Bcl-x₅ Enhances Adenoviral Vector-induced Apoptosis in Neuroblastoma Cells¹

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ABSTRACT

bcl-x is a member of the bcl-2 family of genes and by alternative splicing gives rise to two distinct mRNAs: bcl-x₅ and bcl-x₇. We have previously investigated the expression of Bcl-x in neuroblastoma (NB) cell lines and have shown that Bcl-x₅ is expressed and functions to inhibit chemother-apy-induced apoptosis. However, none of the NB cell lines expressed Bcl-x₇. The aim of the present study was to determine the effects of Bcl-x₅ expression on the viability of NB cells. A panel of NB cell lines (CHP-382, GOTO, SHEP-1, SH-SY-5Y, and GI-CA-N) were infected with either a bcl-x₅ adenovirus (pAdRSV-bcl-x₅) or a control virus (pAdRSV-lac-z). NB cells showed loss of viability with both viruses, although the bcl-x₅ virus was most toxic. Importantly, infection with the bcl-x₅ adenovirus resulted in rapid loss of cell viability, DNA fragmentation, and morphological features of apoptosis even in NB cells transfected to overexpress Bcl-2 and Bcl-x₇. These findings suggest that deregulated expression of Bcl-x₅ using an adenovirus may provide a novel mechanism for initiating cell death in tumors that express Bcl-2 or Bcl-x₇.

INTRODUCTION

PCD³ or apoptosis is an essential feature of the regulation of eukaryotic cell number. It is characterized by the activation of an intrinsic genetic program leading to typical morphological changes, such as cytoplasmic contraction, plasma membrane blebbing, nuclear condensation, and DNA degradation, culminating in cell death (1). The biochemical hallmark of this process in epithelial cells is high molecular weight DNA fragmentation best demonstrated by PFGE (2, 3). bcl-2 was the first member of a family of genes, the products of which function to modulate PCD pathways. Initially identified at the t(14;18) translocation in follicular B-cell lymphomas (4), subsequent work showed that overexpression could promote cell survival (5) by inhibiting PCD induced by growth factor withdrawal (6). A number of studies by numerous investigators have now determined that Bcl-2 delays or prevents PCD induced by a variety of stimuli, including growth factor deprivation, γ-radiation, glucocorticoids, heat shock, and multiple chemotherapeutic agents (5, 7–9).

bcl-x is a new member of the bcl-2 family of genes and was isolated by cross hybridization with a bcl-2 probe (10; reviewed in Ref. 11). As a result of alternative splicing, this gene gives rise to two distinct mRNAs designated as bcl-x₅ and bcl-x₇. The larger transcript encodes a 241-amino acid protein (Bcl-x₅), which displays a high level of amino acid and structural homology to Bcl-2. Bcl-x₅, like Bcl-2, functions to inhibit PCD induced by growth factor withdrawal in hematopoietic cells (10). The smaller transcript, bcl-x₇, encodes a 178-amino acid protein, which lacks an internal 63-amino acid domain in the region of highest homology to Bcl-2. In single-gene transfection experiments in hematopoietic cells, Bcl-x₇ had no effect on cell growth in the presence of growth factor but facilitated PCD by inhibiting the death-suppressing activity of Bcl-2 (10). Boise et al. (10) have proposed that Bcl-x₇ acts in a dominant fashion by forming inactive heteromeric complexes with Bcl-2 or Bcl-x₅. In support of this hypothesis are studies by Sato et al. (12) showing that Bcl-x₅ and Bcl-x₇ can combine to form heterodimers or heteromultimers with Bcl-2 in a yeast two-hybrid system. Recent studies have indicated that Bcl-x₅ is widely expressed (13, 14), and the pattern of expression varies from that of Bcl-2, and other bcl-2 gene family members (14–16). The Bcl-x₅ protein was detected in the thymus, lymph nodes, tonsils, and reproductive tissues such as testes, prostate, and mammmary epithelia. Bcl-x₇ levels were variable, and in some tissues, both Bcl-x₅ and Bcl-x₇ were detected, suggesting a hypothesis that the ratio of Bcl-x₅:Bcl-x₇ may modulate PCD induction in vivo (13).

