# Inhibition of Caspases Inhibits the Release of Apoptotic Bodies: Bcl-2 Inhibits the Initiation of Formation of Apoptotic Bodies in Chemotherapeutic Agent-induced Apoptosis

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*Abstract.* During apoptosis, the cell actively dismantles itself and reduces cell size by the formation and pinching off of portions of cytoplasm and nucleus as "apoptotic bodies." We have combined our previously established quantitative assay relating the amount of release of [$^3$H]-membrane lipid to the degree of apoptosis with electron microscopy (EM) at a series of timepoints to study apoptosis of lymphoid cells exposed to vincristine or etoposide. We find that the [$^3$H]-membrane lipid release assay correlates well with EM studies showing the formation and release of apoptotic bodies and cell death, and both processes are regulated in parallel by inducers or inhibitors of apoptosis. Overexpression of Bcl-2 or inhibition of caspases by DEVD inhibited equally well the activation of caspases as indicated by PARP cleavage. They also inhibited [$^3$H]-membrane lipid release and release of apoptotic bodies. EM showed that cells overexpressing Bcl-2 displayed near-normal morphology and viability in response to vincristine or etoposide. In contrast, DEVD did not prevent cell death. Although DEVD inhibited the chromatin condensation, PARP cleavage, release of apoptotic bodies, and release of labeled lipid, DEVD-treated cells showed accumulation of heterogeneous vesicles trapped in the condensed cytoplasm. These results suggest that inhibition of caspases arrested the maturation and release of apoptotic bodies. Our results also imply that Bcl-2 regulates processes in addition to caspase activation.

Key words:   apoptosis • bcl-2 • chemotherapy • electron microscopy • lipids

A POPTOSIS was recognized in the early 60's when Kerr and Wyllie described a form of cell death different from necrosis (Kerr and Harmon, 1991). Apoptosis is an important process in normal development, tissue homeostasis, immune responses and cancer pathogenesis and treatment. Apoptotic cells show distinct morphological features not found in necrotic cells, including membrane blebbing, chromatin condensation, and cell shrinkage (Kerr and Harmon, 1991). An important feature of apoptosis is the formation and release of apoptotic bodies: membrane bound remnants of varying sizes containing cellular organelles and/or fragments of chromatin. During the process of apoptosis, cells dismantle themselves by packaging cell remnants and condensed chromatin into apoptotic bodies that are recognized and engulfed by neighboring cells, thereby avoiding an inflammatory response characteristic of necrosis (Kerr and Harmon, 1991). Although formation of apoptotic bodies is accepted as a distinct feature of apoptosis, few studies coordinate biochemical and ultrastructural studies of the process of apoptosis.

Several activators and inhibitors of apoptosis have been studied, including Bcl-2 (Reed, 1997a) and the caspases (Cohen, 1997), whose activities regulate the execution of apoptosis. Bcl-2 is a 26-kD protein distributed in mitochondrial membranes, endoplasmic reticulum, and the nuclear envelope (Reed, 1994). Overexpressing Bcl-2 protects cells from apoptotic inducers including TNF-$\alpha$, $\gamma$ irradiation, serum deprivation and chemotherapeutic agents such as vincristine and etoposide (Reed, 1994, 1997a).

Caspases are a family of cysteine-directed proteases including 10 subgroups, each recognizing a distinct group of substrates. Caspase 3 (CPP32/YAMA/Apopain) is activated in chemotherapy-induced apoptosis (Cohen, 1997). Protein substrates of caspases include poly (ADP-ribosyl) polymerase (PARP), lamins, retinoblastoma protein (*Rb*), the 70-kD protein component of the U1 small ribonucleoprotein, fodrin, and actin (Cohen, 1997).

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In addition to Bcl-2 and caspases, studies have implicated mitochondrial function, ceramide and phospholipase $A_2$ (the enzyme that causes generation of arachidonic acid) in the regulation of apoptosis (Dennis, 1994). In a previous study evaluating the role of arachidonic acid (AA) and phospholipase $A_2$ in apoptosis (Zhang et al., 1998), we demonstrated that the release of [$^3$H]arachidonic acid (or [$^3$H]palmitic acid) during chemotherapy-induced apoptosis did not represent release of free fatty acid. Instead, release of [$^3$H]-label reflected the release of intact membrane lipids. The release of [$^3$H]-labeled membrane lipids, therefore, did not represent activation of the enzyme $PLA_2$ but probably represented release of membrane vesicles and fragments.

Using this release assay we demonstrate here that membrane lipid release is a regulated aspect of apoptosis in lymphoid cells. Chemotherapy-induced membrane lipid release is inhibited by Bcl-2 and by the caspase 3 inhibitor peptide, DEVD. In contrast, cell death is inhibited by Bcl-2 but not by DEVD. EM shows several stages of apoptosis and apoptotic body formation. EM demonstrates that Bcl-2 inhibits all stages of apoptosis. In contrast, the caspase inhibitor, DEVD, does not inhibit all morphologic aspects of apoptosis, but does inhibit the release of apoptotic bodies as measured by biochemical assay and by EM studies. Thus, coupling membrane lipid release assay with electron microscopy allows new insight into the process of apoptosis. These data implicate for the first time caspases in the blebbing and pinching off of apoptotic bodies and vesicles. The results also support a more generalized role for Bcl-2 in regulating apoptosis that transcends inhibition of caspases.

## Materials and Methods

### Cell Lines and Reagents

Pre-B leukemia cell line ALL-697 containing a mock vector (neo) or a Bcl-2 overexpression vector (Bcl-2) were kindly provided by Dr. John Reed (Burnham Institute, La Jolla, CA). T cell leukemia Molt-4 cells stably transfected with the vector carrying a hygromycin-resistant cDNA or a murine Bcl-2 overexpression vector were also used in this study (Zhang et al., 1996). Molt-4 and ALL-697 cell lines were maintained in RPMI 1640 medium supplemented with 2.5% Hepes buffer and 10% fetal bovine serum (GIBCO BRL). All experiments were conducted under serum-free conditions. Vincristine (VCR) and etoposide were purchased from Sigma Chemical Co. [$^3$H]Arachidonic acid and [$^3$H]palmitic acid were purchased from NEN™ Life Science Products.

### Measurement of Release of Radioactivity

Cells were seeded at $1.2 \times 10^6$ cells/ml in serum-free medium and labeled for 16 h with [$^3$H]-labeled free fatty acid (1.5 μCi/ml). The cells were washed twice with sterile PBS (Sigma Chemical Co.), seeded at $5 \times 10^5$ cells/ml in serum-free medium, and treated under various conditions as indicated in the text. The cells were collected in a microtube afterward and resuspended evenly. Sample of 200 μl was counted and labeled as total counts (A). The remaining cells in the microtube were pelleted at 2,000 $g$ in a micro centrifuge for 5 min. The supernatant (200 μl) was transferred to another scintillation vial, and the radioactivity was counted as the

amount of radioactivity release into the medium and termed B. The amount of release of radioactivity was expressed as percentage of B over A.

