clone and CH3I clones with human bcl-2 sense or antisense plasmids. These findings indicate that (a) apoptosis or cytotoxicity induced by etoposide can be separated into early events (formation of double-strand breaks, DNA single-strand breaks, and double-strand breaks) and later events (secondary DNA fragmentation or cell death) and (b) bcl-2 inhibits apoptosis and cytotoxicity induced by etoposide at some steps between these events.

INTRODUCTION

Cell death occurs via two distinct mechanisms: necrosis and apoptosis (1). While necrosis is a degenerative phenomenon produced by severe environmental disturbances, apoptosis is regarded as an active suicidal response to various physiological or pathological stimuli. Recent studies have shown that a variety of DNA-damaging agents initiate pathways leading to apoptosis. X-irradiation and several chemotherapeutic drugs (alkylating agents, topoisomerase II inhibitors, etc.) are known to trigger apoptosis, although the mechanism by which apoptosis is induced by these agents is not known (2-4). bcl-2 is a protooncogene involved in the t(14;18) translocation found in human malignant lymphomas, especially follicular lymphomas (5). It encodes a 26-kDa protein that extends cell survival through inhibition of apoptosis induced by various stimuli, including chemotherapeutic drugs (glucocorticoids, alkylating agents, topoisomerase II inhibitors, etc.) (6-10). Thus, deregulated expression of bcl-2 protein may protect lymphoma cells from chemotherapeutic drugs. Indeed, the presence of t(14;18) translocations or bcl-2 rearrangements seems to be associated with shorter disease-free survival and failure to achieve complete remission, although large-scale controlled clinical trials are necessary to confirm this association (11, 12). Therefore, strategies to develop new drugs against malignant lymphomas with deregulated bcl-2 genes must account for the mechanism by which bcl-2 protects against apoptosis induced by cytotoxic drugs.

In this study, we used the CH3I murine B-cell lymphoma line transfected with human bcl-2 expression plasmids to examine the mechanism by which bcl-2 protein inhibits apoptosis induced by etoposide (an epipodophyllotoxin). We chose etoposide as a DNA-damaging agent for the following reasons: (a) the mechanism of DNA strand break induction by etoposide is well-characterized (topoisomerase II inhibition) (13, 14), and (b) DNA strand breaks induced by etoposide are usually rapidly repaired after drug removal (15, 16). Thus, DNA repair can be examined before apoptosis (secondary DNA fragmentation) occurs. We show here that bcl-2 overexpression has no or little effect on the formation and disappearance of DNA strand breaks (initial DNA damage), although it strongly inhibits apoptosis (secondary DNA fragmentation) induced by the cytotoxic agent, etoposide.

MATERIALS AND METHODS

Chemicals. Etoposide was purchased from Sigma (St. Louis, MO) and was dissolved in dimethyl sulfoxide at 20 mg/ml and stored at -20°C. It was diluted in complete medium before each experiment. [3H]Thymidine and [14C]thymidine were purchased from Amersham (Arlington Heights, IL). All other chemicals were from Sigma.

Cell Culture. The CH3I cell line (17) (a generous gift from Dr. G. Haughton, University of North Carolina at Chapel Hill, NC) was maintained at 37°C in a 5% CO2 atmosphere in RPMI 1640 medium supplemented with 2 mm L-glutamine, 50 μm 2-mercaptoethanol, 1 mm sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum (GIBCO-BRL, Gaithersburg, MD). Cell morphology was evaluated on Diff Quick (American Scientific Products, McGaw Park, IL) stained cytocentrifuge cell preparations.

Transfection of bcl-2 Plasmids. We stably introduced human bcl-2 sense and antisense expression plasmids into CH3I cells using a retroviral vector (18, 19). We also prepared a CH3I clone stably transfected with parental plasmids (control 1) as a control. Neomycin-resistant clones were selected and used for further experiments. Expression of bcl-2 sense plasmids in CH3I cells was confirmed by immunoblotting using rabbit antisera specific for the human bcl-2 protein, as described in detail elsewhere (20) (Fig. 1). Expression of bcl-2 antisense plasmids was confirmed by RNA blotting (data not shown).

The cell-doubling time of control, sense, and antisense clones was 18–22 h.

