Bcl-x\textsubscript{L} Inhibits Cytochrome \textit{c} Release but Not Mitochondrial Depolarization during the Activation of Multiple Death Pathways by Tumor Necrosis Factor-\textit{α}\textsuperscript{*}

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Bryan W. Johnson‡‡§§, Enrique Cepero‡‡§§, and Lawrence H. Boise‡¶

From the ‡Department of Microbiology and Immunology and the ¶Sylvester Comprehensive Cancer Center, University of Miami School of Medicine, Miami, Florida 33101

Cells can respond differently to anti-CD95 antibody treatment. Type I cells show strong activation of caspase-8 and directly activate caspase-3. Type II cells weakly activate caspase-8 and must amplify their death signal through the mitochondria. These cells can be rescued by Bcl-x\textsubscript{L}. Here we show that tumor necrosis factor-\textit{α} induces both Type I and II pathways, which can be inhibited by benzylxycarbonyl-Val-Ala-Asp-fluoromethyl ketone (Z-VAD-fmk) and Bcl-x\textsubscript{L} in a cooperative fashion. Death induced in the presence of Z-VAD-fmk was associated with a partial inhibition of caspase-8, whereas no effects on cytochrome \textit{c} release, DEVDase activity, and intranucleosomal DNA cleavage were observed. Thus, Z-VAD-fmk is likely weakening the death-inducing signaling complex-mediated activation of caspase-8 and diverting cells to a Type II pathway. Bcl-x\textsubscript{L} cooperates with Z-VAD-fmk by blocking the Type II pathway at the level of cytochrome \textit{c} release. Surprisingly, although Bcl-x\textsubscript{L} was able to block cytochrome \textit{c} release, it was unable to block mitochondrial depolarization, suggesting that these are separate events. This suggests that mitochondria occupy two places in apoptotic signaling, as initiators of apoptosis through the release of cytochrome \textit{c} as well as a target for effector caspases.

Apoptosis, or programmed cell death, is an evolutionarily conserved mechanism through which normal cellular development and homeostasis are maintained. Only recently have the mechanisms of apoptosis begun to be elucidated at the signal transduction level. Apoptosis can be induced by numerous stimuli, including members of the tumor necrosis factor receptor (TNFR)\textsuperscript{1} superfamily (1). The two well characterized prototypes of the TNFR superfamily that have been shown to induce apoptosis are CD95 (Fas/Apo-1) and the p55 Type 1 TNFR (TNFR1). Ligation of these receptors leads to oligomerization and subsequent recruitment of cytosolic adapter and effector proteins to the receptor complex (also termed the death-inducing signaling complex (DISC)) (2). CD95 contains a stretch of amino acids on its cytoplasmic tail termed the “death domain” that binds to the adapter molecule Fas-associated death domain (FADD) protein and is essential for CD95-mediated apoptosis (1–3). FADD then binds to the upstream initiator molecule procaspase-8 through death effector domains contained in each protein; and upon receptor oligomerization, adjacent procaspase-8 molecules are activated to initiate the apoptotic signaling cascade (4, 5).

Cell lines can respond differently to anti-CD95 antibody treatment (6). Type I cells induce strong activation of the DISC, leading to a mitochondria-independent pathway resulting in activation of caspase-3. Thus, Bcl-2 cannot rescue these cells from CD95-induced apoptosis. Conversely, Type II cells weakly activate their DISC and induce death through a mitochondria-dependent pathway that is inhibited by Bcl-2. Parameters of mitochondrial dysfunction that have been demonstrated to occur during apoptosis include loss of mitochondrial membrane potential ($\Delta\Psi_{m}$) (7–10), release of cytochrome \textit{c} from the mitochondrial intermembrane space to the cytosol (9, 11–14), and the generation of reactive oxygen species (9, 10). Some have suggested that these events are mediated by the irreversible opening of the mitochondrial permeability transition pore (PTP), which consists of a complex of proteins that spans the inner and outer mitochondrial membranes (15–18). The role of the PTP and the ordering of these mitochondrial events in the apoptotic cascade are unclear and may be both stimulus- and cell-specific.