### Western Blot Analysis

Cells were seeded at $5 \times 10^5$/ml in serum-free medium. Cells of $1 \times 10^6$ were treated under conditions indicated in the text, and were collected and washed once with phosphate buffered saline (pH 7.4). These cells were resuspended in the sample buffer. The whole cell extracts were separated by 6% SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked with 5% dry milk and detected by anti-PARP antibody in a 1:2,000 dilution (Transduction Laboratories).

### Electron Microscopy

ALL-697 pre-B cell line with and without human Bcl-2 overexpression vector and Molt-4 T cells with and without murine Bcl-2 overexpression vectors were prepared for EM after 3, 6, 12, and 24 h of exposure to the selected apoptotic inducer. Controls were identical cells grown for the same length of time without the inducer. Cells were spun at the relatively high speed of 2,000 $g$ for 5 min to pellet cell fragments as well as intact cells (one set of experiments was conducted using a low speed spin just sufficient to pellet the cells). Cell pellets were immediately fixed at room temperature in 3% glutaraldehyde tousimis, 0.2% tannic acid (Electron Microscopy Sciences) in 10 mM MOPS (3-[$N$-Morpholino]propanesulfonic acid) buffered mammalian ringer, pH 7.2 for 30 min. After rinsing in buffered ringer and then in $PO_4$ buffer, cells were post fixed in 1% $OsO_4$ (100 mM $K_3PO_4$, 10 mM $MgCl_2$, pH 6.1) ice-cold for 20 min. Cells were block-stained with 2% aqueous uranyl acetate for 30 min at room temperature after rinsing twice in water, and dehydrated in a graded ethanol series 50–100% and embedded in Araldite 506 (Reedy et al., 1988; Novak et al., 1995). Sections were cut with a diamond knife on a Reichert OMU3 ultramicrotome and stained with $KMnO_4$ and lead (Reedy et al., 1988) and photographed on a Philips EM 420.

### Trypan Blue Exclusion Experiments

Cells were seeded at $5 \times 10^5$ cells/ml in RPMI 1640 serum-free medium. After the indicated times of treatments under conditions, as indicated in the text, cells were resuspended in 10 μl PBS, diluted at 1:1 ratio with Trypan Blue solution (0.4%; Sigma Chemical Co.). The dead and live cells were counted using a hemocytometer.

## Results

### Caspase Inhibitors Inhibit Chemotherapeutic Agent-induced Release of Membrane Lipid Release

To study the regulatory mechanism of chemotherapy-induced apoptosis, we took advantage of the membrane lipid release assay (Zhang et al., 1998). Pre-B cell leukemia ALL-697 cells were prelabeled overnight with [$^3$H]palmitate and treated with etoposide (10 mM), alone or together with either 60 μM Z-Asp-Glu-Val-Asp-$CH_2$F (DEVD; an irreversible inhibitor of caspase 3 and related caspases), or the same concentration of N-Tyr-Val-Ala-Asp-aldehyde (YVAD; a reversible inhibitor of caspase 1 and related caspases). Fig. 1 A demonstrates that etoposide induced release of [$^3$H]palmitate in the form of membrane lipid such that by 12 h there was more than twofold increase of release of radioactivity as compared with control cells. In contrast, in the presence of DEVD, the etoposide-induced release of radioactivity was inhibited throughout the time course evaluated, and remarkably, this inhibition continued up to 72 h of etoposide treatment. The inhibitor of caspase 1, YVAD, could not effectively inhibit etoposide-induced release of labeled membrane lipid in ALL-697
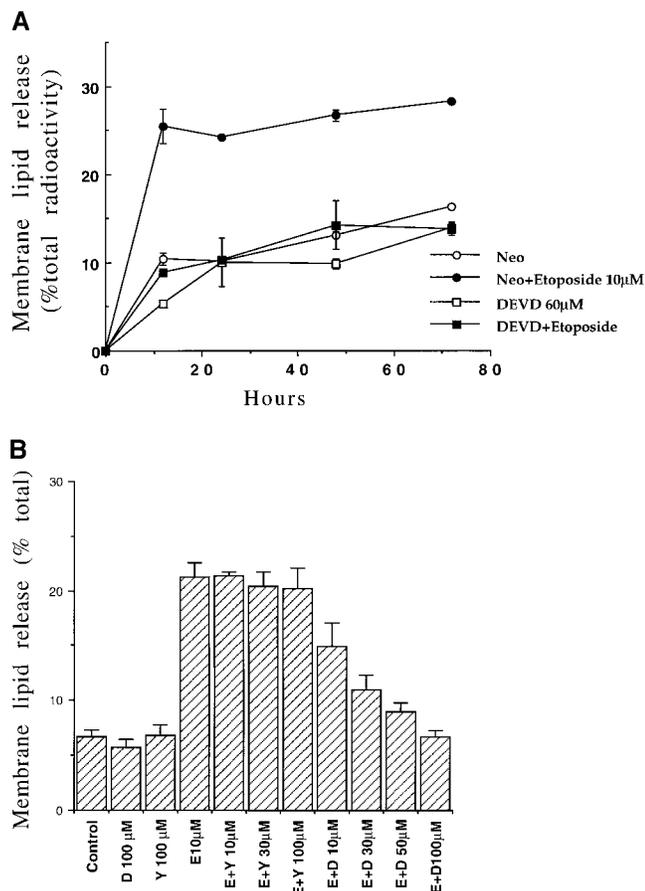
## A



## B



*Figure 1.* The caspase inhibitor DEVD prevents membrane lipid release induced by etoposide. ALL-697 cells were seeded at $1.2 \times 10^6$ cells/ml in serum-free RPMI 1640 medium. These cells were pre-labeled with [$^3$H]palmitic acid (see Materials and Methods). The pre-labeled cells were seeded at $5 \times 10^5$ cells/ml in serum-free medium, and treated under various conditions. At the indicated time, samples (including medium and cells) were collected and the released radioactivity was measured following the method described in Materials and Methods. (A) Time course of etoposide treatment. (B) Dose response of inhibitors DEVD and YVAD.

cells even at the earliest time point (12 h). We used both reversible and irreversible forms of DEVD and YVAD in this assay. Both forms of DEVD were inhibitory, whereas neither form of YVAD was inhibitory (data not shown).

To establish the effective concentration for the caspase inhibitors, we conducted a dose response study using varying concentrations of either DEVD or YVAD on the release of membrane lipids, as an indicator of release of apoptotic bodies. DEVD or YVAD ranging from 1 μM to 100 μM were used. Fig. 1 B demonstrates that etoposide treatment (10 μM) induced release of membrane lipids within 6 h. The addition of YVAD up to 100 μM had no effect on the release of membrane lipids. In contrast, the addition of DEVD effectively inhibited the release of membrane lipids at a concentration as low as 10 μM. At 50 μM, the release of membrane lipid was completely inhibited (Fig. 1 B). Thus, in the following experiments, we

chose 60 μM as our effective concentration of caspase inhibitor on the regulation of apoptosis induced by chemotherapeutic agents.