Colonization Formation Assay. Etoposide-induced cytotoxicity was determined by colony formation assays following drug removal. After treatment with 10 μg/ml (17.3 μM) etoposide for 1 h, cells were washed twice and plated in triplicates at a density of 102, 103, and 104 cells/cm2 in 5 ml of complete medium containing 0.3% soft agar. Colonies were counted after 10 days of incubation at 37°C. The results were expressed in percentages as the ratio of the plating efficiencies of etoposide-treated cells to the plating efficiencies of untreated cells. The plating efficiencies of untreated cells varied between 26 and 38%.

DNA Fragmentation Assay. This assay [a modification of protocol described by Duke et al. (21)], 5 × 105/ml cells were labeled with [3H]- thymidine (0.5 μCi/ml) for 2 h at 37°C, washed three times with complete medium, and treated with 10 μg/ml etoposide for 1 h. After 1 h incubation, cells were washed twice, and 2 × 108 cells in 1 ml complete medium were incubated in 24-well plates (Costar, Cambridge, MA). At various times, the medium was collected. Cells were lysed in 600 μl lysis buffer containing 5 μM Tris (pH 8.0), 20 mM EDTA (pH 8.0), and 0.5% Triton X-100. The lysate was dissolved in 10 ml of 0.4 M KOH and incubated for 5 h at 37°C. The DNA was precipitated with 20% PCA and washed three times with 100% ethanol.

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3 H. Kamesaki, J. A. Zwiebel, J. C. Reed, and J. Cossman, Role of bcl-2 in the cooperative regulation of anti-IgM induced apoptosis in a Ly-1 B-cell line, submitted for publication.
To each vial of double-stranded DNA, 1.5 ml distilled water were added. The alkali-unwinding assay described by Ahnstrom and Erixon was used. Briefly, 1 x 10^6 cells in 10 ml medium were labeled with [14C]-thymidine (0.04 μCi/ml) for 24 h at 37°C and chased in isotope-free medium for 20 h. Cells were treated with the indicated concentration of etoposide for 1 h, washed 2 times, and assayed for DSB. Approximately 2.5 x 10^5 [14C]-labeled cells were loaded onto polycarbonate filter (2-μm pore size; Nucleopore, Pleasanton, CA) and lysed with 5 ml of 2% SDS and 0.5 mg/ml proteinase K at pH 10.0. Elution was performed with tetrapropylammoniumhydroxide/EDTA solution (pH 9.6) containing 0.1% SDS. Flow rate was 0.03–0.04 ml/min, and fractions were collected at 3-h intervals for 15 h. We used the filter-retained fraction of DNA from drug-treated cells at 10 h elution as a measure of DSB frequency.

Measurement of DNA-Protein Cross-Links by Alkaline Elution Assay. Cells were labeled and treated with etoposide as described above. Internal standard cells were labeled with [3H]thymidine (0.2 μCi/ml) for 24 h at 37°C and chased in isotope-free medium for 20 h. 14C-Labeled control and drug-treated cells and 3H-labeled internal standard cells were irradiated on ice with 3000 rads using 140Cs source. Cells were loaded onto vinyl acrylic copolymer filters (Metrical membrane, 0.8–μm pore size; Gelman Science Inc., Ann Arbor, MI), lysed with 5 ml of solution containing 0.2% sodium sarcosyl-2 M NaCl-0.04 M EDTA (pH 10.0), and washed with 5 ml of 0.02 M EDTA (pH 10.0). Elution was performed with tetrapropylammoniumhydroxide/EDTA (pH 12.1) without SDS at a flow rate of 0.03–0.04 ml/min. DPC were then calculated using the "bound-to one terminal" model and expressed in Rad equivalents (23).

Measurement of Intracellular NAD*. To extract NAD* + 2 x 10^6 cells were washed with cold phosphate-buffered saline. Cells were incubated in 3 ml of 0.5 M perchloric acid at 0°C for 15 min. The solution was then adjusted to pH 7.5 by adding 1.5 ml of 1.0 M KOH, 0.33 M K2HPO4-KH2PO4. After 15 min of incubation at 0°C, the insoluble KClO4 was removed by centrifugation at 1500 x g for 10 min. The final supernatant was stored frozen at −20°C and assayed for NAD* by the method of Jacobson et al. (25).