Anti-apoptotic members of the Bcl-2 family reside on the outer mitochondrial membrane and have been implicated in maintaining normal mitochondrial function (19–22). The ability of anti-apoptotic Bcl-2 family members (such as Bcl-2 and Bcl-x\textsubscript{L}) to inhibit apoptosis appears to depend on both the stimulus as well as the cell tested. Bcl-2 family members can inhibit apoptosis by many mechanisms such as homo- or heterodimerization with other family members (19–22), maintenance of mitochondrial homeostasis resulting in the prevention of cytochrome \textit{c} release and subsequent caspase activation (11–14, 19–22), and inhibition of the production of reactive oxygen species (9, 10, 19–22). These mechanisms may explain, at least in part, why Bcl-2 is unable to block apoptotic signals that bypass the mitochondria.

Although the Type I/II cell death pathways induced by CD95 have been studied in some detail, the pathways generated in response to TNF-\textit{α}-induced apoptosis are not as well charac-
terized. In some cells, TNF-α treatment alone does not lead to apoptosis due to the proliferation and survival effects of TNFR1 signaling. These signals require de novo protein synthesis. For this reason, these cells can be sensitized to TNF-signaling. These signals require Bcl-2 cleavage-independent fashion (23). In this report, we show that Z-VAD-fmk has no effect on cytochrome c release or caspase-3 activation, but partially inhibits caspase-8 activation. Conversely, Bcl-xL can block cytochrome c release from the mitochondria and decrease effector caspase activity. Interestingly, neither Bcl-xL nor Z-VAD-fmk alone blocks loss of ΔΨm. Furthermore, the loss of ΔΨm is not inhibited by cyclosporin A, an inhibitor of the PTP (24, 25). Based on the data we have obtained, we propose a model of TNF-α-induced apoptosis whereby both Type I and II signals are utilized. Z-VAD-fmk inhibits the Type I signal through a partial inhibition of caspase-8, and Bcl-xL blocks the Type II signal. Activation of effector caspases through either apoptotic pathway results in loss of ΔΨm in a Bcl-xL-, Z-VAD-fmk-, and PTP-independent fashion.

EXPERIMENTAL PROCEDURES

Cell Culture—The interleukin-3-dependent murine pro-B cell line FL5.12 was cultured and transfected as described previously (23). All results shown in the figures were from experiments performed with Bcl-xL-expressing cells, but identical results were obtained using Bcl-2 expressing cells. Results shown were derived from bulk-transfected cultures, but similar results were obtained using single-cell clones.

Generation of Cowpox Virus Protein CrmA and Murine Dominant-negative FADD (FADD-DN)—The crmA construct was generated by polymerase chain reaction using a crmA cDNA construct (obtained from Colin S. Duckett, National Institutes of Health) and the following primers: 5′-CGAATTCAATTTCATATGGCGATCTCGAGGCGAT-3′ and 5′-CCGAAATCATCTAATAGTTGTTGGAGAGCAATATCTAC-3′. The polymerase chain reaction product was cloned into the EcoRI site of pBluescript SK+ (Stratagene, La Jolla, CA), verified by DNA sequencing, and subsequently cloned into the expression vector pSPFV-Neo (23). A dominant-negative FADD molecule was generated by reverse transcription-polymerase chain reaction using mRNA from a mouse pre-B cell line (A50C μ) with the following primers: 5′-CCGAAATCTCAGGATTTTCATTGGCAAGCGAC-3′ and 5′-CCGAAATCAGAGATCTTCCTGCAGGGAGC-3′. All cDNA constructs were digested with EcoRI (New England Biolabs Inc., Beverly, MA) and subcloned into the phagemid vector pBluescript SK+ (Stratagene). The sequenced insert was then cut out with EcoRI and subcloned into the expression vector pSPFV-Neo, which has been described previously (23), and used for cell transfections. As a negative control, cells were transfected with the FADD-Neo cDNA cloned into pSPFV-Neo in the reverse (antisense) orientation.

Cell Death Assays—Cell death assays were performed as described (23), and percent viability was assessed by annexin V-FITC (5 μl; Pharmingen, San Diego, CA) and propidium iodide (PI; 2 μg/ml; Sigma) staining on a FACSscan flow cytometer (Becton Dickinson).