These results indicate that the release of membrane lipids in chemotherapeutic agent-induced apoptosis could be effectively inhibited by DEVD, the inhibitor of caspase 3.

### Caspase Inhibitors, Unlike Bcl-2, Cannot Prevent Cell Death

In an effort to understand the relationship between the release of labeled membrane lipids and cell death, we next evaluated cell viability by Trypan Blue exclusion analysis. ALL-697 cells transfected with Bcl-2 or with a vector carrying the neomycin gene (neo) were evaluated for viability in response to etoposide (10 mM). Fig. 2 demonstrates that cells overexpressing Bcl-2 no longer proliferated in the presence of etoposide, but these cells were alive even up to 72 h of etoposide treatment, as no dead cells were present upon Trypan Blue exclusion analysis. In contrast, cells transfected with vector control started to die as early as 6 h, and by 24 h all the cells were dead.

On the other hand, despite the fact that DEVD blocked membrane lipid release, it was unable to prevent cell death. ALL-697 cells pretreated with DEVD (60 μM) and then with etoposide initially displayed a slower rate of cell death as measured by Trypan blue exclusion assay, but by 24 and 48 h cell death increased to the same level as the cells that had not been exposed to DEVD (Fig. 2).

To demonstrate that cell death in response to etoposide in the ALL-697 was indeed apoptotic and was mediated by caspases, we next evaluated cleavage of the caspase 3 substrate poly (ADP-ribose) polymerase (PARP). PARP was cleaved from the intact 116-kD protein into the expected 85-kD proteolytic fragment after treatment with etoposide at 12 h (Fig. 3). As expected, cells overexpressing Bcl-2 were protected from etoposide-induced PARP cleavage, consistent with the lack of cell death seen in Fig. 2. Similarly, the addition of DEVD almost completely protected cells from PARP cleavage by 12 h, and significantly protected cells from PARP cleavage by 48 h. Under the same
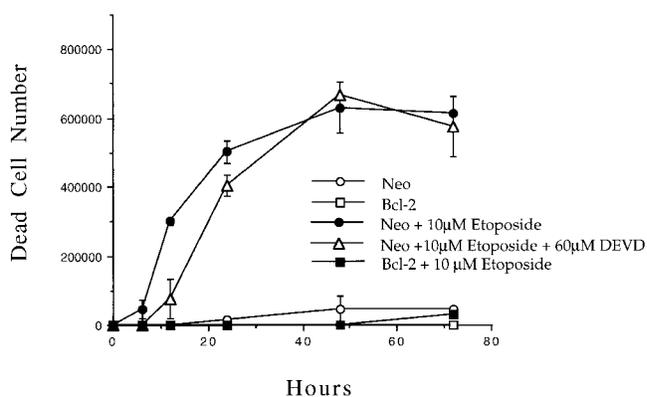


*Figure 2.* DEVD, unlike Bcl-2, does not prevent loss of viability by etoposide treatment. ALL-697 cells were seeded at $5 \times 10^5$ cells/ml in serum-free RPMI 1640 medium. These cells were treated as described in the text, and the loss of cell viability was measured using Trypan Blue exclusion assay.
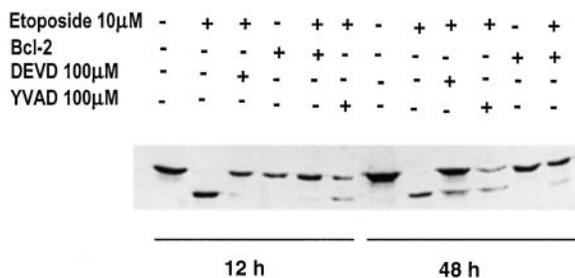
| Etoposide 10μM | - | + | + | - | + | + | - | + | + | + | - | + |
| Bcl-2 | - | - | - | + | + | - | - | - | - | - | + | + |
| DEVD 100μM | - | - | + | - | - | - | - | - | + | - | - | - |
| YVAD 100μM | - | - | - | - | - | + | - | - | - | + | - | - |



12 h          48 h

*Figure 3.* DEVD prevents PARP cleavage for up to 48 h. ALL-697 cells were seeded at $6 \times 10^5$ cells/ml in serum-free RPMI 1640 medium. These cells were treated under various conditions indicated in the text. At the indicated times the cells were harvested and suspended in 40 μl homogenizing buffer (50 mM Tris-HCl, pH 7.4, 20 mM EDTA, pH 7.4; 10 mM EGTA, and 20 μg/ml PMSF). These cells were resuspended in $2\times$ sample buffer, boiled for 10 min, and loaded on 7.5% SDS-PAGE. The Western blot analysis was performed using rabbit anti–human PARP antibody.

condition, the cells were beginning to die by 12 h, and were completely dead by 48 h. These data confirm that the activation of caspase 3 and the related enzymes were significantly inhibited by DEVD, but DEVD only slightly delayed but did not protect from cell death. Since DEVD inhibited membrane lipid release, these results also imply that caspase 3 activity is required in the release of membrane lipids.

Bcl-2 inhibits the process that initiates apoptotic body formation whereas DEVD, the inhibitor of caspase, arrests the subsequent maturation and release of apoptotic bodies in chemotherapeutic agent-induced apoptosis.

To gain understanding of the processes involved in apoptotic body formation and release, we next used EM to identify the structures and materials associated with these processes. Using high speed centrifugation (10,000 *g* for 30 min), we pelleted all the components released into the medium from either control or etoposide-treated cells. Our previous studies showed that under high speed centrifugation, more than 80% of the radioactivity released from the apoptotic cells could be pelleted. Thin section EM on the 10,000 *g* pellet showed heterogeneous vesicles, membrane fragments, condensed chromatin, and small debris containing some recognizable portions of mitochondria and parts of other organelles (Zhang et al., 1998). Hardly any of these components were seen in the high speed pellet from the control cells, or in Bcl-2 overexpressing cells treated with etoposide. These studies indicated that the released membrane lipid was probably incorporated into these heterogeneous cellular fragments.

We next processed whole cells for EM in an effort to capture and visualize the several stages in the process of apoptotic body formation, and gain insight into the mechanism by which membrane lipids are released. ALL-697 and Molt-4 cells carrying an empty vector or a Bcl-2 overexpressing vector were fixed at a series of time points with and without exposure to several apoptotic inducers. After treatment, whole cells were centrifuged at 12,000 *g* for 5 min and the cell pellet was fixed. In one experiment, we used 2,000 *g* for 5 min (just sufficient to pellet the cells)

and in all other experiments, we centrifuged cells at 12,000 *g* to ensure that as much of the material released from the cells as possible would be brought down with the whole cell pellet.