Our laboratory has been investigating whether a failure of PCD contributes to the genesis or progression of the pediatric malignancy NB. In previous studies, we have determined that the majority of NB cell lines express Bcl-2 and Bcl-x₅, and that deregulated expression of these gene products confers resistance to a variety of chemotherapeuti-ic agents (17, 18). Most importantly, none of the NB cell lines expressed Bcl-x₇. The aim of the present study was to determine whether expression of Bcl-x₇ could induce apoptosis in NB cells. Using an adenoviral bcl-x₇ expression vector, we were able to induce apoptosis in NB cells even in the context of high levels of Bcl-2 and Bcl-x₅ expression. Analysis of cells expressing high levels of Bcl-x₇ revealed high molecular weight DNA degradation and morphological features of apoptosis. These results suggest that deregulated Bcl-x₇ expression using an adenoviral expression system can abrogate the protective effects of Bcl-2 and Bcl-x₅ and may provide a novel mechanism for increasing the sensitivity of NB cells to PCD.

MATERIALS AND METHODS

Cell Lines. NB cell lines used in the following study included CHP-382, GI-CA-N, GOTO, SH-SY-5Y, and SHEP-1. Infection studies were also performed in SHEP-1 cells transfected to express high levels of Bcl-2 or Bcl-x₅. These lines included vector control (pooled SHEP-1 cells transfected with the pSFFVneo expression vector alone), SHEP-1 Bcl-2⁺ (a cloned cell line transfected with the expression vector pSFFV-bcl-2), and SHEP Bcl-x₅⁺ (a cloned cell line transfected with the expression vector pSFFV-bcl-x₅). The method of transfection and the characterization of the in vitro growth of the transfected cell lines has been previously reported (17, 18). Cells were maintained in MEM supplemented with 10% fetal bovine serum, 2 mM glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml). Transfected cells were maintained in the same media supplemented with G418 (Geneticin; Life Technologies, Inc., Gaithersburg, MD) at 500 μg/ml.
Preparation of Adenovirus and NB Cell Infection. The bcl-x<sub>S</sub> adenoviral vector (pAdRSV-bcl-x<sub>S</sub>; Ref. 19) was constructed by cloning a full-length bcl-x<sub>S</sub> cDNA into the pAdRSV expression vector (20). This vector uses an HSV promoter and SV40 polyadenylation signal, allowing high-level expression of inserted sequences. Recombinant adenovirus was produced by cotransfecting pAdRSV-bcl-x<sub>S</sub> with the replication-deficient sub360 adenovirus into 293 human kidney cells (20). Viral titers were determined by limiting dilution and plaque formation of 293 cells. Each infection was performed in duplicate with at least three viral stock preparations. Controls for these experiments included a mock infection (no virus) and infection with the same adenovirus construct containing lac-z (pAdRSV-lac-z; Ref. 19). For adenoviral infection, the cell lines were rinsed with serum-free DMEM before adding virus (pAdRSV-bcl-x<sub>S</sub> or control virus pAdRSV-lac-z). Following a 3-h incubation, the virus-containing medium was replaced with DMEM containing 2% fetal bovine serum and incubated overnight. The next day, the medium was replaced with DMEM containing 10% serum. The virally transduced cells were grown for an additional 24 h prior to subsequent experimental manipulation. Mock-infected cells were subjected to the same culturing manipulations without the addition of virus-containing medium.

Detection of Bcl-x<sub>S</sub> Expression by Western Analysis. Bcl-x<sub>S</sub> was identified by Western analysis using the enhanced chemiluminescence technique (Amerham International, Buckinghamshire, United Kingdom). Twenty-four hour following infection, cells were harvested and lysis buffer containing 50 mM Tris, 2% SDS, and 10% glycerol, boiled for 10 min, and centrifuged at 10,000 × g. Following protein concentration, 20 μg of protein were electrophoresed on 12% SDS-polyacrylamide gels and transferred to nitrocellulose by electroboblotting. Membranes were blocked and then incubated with an anti-Bcl-x<sub>S</sub> polyclonal antibody that is known to recognize both Bcl-x<sub>S</sub> and Bcl-x<sub>L</sub> (21). Following incubation with a rabbit secondary antibody, antigen-antibody complexes were detected by exposing the membrane to equal volumes of reagents 1 and 2 from the enhanced chemiluminescence kit. Blots were developed by exposing the membrane to Kodak XAR film. Controls for Western analysis included a NB cell line transfected to express Bcl-x<sub>S</sub> (FL5.12 bcl-x<sub>S</sub>-Ha tag; Ref. 16).