DNA Extraction and Electrophoresis. Cells were treated with 10 μg/ml etoposide for 1 h, washed 2 times, and incubated in drug-free medium. After 6 h incubation, 2 x 10^6 cells were lysed and centrifuged as described for the DNA fragmentation assay. The supernatant was extracted with phenol/chloroform (1:1) and then with chloroform/isomyl alcohol (24:1). The DNA was precipitated with 0.1 volume 3 M sodium acetate (pH 5.2) and 2.0 volumes 100% ethanol at −20°C overnight. After the cells were centrifuged, the pellet was dried and resuspended in 20 μl Tris-EDTA buffer (pH 8.0). The sample was treated with RNase (1 μg/ml) for 2 h at 37°C. Electrophoresis was performed in 1.8% agarose gel at 50 V for 4 h.

All experiments were performed using CH31 wild type (CH31-WT), two clones stably transfected with a bcl-2 sense expression plasmid (sense 1 and sense 4), and at least one clone stably transfected with a bcl-2 antisense expression plasmid (antisense 1). For DNA fragmentation and colony formation assay, we also examined a CH31 clone transfected with plasmids lacking bcl-2 sequence (control 1). Because the two sense clones behaved in the same manner in all experiments, only the results of Sense 4 are shown.

RESULTS

Induction of Apoptosis in CH31 Cell by Etoposide. Treatment with 10 μg/ml (17.3 μμ) etoposide for 1 h induced apoptosis in CH31-WT. Six h after drug removal, microscopic examination showed typical "apoptotic" cells with chromatin condensation, nuclear fragmentation, and cytoplasmic vacuolation (Fig. 2A). Oligonucleosomal length DNA fragments were also observed in agarose gel electrophoresis (Fig. 2B).

Inhibition of Etoposide-induced DNA Fragmentation by bcl-2 Overexpression. Cells labeled with [3H]thymidine were treated with 10 μg/ml etoposide for 1 h. After drug removal, the DNA fragmentation assay was performed at the specified times. In CH31-WT, DNA fragmentation became apparent (12.3%) at 2 h after drug removal. DNA fragmentation then increased to 42.1 and 48.1% at 6 and 12 h, respectively (Fig. 2C). In this study, we used this result to define the onset of apoptosis because the DNA fragmentation assay is the most sensitive assay to detect early events of apoptosis.

4 The abbreviations used are: SDS, sodium dodecyl sulfate; DPC, DNA-protein cross-links; SSB, single-strand breaks; DSB, double-strand breaks; IL, interleukin.
To examine the effect of bcl-2 overexpression on etoposide-induced DNA fragmentation, we compared DNA fragmentation of sense 4 with that of CH31-WT, control 1, and antisense 1 (Table 1). DNA fragmentation of sense 4 was reduced 10- to 20-fold by bcl-2 overexpression to only 2.3% (5.5, 8.1, and 10.3% of that of CH31-WT, control 1, and antisense 1, respectively) at 6 h and 5.7% (11.8, 11.7, and 13.5% of that of CH31-WT, control 1, and antisense 1, respectively) at 12 h.

**Etoposide-induced Cytotoxicity in CH31 Cells.** Cytotoxicity was determined by colony formation assays. Two independent experiments for each clone were performed in triplicates. As shown in Table 2, sense 4 clone was resistant to etoposide compared with CH31-WT, control 1, or antisense 1. Therefore, bcl-2 overexpression reduced etoposide-induced cytotoxicity as well as apoptosis.

**Production of DPC, SSB, and DSB by Etoposide.** The formation of covalently linked complexes between topoisomerase II and cellular DNA is regarded as the first step of cell killing by etoposide (26-28). The stabilization of this covalent complex is thought to induce concomitant SSB and DSB (29, 30). In order to investigate the effects of bcl-2 overexpression on DNA damage, we examined DPC, SSB, and DSB induced by various concentrations of etoposide in all CH31 cell clones. Cells were treated with several concentrations of etoposide for 1 h and assayed for DNA fragmentation at the specified incubation time. (See "Materials and Methods."