Consistent with the [³H]-membrane lipid release assay, the extracellular space around cells treated with either etoposide or vincristine showed grossly more debris, membrane profiles, and vesicles than the untreated cells. Cells undergoing apoptosis also showed membrane blebbing (Figs. 4 B and 5 B). The apoptotic cells were different from the untreated cells (Figs. 4 A and 5 A) in the following aspects: (a) The cytoplasm of apoptotic cells contained heterogeneous vesicles in significantly greater abundance than the control cells. The vesicles were of varying sizes and of varying content; luminal profiles varied from empty to dense, amorphous to particulate. Frequently, the contents of vesicles appeared to be in the process of being released from the apoptotic cells (Figs. 4 B and 5 B). There was a class of lysosome-like vesicles (Fig. 6 A, arrowhead) that were not seen in the untreated cells (Figs. 4 A and 5 A). (b) At early time points in apoptotic cells, the mitochondria were not disrupted, but at later time points, they appeared to be in various stages of involution. In some cases, it was clear that mitochondria were being converted to mid-size vesicles containing an amorphous substance of medium density (Fig. 4 B). (c) The release of vesicles (Figs. 4 B, 5 B, and 6 C) and the formation of apoptotic bodies (Fig. 6 A) were evident. Some of these cells were clearly pinching off portions of themselves (Fig. 6 A). These budding portions fit all the descriptions of apoptotic bodies that include cytoplasm, vesicles, and fragmented nucleus. At early time points (3 h, Fig. 6 B) after exposure to apoptotic inducers, the majority of the cells showed irregular shapes and the nuclei were polymorphic (lobulated). Although the nuclear chromatin was denser than control cells, complete condensation of chromatin was seen more frequently at later time points (Figs. 5 B and 6 C). By 6 h of exposure to 10 μM etoposide, many cells showed completely condensed chromatin with no visible sub-structure and the surrounding cytoplasm was pale and granular, without organelles (Fig. 6 C). These cells were classic apoptotic cells.

Cells overexpressing Bcl-2 (Figs. 4 C and 5 C) responded differently to inducers of apoptosis from untreated cells (Figs. 4 A, 5 A, and Table I). Consistent with the experiments on release of [³H]-membrane lipids, there were no apoptotic bodies formed in the Bcl-2–transfected cells. The extracellular space around the treated Bcl-2 cells was clean, with no heterogeneous vesicles, organelle debris, or electron dense substances. There was no recognizable change in the morphology of the nucleus compared with the control cells, i.e., chromatin condensation was inhibited completely, consistent with previous reports (McCarthy et al., 1997). However, some large vesicles with dense contents that appeared similar to lysosomal vesicles, seen in apoptotic cells, also appeared in the Bcl-2 cells exposed to vincristine or etoposide (Figs. 4 C, 5 C, and Table I). The overall morphology of Bcl-2 cells under vincristine and etoposide treatment was similar to that of untreated Bcl-2 cells and untreated control cells.

The effects of the protease inhibitor DEVD on the morphology of treated cells were quite different from that of

Bcl-2 overexpression. We treated the cells with DEVD alone or in combination with vincristine or etoposide. In cells treated with DEVD alone, the cells looked identical to the control cells (data not shown). When the cells were treated with vincristine or etoposide in the presence of DEVD (Figs. 4 D and 5 D), their morphology changed dramatically. The extracellular space around the cells treated with chemotherapeutic agent plus DEVD was much cleaner than the cells treated with chemotherapeutic agent alone. Also, there were no broken vesicles surrounding the cell membrane. The major features of a typical response were: (a) Many cells treated with vincristine and DEVD became shrunken and had dark, condensed cytoplasm. The cells showed membrane-bound finger-like projections. (b) Abundant accumulation of various vesicles crowded the condensed cytoplasm, suggesting that these vesicles were trapped inside the cells. (c) The caspase inhibitor DEVD did prevent complete condensation of chromatin into dense pools; chromatin in the nuclei remained recognizable as dark masses against a somewhat lighter background. There was no fragmented chromatin in the presence of DEVD. However, contrary to what was expected, nuclei and chromatin appeared more dense and compacted than normal (Table II and Figs. 4 D and 5 D vs. Figs. 4 A and 5 A).

The great density and shrinkage of the DEVD cells and the crowding of the remaining cytoplasm with accumulations of various vesicles, and the lack of apoptotic bodies in the extracellular space suggested that cells were beginning the process of formation of apoptotic bodies. How-
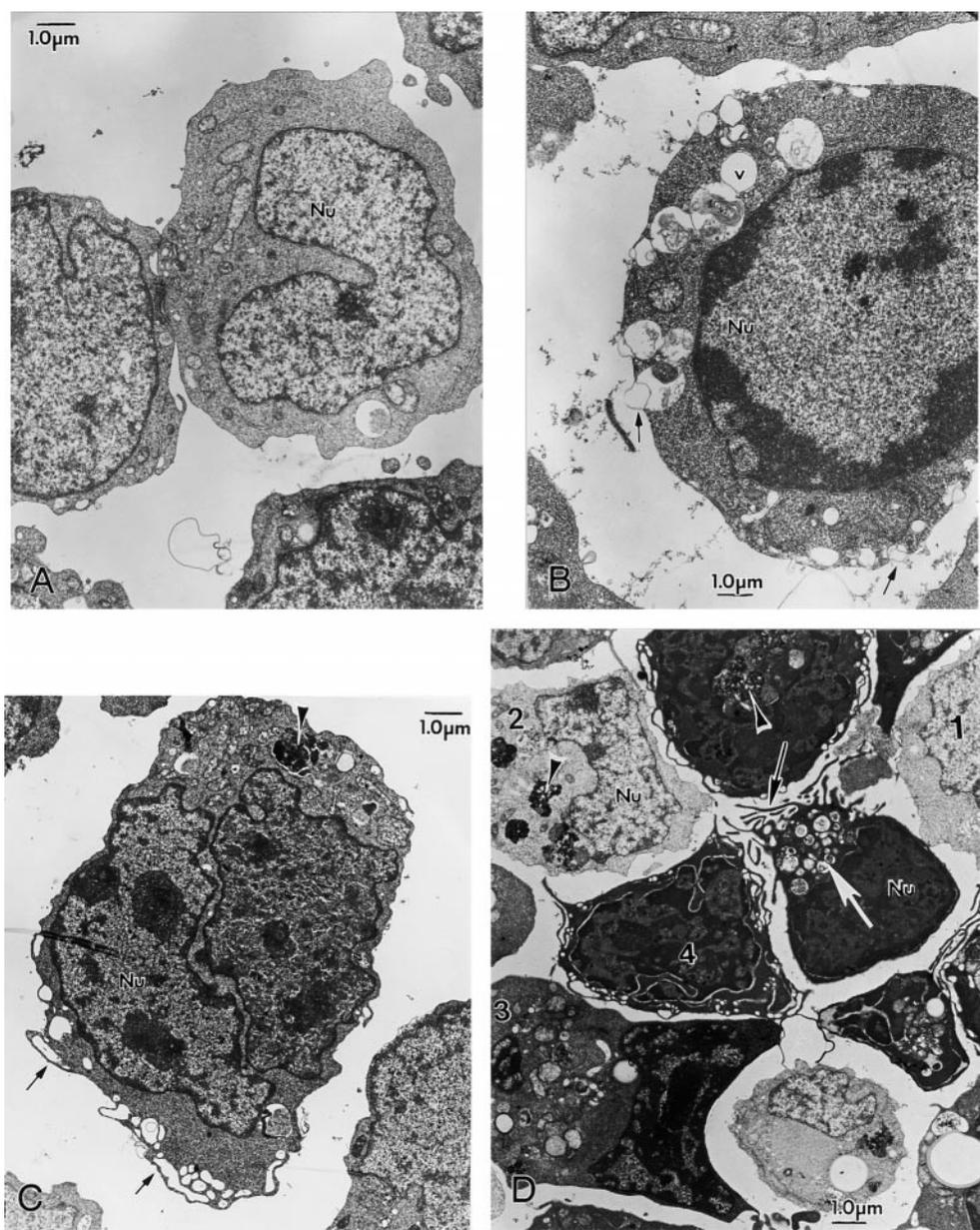