Cell Viability. In vitro cell viability was determined by the MTT dye reduction assay (22). Following infection, 10<sup>5</sup> cells were plated in triplicate in a 96-well microtiter plates overnight. Plates were harvested daily for 7 days. Media were removed and replaced with 125 μl of complete media containing 1 mg/ml MTT. Plates were incubated at 37°C for 4 h. Following incubation, cells were lysed in a buffer containing 20% (w/v) SDS and 50% (v/v) N,N-dimethylformamide (pH 4.5). Absorbance at 570 nm was determined for an additional 24 h prior to subsequent experimental manipulation. Mock-infected cells were subjected to the same culturing manipulations without the addition of virus-containing medium.

RESULTS

Bcl-x<sub>S</sub> Expression in NB Cells. Previous studies have indicated that NB cell lines including SHEP-1 do not express endogenous Bcl-x<sub>S</sub> (17). To determine the effects of Bcl-x<sub>S</sub> expression in NB cells, we used an adenoviral expression vector to overexpress Bcl-x<sub>S</sub> in the NB cell lines CHP-382, GI-CA-N, GOTO, SH-SY-5Y, and SHEP-1. In a separate set of experiments, SHEP-1 cells transfected to express high levels of Bcl-2 or Bcl-x<sub>L</sub> were also infected with the Bcl-x<sub>S</sub> adenovirus. These lines were chosen because previous studies had determined that deregulated expression of Bcl-2 and Bcl-x<sub>L</sub> in SHEP-1 cells could inhibit apoptosis induced by a variety of chemotherapeutic agents, and also that these cells were relatively resistant to the toxic effects of the virus alone. The pAdRSV-bcl-x<sub>S</sub> expression vector is a replication-incompetent virus that has an infection efficiency of greater than 99%, as determined by staining for β-galactosidase (19). The relative efficiency of adenoviral infection was determined by 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside staining after inoculation of NB target cells with a lac-z virus. For each line, viral titers that would result in >99% infection were used. NB cells were infected either with pAdRSV-bcl-x<sub>S</sub> or pAdRSV-lac-z (adenoviral control) or underwent a mock infection (no virus). A high level of Bcl-x<sub>S</sub> protein was detected in pAdRSV-bcl-x<sub>S</sub>-infected cells 24 h after infection by Western blot analysis (Fig. 1). Mock-infected and lac-z-infected cells were negative for Bcl-x<sub>S</sub>. Expression of β-galactosidase in control infected cells was confirmed by immunohistochemical analysis (data not shown).

The Adenoviral Vector Promotes NB Cell Death, Which Is Enhanced by Bcl-x<sub>S</sub> Expression. Cell viability of virally infected NB cells was determined by the MTT viability assay. This assay relies on the ability of intact mitochondria to reduce the yellow MTT compound to a blue formazan derivative. NB cells infected with the adenoviral vectors exhibited one of three patterns of loss of cell viability. In some cells (CHP-382 and GOTO) the lac-z and bcl-x<sub>S</sub> viruses were equally toxic and resulted in rapid and complete loss of cell viability within 72 h of infection (Fig. 1A). A second pattern of cell death was seen in SH-SY-5Y cells. Here, both viruses induced equivalent loss of viability over the duration of the experiment, with the bcl-x<sub>S</sub> virus inducing the most rapid onset of cell death (Fig. 1B). A third pattern was seen in GI-CA-N and SHEP-1 cells. In these cells, the lac-z virus caused minimal loss of viability, whereas the bcl-x<sub>S</sub> virus caused the cells to die in a dose-dependent manner over time. Cell viability was calculated as the percentage of viable cells compared with the start of treatment and was expressed as the VCI (VCI = (A<sub>570 virus or mock</sub>/A<sub>570 day 0</sub>) × 100). The day-0 reading was obtained 6 h after cells were counted and plated onto the 96-well dishes. Student's paired t test was used to test the significance of the difference in cell viability following infection with control virus (pAdRSV-lac-z) or bcl-x<sub>S</sub> virus (pAdRSV-bcl-x<sub>S</sub>.

FACS Analysis for DNA Damage. FACS analysis of propidium iodide-stained nuclei was performed as described previously (23). This assay is rapid and provides an excellent method for assessing DNA damage in apoptotic cells (24). Cells were plated at a density of 1 × 10<sup>6</sup> cells/well in six-well dishes overnight. At indicated time points, cells were harvested by trypsinization, suspended in hypotonic lysis buffer (0.1% sodium citrate, 0.01% Triton X-100, and 0.1 mg/ml propidium iodide) and incubated at 4°C for 4 h. Cells were analyzed for DNA content on FACS (Becton Dickinson, Mountain View, CA) using Lysis-II software (Becton Dickinson). Differences in percentage of DNA fragmentation between lac-z- and bcl-x<sub>S</sub>infected cells was determined using Student's paired t test.