**Table 1** Etoposide-induced DNA fragmentation

<table>
<thead>
<tr>
<th>Clones</th>
<th>Time after drug removal (h)</th>
<th>6 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>42.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(41.1-43.7)</td>
<td>(46.8-49.1)</td>
<td></td>
</tr>
<tr>
<td>Control 1</td>
<td>28.4</td>
<td>48.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(27.9-28.8)</td>
<td>(47.9-49.5)</td>
<td></td>
</tr>
<tr>
<td>Antisense 1</td>
<td>21.3</td>
<td>42.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(20.0-22.3)</td>
<td>(42.3-42.9)</td>
<td></td>
</tr>
<tr>
<td>Sense 4</td>
<td>2.3</td>
<td>5.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(6.4-7.1)</td>
<td>(4.9-6.2)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Mean percentages (range) of triplicates.

**Table 2** Colony formation assay

<table>
<thead>
<tr>
<th>Clones</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.31 ± 0.05*</td>
<td>0.11 ± 0.10</td>
</tr>
<tr>
<td>Control 1</td>
<td>0.08 ± 0.08</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>Antisense 1</td>
<td>0.42 ± 0.08</td>
<td>0.50 ± 0.14</td>
</tr>
<tr>
<td>Sense 4</td>
<td>1.04 ± 0.07</td>
<td>0.70 ± 0.12</td>
</tr>
</tbody>
</table>

<sup>*</sup> E, plating efficiencies of etoposide-treated cells; C, plating efficiencies of untreated cells. The values are means ± SD of triplicates.
EFFECTS OF bcl-2 OVEREXPRESSION ON DNA DAMAGE

measured DPC, SSB, and DSB up to 2 h after drug removal. DNA repair was assayed for 2 h after drug removal in order to limit the effect of secondary DNA fragmentation. Cells were treated with 10 μg/ml etoposide for 1 h, washed 2 times, and incubated in drug-free medium. At the indicated time, cells were assayed for DPC, SSB, and DSB.

As shown in Fig. 4A, DPC were repaired quickly and almost completely within 2 h in all CH31 cell clones. There was no significant difference in DPC repair kinetics among these clones, although DPC in antisense 1 disappeared more rapidly during the first 30 min.

DNA single-strand breaks were also repaired quickly after drug removal without a significant difference among the three clones (Fig. 4B).

In contrast, DNA double-strand breaks were slowly and not completely repaired after drug removal (Fig. 4C). DSB in sense 4 were not fewer than those of CH31-WT or antisense 1 at 30 and 60 min after drug removal. However, DSB in sense 4 reversed more extensively than those in CH31-WT or antisense 1 at 120 min. The finding of slower reversal of DSB than DPC or SSB is probably due to the fact that the DSB assay measures both the topoisomerase II-induced DNA strand breaks and apoptosis-associated DNA fragmentation. The more extensive reversal of DSB in sense 4 is consistent with this explanation.

Therefore, inhibition of etoposide-induced apoptosis and cytotoxicity by bcl-2 overexpression cannot be explained by differences in the extent of DNA damage or the rate of its repair since bcl-2 had no effect on etoposide-induced DNA damage and its repair.

NAD⁺ Levels after Etoposide Treatment. DNA strand breaks are known to induce NAD⁺ depletion through activation of poly(ADP-ribose) polymerase. To examine the role of NAD⁺ depletion in apoptosis, we measured NAD⁺ levels in CH31-WT with or without etoposide treatment. Cells were treated with 10 μg/ml etoposide for 1 h, washed 2 times, and incubated in drug-free medium. Because DNA fragmentation occurred at 2 h after drug removal, NAD⁺ levels were monitored up to 2 h.

NAD⁺ levels (means ± SD) in untreated cells were 67.3 ± 3.8, 67.3 ± 2.0, and 70.7 ± 1.4 pmol/10⁶ cells at 0, 1, and 2 h, respectively, while those in etoposide-treated cells were 71.2 ± 3.2, 59.1 ±
EFFECTS OF bcl-2 OVEREXPRESSSION ON DNA DAMAGE

Fig. 4. Reversal of etoposide-induced DNA damage. Cells were treated for 1 h with 10 \( \mu \text{g/ml} \) etoposide, washed twice, and incubated in drug-free medium. At the specified time, cells were assayed for DPC, DNA SSB, and DNA DSB. A, DPC assayed by alkaline elution. Points, means of 2–5 experiments (bars, range). B, SSB assayed by alkali-unwinding method. Points, means of triplicate measurements (bars, SD). C, DSB assayed by alkaline elution. Points, means of 2–4 experiments (bars, range). Hatched area, range of DNA filter retention after 10 h of elution in untreated cell lines. □, CH31-WT; ●, sense 4; ○, antisense 1. Sense 1 showed similar results as sense 4.