*Figure 4.* Electron micrographs comparing the effects of DEVD and Bcl-2 on the morphological changes of ALL-697 cells treated with chemotherapeutic agent. (A) Untreated ALL-697 cells show very few vesicles, a rather uniform rounded shape and no condensation of nuclear chromatin. Typically, the extracellular space shows little debris. (B) ALL-697 cells treated with 1 μM vincristine for 6 h. A typical apoptotic cell showing some chromatin condensation around the periphery of the nucleus and numerous vesicles, several of which appear to be releasing to the extracellular space (arrows). (C) After 6 h of 1 μM vincristine treatment, ALL-697 Bcl-2 cells show only a few lysosomal-type vesicles (black and white arrowhead) in the cytoplasm, and some cells show clear vesicles at the cell surface (arrows); in contrast to the releasing vesicles in apoptotic cells (B), these cells have a relatively thick strand of cytoplasm still separating them from the extracellular space. (D) ALL-697 cells treated with 1 μM vincristine in the presence of DEVD (60 μM). Morphologic changes include partial condensation of chromatin and a striking accumulation of various vesicles in the cytoplasm. The overall condensation of both cytoplasm and nucleus caused shrinkage of cell size compared with the untreated cells. Heterogeneous vesicles are present and dense lysosomal vesicles are seen in cells labeled 2 as well as cells that are very dark and shrunken labeled 3 and 4. Note that the cell surface shows finger-like projection and the extracellular space is relatively clean.

ever, the inhibition of caspase activation apparently arrested the formation of apoptotic bodies in a intermediate stage (Figs. 4 D and 5 D). This "intermediate stage" of apoptosis distinguished itself from other stages of apoptotic body formation by the apparent intracellular accumulation of various vesicles in the condensed cytoplasm. Comparison of Figs. 4 B vs. 4 D and 5 B vs. 5 D, together with our studies on membrane lipid release (Zhang et al., 1998) suggested that the heterogeneous vesicles that appear to accumulate in the cytoplasm of the cells treated with DEVD and chemotherapeutic agents are possible precursors of apoptotic bodies normally released in the extracellular space.

The regulatory role of DEVD on the formation of apoptotic bodies suggested to us that the formation of apoptotic bodies is a multi-staged process. Thus, we sequentially investigated the stages of apoptotic body formation by studying the cellular morphological changes at various time points (3, 6, and 24 h) in response to treatment of chemotherapeutic agents (Fig. 6). The various stages of apoptotic body formation can be best illustrated in Fig. 6

A (stage 1 though stage 4). The early stage of apoptotic body formation is characterized by the initial condensation of chromatin (Fig. 6 A, labeled 1). In this initial phase, the overall structure of chromatin is still recognizable, although compared with the normal nucleus (Fig. 4 A), the chromatin is already somewhat condensed. The cell membrane is intact at this stage. The next stage (Fig. 6 A, labeled 2) shows the intermediate stage of apoptotic body formation, where various vesicles are formed and their contents are being released into the extracellular space, together with the striking condensation of chromatin. Clearly, the activation of caspases is required in the intermediate step between the formation of heterogeneous vesicles and the release of apoptotic bodies. The final stages of apoptotic body formation are illustrated in Fig. 6 A (labeled 3 and 4), where the cells appear as ghost structures in which all vesicles and apoptotic bodies have been released and the cytosol is uniformly granular and only a small dense pool indicates the former nucleus. At this stage, the extracellular space is littered with granular ghosts of cells that completed apoptosis at earlier times. In fact, at this late
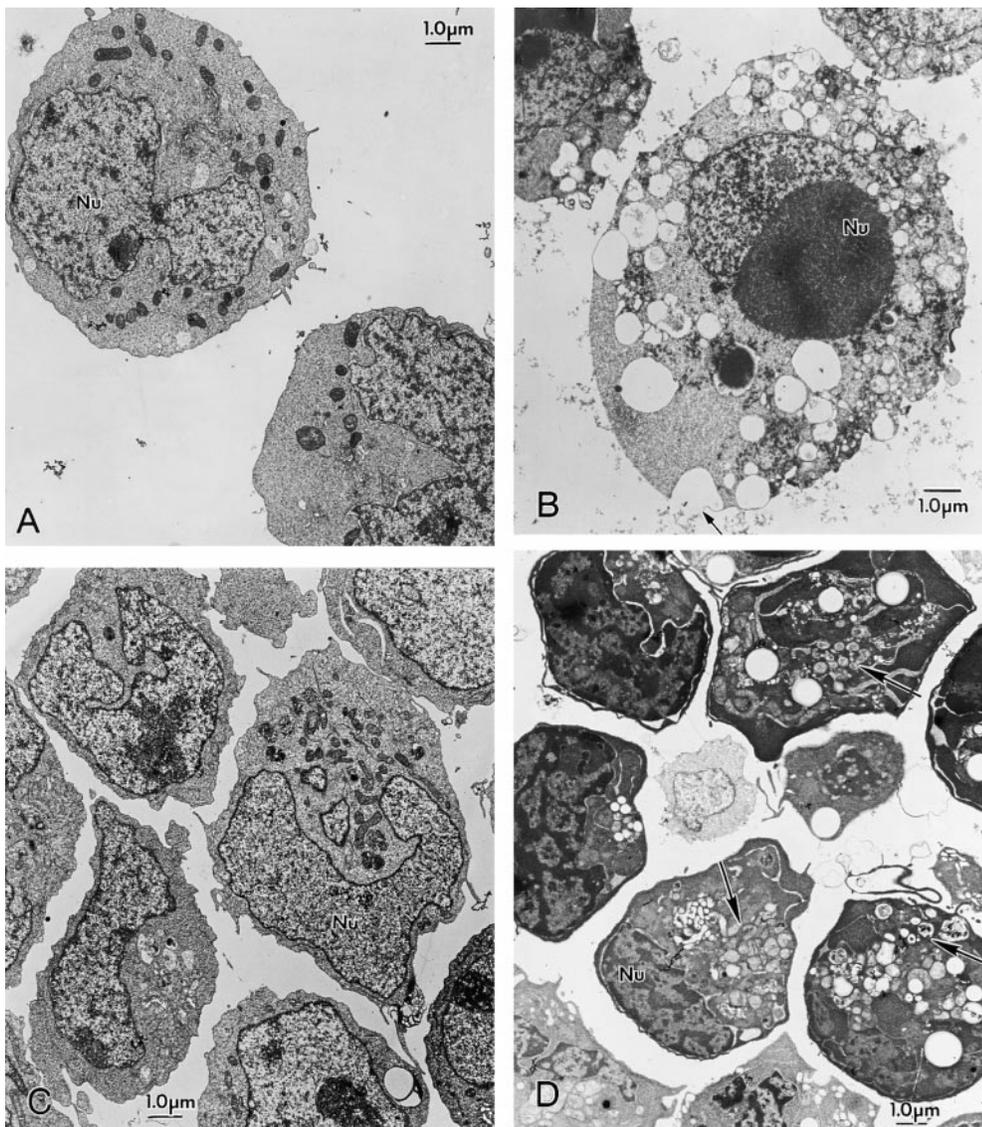