PFGE Analysis for DNA Fragmentation. In addition to FACS analysis, DNA integrity was also assessed by PFGE. This technique has been shown to correlate with morphological changes (3) and is capable of detecting the early phases of DNA degradation in apoptotic cells (25). Following infection, cells were harvested by trypsinization, and pellets were prepared in low-melting point agarose as described previously (17). Cell pellets were loaded into 1.2% agarose gels (Fastlane; FMC Bioproducts, Rockland, ME) and run at 6 V/cm with ramped switch times from 26 to 30 s over 20 h using the Chef DR-III system apparatus (Bio-Rad Laboratories, Hercules, CA). Following electrophoresis, gels were stained in ethidium bromide and destained in distilled water. λ DNA digested with HindIII (Life Technologies) and Coliphage λ DNA concatamers (MegaBase II; Life Technologies) were used as size standards.

Preparation of Monolayer Cells for Electron Microscopy. Following infection, cells were harvested by trypsinization and washed in serum-free media. Cells were pelleted by centrifugation and in an equal volume suspended in 25% BSA and 6% glutaraldehyde. Pellets were isolated, cut into cubes, and fixed in 0.1 M sodium cacodylate buffer (pH 7.4). Fixed cubes were dehydrated in increasing concentrations of ethanol. Cubes were polymerized in Epon resin (44.5% poly/bed 812 resin, 24.1% dodecane/Lysin succinic anhydride, 29.8% nadic methyl anhydride, and 1.6% 2, 4, 6-Tri (dimethylaminomethyl)phenol; Polysciences, Inc.) at 60°C overnight. Seventy- to 90-nm ultrathin sections were cut on a Reichert-Jung Utracut E-43 microtome, placed on copper grids, and stained with 4% uranyl acetate in 50% methanol and Reynolds lead citrate. Sample sections were examined in a Phillips Electronics CM-100 transmission electron microscope. Images were digitally recorded using a Kodak Megaplus 1.6 camera with Advanced Microscopy Techniques Digital Montaging System software. Representative grids were scored, and the percentage of apoptotic cells displaying cytoplasmic (retraction, condensation, and organelle segregation) and nuclear changes (folding, segmentation, chromatin condensation, margination, and nuclear membrane degeneration) was determined at 24 and 72 h.

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Fig. 1. Cell viabilities of NB cell lines GOTO (A), SHSY-5Y (B), and GI-CA-N (C) infected with the pAdRSV-lac-z or pAdRS-bcl-x5 virus were determined by MTT conversion. After subtracting background absorbance, the viability of each cell was calculated as VCI = (A370 virus or mock)/A370 day 0) × 100. Data represent the means of triplicate experiments. Bars, SD. The VCI for mock-infected cells ranged from 250 to 800% on day 3 (data not shown). Bcl-x5 protein expression following viral infection was confirmed by Western blot. As shown, Bcl-x5 protein was detected in bcl-x5 virus-infected cells but not following mock or lac-z infection. Positive controls included a cell line transfected to express Bcl-xL (Bcl-xL-FL5.12 bcl-xL- HA Tag; Ref. 16). The protein product of the Bcl-x5 control migrates higher than the viral induced protein as it expresses a flag tag. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control for protein loading.
virus caused rapid and complete loss of viability within 72 h (Fig. 1C). High-level Bcl-x<sub>S</sub> protein was detected in all cells 24 h following infection but was absent in lac-z-infected or mock-infected cells (Fig. 1).