Western Blotting—Western blotting was performed as described (23). The following primary antibodies were used: polyclonal antibodies against caspase-3 (Dr. Anu Srinivasan, Idun Pharmaceuticals), a monoclonal antibody against pig neon cytochrome c (THS.2C12; Pharmingen), a polyclonal antibody against mouse BID (obtained from Dr. Stanley Korsmeyer, Dana-Farber Cancer Institute), a polyclonal antibody against mouse FADD (obtained from Dr. Astar Winoto, University of California, Berkeley, CA), and a polyclonal antibody directed against the C terminus of mouse DNA fragmentation factor-caspase-4 (ICD; Alexis Biochemicals, San Diego, CA). Where indicated, blots were stripped and reprobed with a polyclonal antibody against actin (Sigma) to confirm equal protein loading. The following secondary antibodies were used: horseradish peroxidase-conjugated sheep anti-mouse Igg and horseradish peroxidase-conjugated donkey anti-rabbit Ig (Amersham Pharmacia Biotech). Proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech). Denotometry was performed using the NIH Image 1.61 program. Shorter exposures than those shown were scanned for densitometry to ensure that the values obtained were within the linear range.

DEVD-G, D, Caspase Activity Assay—Cells were treated as described. After 5.5 h, 5 × 106 cells were harvested and stained for 60 min at 37 °C with 10 μM DEVD-G, D, substrate solution (Oncoimmunin, Inc., College Park, MD). Cells were then washed once with ice-cold flow cytometry dilution buffer and resuspended in 500 μl of fresh dilution buffer. Samples were analyzed on the FACScan flow cytometer using the FL1 channel. The mean fluorescence intensity of the DEVD-G, D, positive cells was calculated relative to the mean fluorescence intensity of the DEVD-G, D, positive Neo cells cultured in medium alone (untransfected).

Cytochrome c Release Assay—Cells (5 × 106) were resuspended at 2.5 × 106 cells/ml and treated as indicated. Cells were pelleted, washed once with phosphate-buffered saline, resuspended in 1 ml of ice-cold Buffer A (250 mM sucrose, 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 170 μg/ml phenylmethylsulfonyl fluoride, 16 μg/ml aprotinin, and 4 μg/ml leupeptin), and incubated for 10 min on ice. Cells were lysed in a Wheaton overhead stirrer: seven strokes at the 3.5 setting. Unlysed cells and nuclei were pelleted at 120 × g for 10 min at 4 °C. The supernatant was spun at 10,000 × g for 25 min at 4 °C. The pellet from this spin contained the mitochondrial fraction, and was resuspended in 1 ml of Buffer A. The supernatant was spun at 100,000 × g for 1 h at 4 °C, and the supernatant from this spin contained the cytosolic S100 fraction. Protein concentrations were determined by the BCA assay (Pierce). 150 μg of the mitochondrial fraction and an equal volume of the S100 fraction were loaded on a 12% SDS-polyacrylamide gel and subjected to Western blotting for cytochrome c as described above.

Chromosomal DNA Isoaution and Fragmentation Assay—Genomic DNA was isolated as described (26), and 1 μg of DNA was run on a 1.5% agarose gel to analyze DNA fragmentation.

Flow Cytometric Analysis of ΔΨm—FL5.12 cells were incubated for the last 30 min of cell death experiments with 150 nm tetramethylrhodamine ethyl ester (Molecular Probes, Inc.). Cells were harvested and washed with and then resuspended in fluorescence-activated cell sorting buffer (1 × phosphate-buffered saline with 1% bovine serum albumin and 0.01% sodium azide) supplemented with 15 nm tetramethylrhodamine ethyl ester. Samples were analyzed on the FACScan flow cytometer.

RESULTS

Expression of Bcl-xL Cooperates with Caspase Inhibition by Z-VAD-fmk to Inhibit TNF-α-induced Cell Death—We have shown previously that overexpression of Bcl-2 in the murine pro-B cell line FL5.12 does not inhibit TNF-α-induced cell death unless caspases are also inhibited by the addition of Z-VAD-fmk (50 μM) (23). As shown in Fig. 1, similar results were obtained using Bcl-xL-transfected cells. The addition of Z-VAD-fmk (50 μM) to control (Neo) cells did rescue these cells from TNF-α-induced apoptosis. Similarly, overexpression of Bcl-xL was also insufficient to inhibit cell death. However, as previously shown for Bcl-2, the combination of Bcl-xL and Z-VAD-fmk was sufficient to inhibit TNF-α-induced cell death.