*Figure 5.* Electron micrographs comparing the effects of Bcl-2 and DEVD on the morphological changes of Molt-4 T cells to etoposide. (A) Untreated Molt-4 cells display a rather uniform rounded shape and no condensation of chromatin. There were very few vesicles in the cytoplasm of the cells. Typically, the extracellular space shows little debris. (B) Molt-4 T cells after 14 h exposure to etoposide show many vesicles, some of which are releasing to the medium and a classic apoptotic nucleus with completely condensed chromatin. (C) After 14 h exposure to etoposide, Molt-4 Bcl-2 cells appear identical to untreated control cells. Note that the extracellular space is clean. (D) Caspase inhibition produces strikingly condensed cells overall, but nuclear chromatin is only partly condensed (compare to cells in B), the remaining cytoplasm is crowded with vesicles (arrows). Finger-like projections are prominent at the cell surface.

*Table I. Morphometric Analysis of EM Data*

| | Vector | | | | Bcl-2 | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | Vcr (6 h) | Vcr (12 h) | Vcr (24 h) | Control | Vcr (6 h) | Vcr (12 h) | Vcr (24 h) |
| Number of cells counted | 23 | 50 | 18 | 39 | 14 | 54 | 33 | 23 |
| Number of apoptotic cells | 0 | 20 | 13 | 37 | 0 | 0 | 0 | 0 |
| % Apoptotic cells | 0 | 40 | 72 | 95 | 0 | 0 | 0 | 0 |
| Number of vesicles counted | 47 | 367 | 210 | All released | 39 | 133 | 84 | 49 |
| Vesicles per cell | 2.04 | 7.34 | 11.67 | | 2.78 | 2.46 | 2.50 | 2.13 |
| Number of apoptotic bodies | 0 | 34 | 35 | 74 | 0 | 0 | 0 | 0 |
| Apoptotic bodies per cell | 0 | 0.68 | 1.94 | 1.90 | 0 | 0 | 0 | 0 |

Time course of Vcr (1 μM) meanment of ALL-697 cells stably transferred with vector or Bcl-2.

stage, the cells resembled those described by Wyllie and Kerr as "secondary necrosis," probably since "in vitro" the dead cells are not absorbed by surrounding cells. These stages of apoptosis are also illustrated in the time course of treatment (Fig. 6, B–D). Fig. 6 B shows the typical cell morphology after 3 h of etoposide treatment, as chromatin condensation has already started at this time (arrow). After 6 h of etoposide treatment, various vesicles have formed in the cytoplasm, combined with the striking condensation of chromatin (Fig. 6 C). After 24 h of vincristine treatment (Fig. 6 D) or etoposide (data not shown), apoptotic cells have completed the release of apoptotic bodies, and the ghost structure of cells are the only remnants.

An interesting observation is that cells that had entered mitosis appeared not to be affected by vincristine, since after 24 h of vincristine treatment, the only surviving cells were those that had entered mitosis (Fig. 6 D). These surviving mitotic cells were numerous in vincristine-treated cells, but appeared far fewer in the presence of etoposide, possibly as a result of the effects of vincristine on $G_2/M$ progression.

Comparing the response of cells overexpressing Bcl-2 to cells treated with DEVD suggested to us that multiple factors regulate the initiation, maturation, and release of apoptotic bodies. The overexpression of Bcl-2 totally inhibited the formation of apoptotic bodies. Compared with control cells, the Bcl-2 cells appeared normal even under electron microscopy after exposure to vincristine and etoposide for extended times. There was no accumulation of vesicles or fragmentation of chromatin in Bcl-2 cells in the presence of chemotherapeutic agents, as an early indicator of the formation of apoptotic bodies. More importantly, treated

Bcl-2 cells maintained their viability for a long time. On the other hand, the inhibitor of caspases could not totally reverse the process of the formation of apoptotic bodies. However, DEVD interfered with the later stage of apoptotic body formation by inhibiting the release of apoptotic bodies, leading to the accumulation of various vesicles inside the cells and somewhat condensed chromatin. Therefore, the activation of caspases appears to be a necessary step in the subsequent maturation and release of apoptotic bodies.

Overall, our studies demonstrate that formation of apoptotic bodies is a multi-staged process, characterized by the appearance of heterogeneous vesicles as the possible precursor of apoptotic bodies. Bcl-2 and the inhibitor of caspases differentially regulate the process of apoptotic body formation. Overexpression of Bcl-2 protein acts upstream of apoptotic body formation by completely inhibiting the initiation of this process, whereas the inhibition of caspase only inhibited the later stage of apoptotic body release.

## Discussion

This study demonstrates that the formation of apoptotic bodies is a multi-staged process that is regulated differentially by Bcl-2 and caspases. Since the results clearly distinguish the action of Bcl-2 from that of caspases, they suggest that the effects of Bcl-2 transcend the regulation of caspases. In essence the results argue against proposed unitary pathways whereby Bcl-2 functions primarily to regulate caspases.