To determine whether expression of Bcl-2 or Bcl-x<sub>L</sub> could overcome the effects of Bcl-x<sub>S</sub> expression, SHEP-1 cells transfected to express high levels of Bcl-2 or Bcl-x<sub>S</sub> were infected in a similar manner. Similar to control transfected SHEP-1 cells, both the bcl-x<sub>S</sub> and lac-z viruses were inherently toxic to the transfected cell lines regardless of the level of Bcl-2 or Bcl-x<sub>S</sub> expression (Fig. 2). The VCI on day 3 following lac-z infection was 31% in vector-transfected controls, 81% in Bcl-2-expressing cells, and 146% in Bcl-x<sub>S</sub>-expressing cells. On day 5, the VCI in lac-z-infected cells was 3% for vector controls, 13% for Bcl-2-expressing cells, and 6% for Bcl-x<sub>S</sub>-expressing cells. Importantly, however, cells infected with the bcl-x<sub>S</sub> adenovirus showed earlier and enhanced loss of cell viability when compared with lac-z-infected cells. The VCI on day 3 following bcl-x<sub>S</sub> infection was 3% (P < 0.001) for vector controls, 13% (P < 0.001) for Bcl-2-expressing cells, and 30% (P < 0.002) for Bcl-x<sub>S</sub>-expressing cells. By day 5, all of the bcl-x<sub>S</sub>-infected cells displayed a VCI of <6%. These results indicate the pAdRSV-lac-z virus is toxic to NB cells and that the degree of toxicity is enhanced by expression of Bcl-x<sub>S</sub>. Additionally, the bcl-x<sub>S</sub> adenovirus induces cell death even in the context of high Bcl-2 and Bcl-x<sub>L</sub> expression.

Adenoviral bcl-x<sub>S</sub> Expression Induces Apoptosis with Loss of DNA Integrity and Morphological Evidence of Apoptosis. The biochemical hallmark of apoptosis in SHEP-1 NB cells and other epithelial cell types is high molecular weight DNA degradation (2, 3, 17, 18). FACS and PFGE analysis were used to assess the integrity of DNA following bcl-x<sub>S</sub> infection. FACS analysis 24 h following infection revealed that 9–18% of the nuclei contained degraded DNA in lac-z-infected cells (Table 1). Once again, the degree of cell toxicity was enhanced following infection with the bcl-x<sub>S</sub> adenovirus. Vector-transfected controls and the Bcl-2-expressing cells showed a marked loss of DNA integrity, with 49–65% of the nuclei containing degraded DNA (P < 0.009).

Analysis of high molecular weight DNA degradation is best demonstrated by PFGE. Twenty-four h following mock and lac-z infection, DNA in vector-transfected and Bcl-2- and Bcl-x<sub>S</sub>-expressing cells remained intact, with most of the DNA being trapped in the well or in the compression zone immediately below it. In contrast, cells expressing Bcl-x<sub>S</sub> showed early loss of DNA integrity with the appearance of high molecular weight DNA (Fig. 3, A and B). Analysis of DNA integrity 72 h following infection with the lac-z virus indicated that the control virus could also induce this pattern of high molecular weight DNA degradation (Fig. 3C). These results support the viability assays and suggest that both viruses are toxic to NB cells, but the toxicity is enhanced by Bcl-x<sub>S</sub> expression.

The loss in DNA integrity following lac-z or bcl-x<sub>S</sub> infection corresponded to morphological changes consistent with apoptotic cellular demise. As shown in Fig. 4, infection with the lac-z-containing virus resulted in no morphological evidence of apoptosis at 24 h (Fig. 4A), but by 72 h, >85% of the cells were apoptotic with evidence of chromatin clumping, cytoplasmic retraction, and nuclear membrane degeneration (Fig. 4B). In comparison, the bcl-x<sub>S</sub> virus induced more rapid onset of apoptosis, with 20% of the cells displaying apoptotic features as early as 24 h (Fig. 4C). By 72 h, >95% of the cells were apoptotic (Fig. 4D). These findings were absent in mock-infected cells (Fig. 4E). These results indicate that Bcl-x<sub>S</sub> expression enhances the inherent toxicity of the adenoviral vector, resulting in rapid onset of DNA degradation and morphological features of apoptosis.