1.0, and 70.0 \( \pm 0.7 \) pmol/10^6 cells at 0, 1, and 2 h, respectively. A slight decrease in NAD^+ level was observed at 1 h. However, no significant decrease in cellular NAD^+ was detected before the onset of secondary DNA fragmentation.

DISCUSSION

Epipodophyllotoxins are potent and widely used antitumor drugs with inhibitory effects on topoisomerase II (13, 14). Extensive studies of their cytotoxic mechanism suggest that stabilization of the covalently linked complexes between topoisomerase II and cellular DNA (cleavable complexes) is the initial event mediating the antitumor effects of these drugs (26, 27). Protein-linked DNA single- and double-strand breaks result from this interaction (29, 30). However, other studies suggest that one or more steps subsequent to the formation of protein-linked DNA strand breaks is important for their cytotoxic effects. (a) DNA strand breaks induced by these drugs are rapidly resealed after drug removal, but the resealing of these strand breaks does not prevent cell death (15). (b) IL-3 inhibits the cytotoxic effects of etoposide (an epipodophyllotoxin), although the extent of DNA strand breaks and their repair are not affected by IL-3 (31). (c) Etoposide-induced DNA strand breaks occur in calcium-depleted cells in the absence of cytotoxicity (32). In fact, our results support this concept based on the following findings: (a) DPC and DNA SSB were rapidly repaired before the onset of apoptosis (secondary DNA fragmentation). DNA DSB were also repaired, although slowly and not completely. (b) bcl-2 protein overexpression inhibited apoptosis and cytotoxicity with little or no effect on DNA strand break formation and repair. Therefore, the cytotoxicity of etoposide could be dissected into early events (DNA strand breaks mediated by topoisomerase II) and later events (secondary DNA fragmentation and cell death), bcl-2 inhibited apoptosis and cytotoxicity at some steps between these two events.

A prediction based on this concept is that bcl-2 overexpression will protect tumor cells from apoptosis and cytotoxicity of topoisomerase II inhibitors even if their kinetics of DNA strand breaks are different. This might also be applied to other DNA-damaging agents with various kinetics of DNA strand breaks. However, we cannot exclude the
EFFECTS OF bcl-2 OVEREXPRESSION ON DNA DAMAGE

possibility that their preferential interaction with some specific genomic site may produce cytotoxic effects irrespective of bcl-2 overexpression (33).

Recent studies suggest that DNA-damaging agents, such as X-irradiation, alkylating agents, and topoisomerase II inhibitors, may initiate common pathways leading to apoptosis: (a) all induce apoptosis (2–4), as well as G2 arrest in the cell cycle (34), and (b) bcl-2 protein and IL-3 are common inhibitors of apoptosis triggered by these agents (8, 9, 35).

Although the mechanism by which initial DNA damage induces apoptosis remains unknown, two major models are suggested. One model is based on the activation of poly(ADP-ribose) polymerase by DNA strand breaks (36, 37). Activation of this enzyme consumes NAD+, leading to a lethal metabolic disturbance (ATP depletion, etc.) and apoptosis. However, we could not detect a significant decrease in cellular NAD+ levels before the onset of apoptosis. Thus, this model seems to be an unlikely explanation for etoposide-induced apoptosis in the CH31 cells. The other model is based on the induction of normal p53 protein after treatment with DNA-damaging agents. The accumulation of normal p53 switches off replication to allow extra time for DNA repair (38, 39). If the repair fails, p53 may trigger cell suicide by apoptosis (40). Although evidence for this model is still circumstantial, further studies will be needed to clarify the role of p53 in apoptosis induced by DNA damage. To define the inhibitory mechanism of bcl-2 protein on apoptosis, it is now necessary to identify the cellular events triggered by DNA damage, determine how they lead to secondary DNA fragmentation, and determine how they are reversed by bcl-2.

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