This study reveals several differences between Bcl-2 and

*Table II. Morphometric Analysis of EM Data for Effects of DEVD on ALL-697 Cells Treated with Etoposide*

| | Control | Etoposide (3 h) | Etoposide + DEVD (3 h) | Etoposide (6 h) | Etoposide + DEVD (6 h) |
|---|---|---|---|---|---|
| Number of cells counted | 100 | 53 | 64 | 100 | 44 |
| Number of apoptotic cells | 0 | 23 | 21 | 76 | 31 |
| % Apoptotic cells | 0 | 43 | 33 | 76 | 70 |
| Number of vesicles counted | 52 | 227 | 284 | 417 | 670 |
| Vesicles per cell | 0.52 | 4.28 | 4.44 | 4.17 | 15.23 |
| Number of apoptotic bodies | 0 | 12 | 4 | 238 | 14 |
| Apoptotic bodies per cell | 0 | 0.23 | 0.06 | 2.38 | 0.32 |
| Cytosolic density* | 61 ± 8 | 121 ± 5 | 130 ± 7 | 87 ± 8 | 124 ± 14 |
| Chromatin density* | 61 ± 6 | 123 ± 4 | 155 ± 7 | 141 ± 5 | 136 ± 14 |

*Arbitrary units based on analysis using NIH Image Software: The EM pictures were scanned into TIFF image, 30 areas were randomly selected in the nucleus and cytosol of the chromatic condensed cells and their average density measured. The results are the averages of these numbers with standard error of mean.

caspases in regulating apoptosis. First, Bcl-2 prevents the loss of viability in chemotherapy-induced apoptosis, but inhibitors of caspases do not. Second, Bcl-2 prevents most of the manifestation of apoptosis at the ultrastructural level, as shown by the near normal appearance of cells in response to chemotherapeutic agent-treatment whereas caspase inhibitors fail to inhibit many features of apoptosis, including cell volume loss and chromatin condensation. Third, Bcl-2 prevents the formation of apoptotic bodies completely, whereas caspase inhibitors do not interfere with the initial process that lead to apoptotic body forma-

tion, but DEVD clearly inhibits release of apoptotic bodies and vesicles.

These results have several important implications in current research on apoptosis. EM studies reveal several phases of apoptosis and apoptotic body formation. Upon induction of apoptosis, the cells enter the initial phase of apoptotic body formation by the beginning of the condensation of chromatin. In this initial phase, the overall structure of chromatin is still recognizable, although compared with the normal nucleus, the chromatin is already somewhat condensed. The cells enter the intermediate stage of
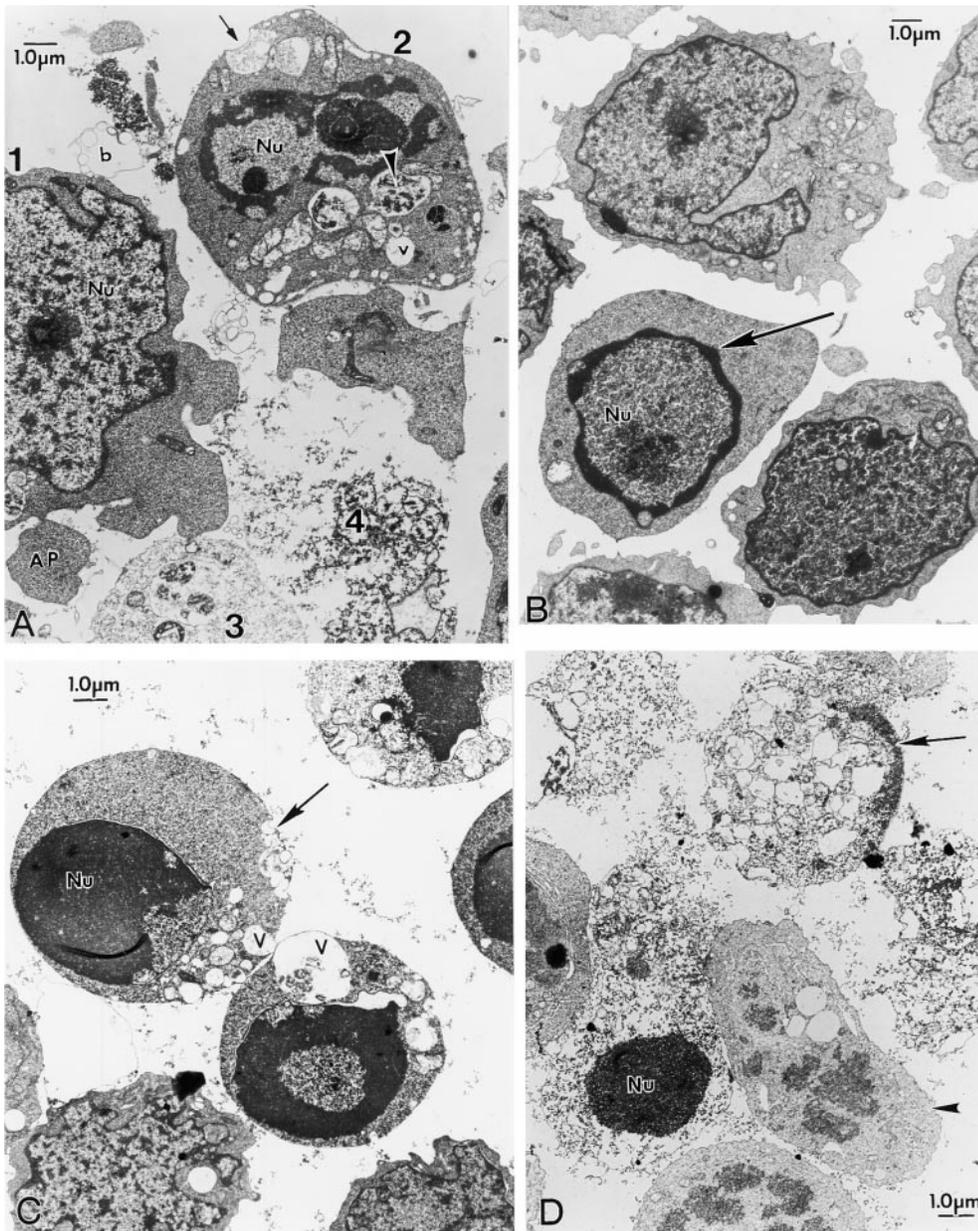


*Figure 6.* The various phases of apoptotic body formation in chemotherapeutic agent-treated ALL-697 cells. (A) ALL-697 cells were treated with 10 μM etoposide for 6 h. Various phases of the formation of apoptotic bodies are evident in this micrograph and are labeled 1–4. The cell labeled 1 is at an early stage and shows very minimal chromatin condensation in the nucleus (Nu), but an apoptotic body (AP) can be seen budding off and another is probably forming adjacent to it. The cell labeled 2 is at an intermediate stage and shows partially condensed chromatin in the nucleus, apoptotic body formation and many heterogeneous vesicles (V). The black and white arrowhead points to a vesicle with dense contents that suggests lysosomal origin. The small arrow points to one vesicle that appears poised to open and release to the extracellular space, which is full of membrane profiles and debris, characteristic of cells exposed to apoptotic inducers. The cells labeled 3 and 4 have completed apoptosis and are dispersing as granular debris (the ghost cell stage). This images clearly demonstrates that induction of apoptosis is asynchronous. (B) The typical morphological changes of the cells after 3 h treatment with 10 μM etoposide. The arrow indicates the beginning of chromatin condensation at the periphery of the nucleus. Of note is that at the early phase of apoptotic body formation, there is not much accumulation of vesicles in the cells. (C) The typical morphological changes of the cells after 6 h treatment with 10 μM etoposide (middle stage). At this stage, the chromatin condensation is very striking, with various vesicles (V) forming and releasing their contents into the extracellular space (arrow). At this stage, the cells are still intact. (D) Typical morphological changes of the cells after 24 h treatment with 1 μM vincristine (latest stage). At this stage, the cellular contents are totally released from the cells, with only ghost cells (arrow) and totally condensed chromatin remaining. Nu, condensed nuclear remnant of ghost cell. Black arrowhead indicates surviving cell in mitosis.
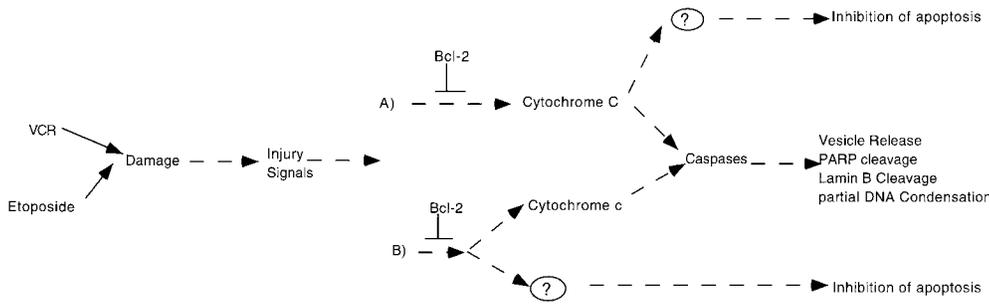
*Figure 7.* Proposed scheme of Bcl-2 regulation of chemotherapy-induced apoptosis.