Inhibition of Upstream Signaling Components Blocks TNF-α-induced Cell Death—One potential explanation for the cooperativity observed is that Bcl-xL and Z-VAD-fmk are inhibiting two independent death pathways initiated by TNFR1 ligation. Thus, we inhibited components of the TNFR1 DISC to determine the farthest downstream molecule common to both pathways. To this end, a dominant-negative murine FADD cDNA construct (FADD-Neo, encoding amino acids 80–205) was generated and stably transfected into FL5.12 cells. FADD-Neo expression was verified by Western blot analysis (data not shown). Expression of FADD-Neo protein was sufficient to block TNF-α-induced cell death, whereas Neo cells, which were transfected with a FADD-Neo cDNA construct in the antisense orientation were not resistant to this stimulus (Fig. 2A). These results are consistent with several previous reports (1–3) and imply that inhibition of FADD signaling by disruption of the FADD/caspase-8 death effector domain binding interaction is sufficient to block TNF-α-induced cell death.

Procaspase-8 binds to FADD, and ligation of the receptor...
causes oligomerization and transactivation of caspase-8 (4, 5). The product of the cowpox virus cytokine response modifier A gene (CrmA) can preferentially inhibit upstream and inflammatory caspases such as caspase-8 (27). As shown in Fig. 2B and consistent with previous findings in other cell types (4), CrmA was sufficient to block TNF-α-induced cell death.

Since CrmA but not Z-VAD-fmk alone is sufficient to block TNF-α-induced cell death, it is likely that Z-VAD-fmk cannot completely inhibit caspase-8. To test the activation of caspase-8, we prepared whole cell lysates after TNF-α treatment and performed Western blotting with a polyclonal anti-caspase-8 antibody. Consistent with the decrease in viability that was observed, the pro-caspase-8 band (~55 kDa) was processed in the presence of TNF-α and CHX in both Neo and Bcl-xL cells (Fig. 2C). However, the addition of 50 μM Z-VAD-fmk to either Neo or Bcl-xL cells had a partial effect on caspase-8 processing (Fig. 2C), CHX had no effect on either the viability of the cells or the intensity of the pro-caspase-8 band (Fig. 2C), suggesting that the disappearance of the 55-kDa proenzyme band was a result of TNF-α signaling and not a lack of de novo protein synthesis. Taken together, these results suggest that if multiple pathways generated by TNF-α treatment are present, they diverge at the level of caspase-8 activation. These data are consistent with the loss of death receptor signaling in FADD- and caspase-8-deficient cells (28–30).

An early event triggered by death receptor signaling is caspase-8-mediated cleavage of the pro-apoptotic Bcl-2 family member BID (31–33). After cleavage, BID translocates to the mitochondria and induces cytochrome c release, ultimately resulting in the activation of effector caspases. We therefore tested the ability of Z-VAD-fmk and/or Bcl-xL to inhibit caspase-8 activity at the level of BID cleavage. As shown in Fig. 2D, treatment of Neo cells with TNF-α and CHX resulted in the appearance of 15-kDa (p15), 13-kDa (p13), and 11-kDa (p11) cleavage fragments of BID. Densitometry indicated that only 20–30% (range from three experiments, normalized to actin) of the full-length p22 BID was present in both Neo and Bcl-xL cells treated with TNF-α/CHX compared with untreated cells. Densitometry also indicated that 50–60% of p22 BID was present in both Neo and Bcl-xL cells treated with TNF-α/CHX/Z-VAD-fmk compared with untreated cells (Fig. 2D). Thus, consistent with a partial inhibition of caspase-8, Z-VAD-fmk could partially inhibit cleavage of BID. Conversely, overexpression of Bcl-xL did not inhibit p22 BID cleavage, suggesting that BID cleavage is upstream of Bcl-xL. The difference in the pattern of BID cleavage products in the presence of Bcl-xL is consistent with previous observations (33). Although only low levels of BID cleavage fragments were detected in Bcl-xL-overexpressing cells treated with Z-VAD-fmk, densitometry demonstrated that the p22 BID band was only 50–60% of untreated levels, suggesting that cleavage had indeed occurred. Again, this change was not induced by treatment of cells with CHX alone (Fig. 2D). We propose that in cells in which apoptosis is blocked, the BID cleavage fragments are unstable and are rapidly degraded.