**DISCUSSION**

PCD or apoptosis in eukaryotes is a genetically regulated process that can be induced by a variety of stimuli (26). Although a number of different genes have been identified that promote cell death in the nematode *Caenorhabditis elegans* and in humans, the precise biochemical events and control of this process has yet to be elucidated. In previous studies we have determined that a majority of NB cell lines express Bcl-x<sub>L</sub> and Bcl-2 and that deregulated Bcl-2 and Bcl-x<sub>L</sub> expression can inhibit apoptosis induced by a number of chemotherapeutic agents in these cells (17, 18). Interestingly, none of the NB cell lines expressed Bcl-x<sub>S</sub> (18), a Bcl-2 family member that functions...
to facilitate apoptosis (10). In this report, we have investigated the effects of adenoviral Bcl-x<sub>S</sub> expression on the in vitro cell viability and DNA integrity of NB cells, including cell lines that have been transfected to express the apoptosis-suppressing proteins Bcl-x<sub>L</sub> or Bcl-2. The results of these studies indicate that the adenoviral vector is inherently toxic to certain NB cells and that expression of Bcl-x<sub>S</sub> enhances this toxicity. Importantly, the bcl-x<sub>S</sub> adenovirus was able to induce rapid onset of apoptosis even in the context of high Bcl-2 or Bcl-x<sub>L</sub> expression. Boise <em>et al.</em> (10) have shown that expression of Bcl-x<sub>S</sub> in IL-3-dependent hematopoietic cells did not affect cell growth in the presence of IL-3 but did abrogate the protective effects of Bcl-2 following growth factor withdrawal in IL-3-dependent hematopoietic cells. In our studies, all cells infected with the bcl-x<sub>S</sub> adenovirus underwent apoptosis, a result that may be related to the high level of Bcl-x<sub>S</sub> expression, which is achieved using an adenoviral expression vector. In support of this interpretation are studies that indicate that MCF-7 cells transfected to express a low level of Bcl-x<sub>S</sub> maintain their viability in culture yet show enhanced chemotherapy-induced apoptosis (27). In contrast, the same cells show marked apoptotic death when high-level Bcl-x<sub>S</sub> expression is achieved using an adenoviral expression system (19).

The mechanism by which the bcl-x<sub>S</sub> adenovirus induced cell death is unknown. It has been shown that Bcl-x<sub>S</sub> fails to interact with Bcl-x<sub>L</sub> and Bcl-2 in vitro, suggesting that Bcl-x<sub>S</sub> promotes cell death independent of its physical interaction with Bcl-x<sub>L</sub> or Bcl-2. It is possible that Bcl-x<sub>S</sub> enhances a death signal from a cellular factor induced by the virus or a virus-encoded protein. The finding that the viability of pAdRSV-lac-z-infected cells was less than that seen in mock-infected control cells lends considerable support for this notion.

The pattern of DNA degradation and the morphological changes seen in the virally infected cells is consistent with death by apoptosis. Recently, several investigators have proposed that DNA fragmentation in apoptosis occurs in two stages, the first being initiated by a distinct, still-undescribed endonucleolytic activity, which cleaves DNA into 50–300-kb fragments, and a second stage catalyzed by a calcium-magnesium endonuclease (28, 29). Our data are in agreement with these findings and suggest that the virus may induce this primary critical endonucleolytic activity, which appears to be sufficient to cause cell death. These large DNA fragments can be explained on the basis of current knowledge of DNA structure. In both interphase nuclei and metaphase chromosomes, DNA is organized into supercoiled, looped domains of approximately 50–100 kb (30), and DNA sequences are nonrandomly organized in these chromosomal loops, with nontranscribed domains localized to the base of these loops, which are attached to the underlying matrix, called matrix-associated regions (reviewed in Refs. 31–33). These matrix-associated region sequences are A/T rich and contain Topo II sites, making it very likely that Topo II participates in binding of loops to the nuclear matrix (34). The appearance of these large fragments is thought to represent the release of loops of chromosomal DNA from their points of attachment to the underlying matrix and nuclear lamina. The sites and the source(s) of endonucleolytic activity induced by the virus are not known at present, but one can speculate that Topo II or other DNA-binding proteins situated at the base of the loops may be involved.

Our findings indicate that adenoviral expression of Bcl-x<sub>S</sub> in NB cells can induce apoptosis even in the context of high-level Bcl-2 and Bcl-x<sub>L</sub> expression. Importantly, Bcl-x<sub>S</sub> induces an onset of cell death that is rapid and complete. This finding suggests that adenoviral bcl-x<sub>S</sub> expression may be a useful therapeutic tool in the treatment of patients with NB, possibly as an <em>ex vivo</em> purging method prior to autologous bone marrow transplantation. Support for this notion is provided by studies indicating that human hematopoietic progenitor cells capable of repopulating the bone marrow of immunodeficient mice are resistant to killing initiated by the bcl-x<sub>S</sub> adenovirus (19). Future studies
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will need to address this possibility and to determine the precise mechanism by which Bcl-x5 induces DNA damage.

Fig. 4. Representative electron micrographs of SHEP-1 cells 24 and 72 h following infection with lac-z or bcl-x5 adenovirus. Apoptotic features could be detected as early as 24 h in bcl-x5-infected cells (B) but not lac-z-infected cells (not shown). By 72 h, the majority of cells infected with either virus (C and D) showed evidence of apoptosis. Arrow, chromatin condensation; • and arrow, cytoplasmic retraction; •• and arrow, nuclear membrane degeneration.
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