apoptotic body formation by forming heterogeneous vesicles in the cytoplasm and release these vesicles into the extracellular space. By this time, the chromatin is condensed to such an extent that no structure could be recognized. The nucleus is fragmented into several pieces. In the final stage of apoptosis, the cells appear as ghost structures in which all vesicles and apoptotic bodies have been released and the cytosol is uniformly granular and only a small dense pool indicates the former nucleus.

This three-phase apoptotic process is regulated at least at two levels: Bcl-2 prevents the initiation of the first phase, whereas caspase inhibitors do not interfere with the initiation of the apoptotic program leading to the accumulation of precursor vesicles, but do prevent the release of apoptotic bodies from apoptotic cells (Fig. 7).

Our study also shows that Bcl-2, compared with DEVD, acts at a proximal step in the apoptotic pathway and that Bcl-2 can maintain cell viability, although it fails to maintain the cells' ability to proliferate. Therefore, Bcl-2 acts upstream of the "commitment site" in apoptosis. In contrast, activation of caspases in chemotherapy-induced apoptosis acts at a point downstream of the commitment step, as inhibition of caspase activity cannot prevent loss of cell viability.

This divergence of the action of Bcl-2 and caspases raises interesting questions about the mechanism of action of Bcl-2 and its relation to caspases: although Bcl-2 has been studied extensively in apoptosis, the exact point at which Bcl-2 works to regulate the apoptotic program is still poorly defined. Bcl-2 has been found to interfere with the apoptotic pathway at a point upstream of caspases, inhibiting caspase activation upon induction of apoptosis (Yuan, 1995). Activation of caspases could also be initiated by cytochrome c, another important regulator of apoptosis (Reed, 1997b). Recent studies suggest that during apoptosis, cytochrome c is released from the mitochondria to the cytosol, and that the increased accumulation of cytochrome c in the cytosol triggers caspase activation. In addition, based on in vitro studies (Li et al., 1997), it was shown that cytochrome c could trigger caspase activation directly by forming a protein complex with Apaf 1 (the human homologue of ced-4), and Apaf 3 (caspase 9; Li et al., 1997). Overexpression of Bcl-2 has been shown to prevent the release of cytochrome c in apoptosis (Yang et al., 1997; Kluck et al., 1997), implying that Bcl-2 acts upstream of the release of cytochrome c in apoptosis. Thus, it has been proposed that Bcl-2 acts primarily to inhibit the release of cytochrome c from mitochondria and the consequent activation of caspases (Kluck et al., 1997; Yang et al., 1997). These and other results have led to the hypothesis that

caspases are the primary executors of apoptosis and that Bcl-2 functions primarily to interfere with caspase activation. The results from our study argue against this simplified model for the regulation of apoptosis, at least in chemotherapy-induced apoptosis in lymphoid cells. Whereas Bcl-2 prevents chemotherapy-induced death, DEVD only inhibits a subset of the manifestations of apoptosis. Thus, Bcl-2 not only acts upstream of caspases, but must also act upstream of other non-caspase effectors of the apoptotic program. Our results are compatible with either of two scenarios: Bcl-2 may function primarily to inhibit cytochrome c release. In this case, the release of cytochrome c causes both activation of caspases required for release of apoptotic bodies and activation of other unknown effectors involved in initiation of the apoptotic program (the early phases of apoptotic body formation; Fig. 7 A). Alternatively, Bcl-2 may inhibit a distinct pathway that activates early events in apoptosis (Fig. 7 B) in addition to inhibiting activation of caspases by cytochrome c (Kluck et al., 1997; Yang et al., 1997).

This study also raises some important considerations as to the role of caspases in chemotherapy-induced apoptosis: (a) caspase inhibitors do not prevent cell death in response to etoposide or vincristine, but they inhibit some of the manifestations of cell death such as PARP cleavage, the release of membrane lipids as the indication of pinching off apoptotic bodies and vesicle release, and partially inhibit the condensation of chromatin. This suggests that caspases are restricted to an execution role. (b) An initial regulatory role for caspases in chemotherapy-induced apoptosis is unlikely to be very significant, as once caspases are activated, the cells have already passed the commitment step in apoptosis. For example, inhibitors of caspases are not expected to ameliorate chemotherapy induced toxicity and side effects.

While studying the role of caspases in apoptosis induced by Bak and DNA-damaging agents, McCarthy et al. (1997) showed that the caspase inhibitors ZVAD.fmk and BD.fmk could inhibit several aspects of cell death but could not inhibit membrane blebbing. The failure to inhibit membrane blebbing caused eventual cell death. In our studies, we have found that DEVD inhibits cellular blebbing effectively (Figs. 4 D and 5 D). It is possible that this difference is due to using different cells (they used Rat-1 fibroblasts where as we used leukemia cells). Therefore, we cannot further relate their observations to ours. Xiang et al. (1996) showed that in Bax-induced apoptosis, the treatment of protease inhibitors partially inhibited the condensation of chromatin and what they termed as "membrane vacuolation," a process similar to what we de-

scribe as the intermediate stage of apoptotic body formation. All these studies however lend support to the notion that caspases have a role only in the execution phase of apoptosis, and after the commitment point has been passed in the signal transduction pathway in apoptosis.

Finally, these observations show that care has to be exercised in interpreting specific assays in the evaluation of apoptosis. In chemotherapy-induced apoptosis in lymphoid cells, caspase inhibitors prevent PARP proteolysis, membrane release, and many nuclear changes, but they clearly do not inhibit ultimate cell death or changes in overall cell morphology and nuclear structure. Therefore, these observations underscore the importance of distinguishing the protection of cell viability from the inhibition of downstream events in apoptosis, such as chromatin condensation, and PARP cleavage.

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