Cell Death in the Presence of Z-VAD-fmk Displays a Caspase-associated Phenotype—Cell death that occurs in the presence of caspase inhibitors can exhibit a necrotic, not apoptotic, phenotype (34). To determine whether the TNF-α-induced cell death observed in the presence of Z-VAD-fmk displays hallmarks of caspase activity, we analyzed the effects of Z-VAD-fmk on caspase-dependent events during TNF-α-induced cell death. One of the hallmarks of apoptosis is the fragmentation of genomic DNA into intranucleosomal fragments. Intranucleosomal DNA fragmentation is achieved through the activity of DFF-40, also called caspase-activated DNase (CAD) (35–37). DFF-40/CAD is normally present in an inactive cytosolic heterodimeric complex with DFF-45, also called inhibitor of CAD (ICAD). Caspase-3-mediated cleavage of DFF-45/ICAD releases DFF-40/CAD, which can then enter the nucleus and degrade the genome. We have tested the ability of Bcl-xL and/or Z-VAD-fmk to inhibit ICAD cleavage and subsequent DNA fragmentation. As shown in Fig. 3A, neither Z-VAD-fmk nor Bcl-xL could inhibit cleavage of DFF-45/ICAD in response to TNF-α. Together, Z-VAD-fmk and Bcl-xL inhibited DFF-45/ICAD cleavage. Consistent with these findings, Z-VAD-fmk alone had no effect on DNA fragmentation (Fig. 3B). Similarly, Bcl-xL had no effect on DNA fragmentation induced by TNF-α. Bcl-xL cooperated with Z-VAD-fmk to inhibit DNA fragmentation and to block cell death. Finally, the nonspecific caspase inhibitor β-butoxycarbonyl-Asp-fluoromethyl ketone (50 μM) inhibited TNF-α-induced cell death in Neo cells (data not shown). These results suggest that cell death that occurs in Neo cells in the presence of Z-VAD-fmk exhibits a caspase-associated phenotype.

Z-VAD-fmk Treatment Has No Effect on Caspase-3 Processing or Effector Caspase Activity, whereas Bcl-xL Decreases Effector Caspase Activity in TNF-α-treated Cells—DFF-45/ICAD cleavage is blocked by caspase-3; therefore, the inability of Z-VAD-fmk to inhibit DNA fragmentation suggests that Z-VAD-fmk may not be inhibiting effector caspases. Therefore, we tested the ability of caspase-3 to be processed in response to TNF-α. Cells were treated for 6 h, and whole cell lysates were analyzed by Western blotting for caspase-3 processing. As shown in Fig. 4, caspase-3 was processed in both Neo- and Bcl-xL-transfected cells. The decrease in the levels of procaspase-3 and the appearance of cleaved caspase-3 indicate that caspase-3 is processed in response to TNF-α/CHX. Z-VAD-fmk alone was unable to inhibit the processing of caspase-3 in Neo cells, consistent with its inability to inhibit cell death. When Z-VAD-fmk was added to Bcl-xL-expressing cells, caspase-3 processing was completely blocked. Although caspase-3 was processed in response to TNF-α treatment, it was unclear as to whether caspase-3 processing correlated with effector caspase activity. We therefore analyzed caspase activity with the caspase-3-like substrate DEVD-Glu-Val-Asp. This rhodamine-based system allows one to determine caspase activity at the cell level by flow cytometry. Therefore, one can monitor the frequency of cells that contain DEVDase (a caspase that cleaves after the sequence Asp-Glu-Val-Asp) activity by
determining the number of cells that display increased fluorescence as well as the level of activity within these cells by determining the intensity of the fluorescence. As shown in Table I, TNFR1 ligation resulted in an increase in the frequency of cells displaying DEVDase activity. The addition of Z-VAD-fmk to Neo cells had no effect on apoptosis, the number of DEVD-G1D2-positive cells, or the mean fluorescence intensity of DEVD-G1D2-positive cells. Taken together with Figs. 3 and 4, these data suggest that Z-VAD-fmk cannot inhibit caspase-3 processing or activity.

Surprisingly, Bcl-xL-expressing cells displayed a different pattern of DEVDase activity. Although Bcl-xL expression had no effect on apoptosis or the number of DEVD-G1D2-positive cells, the mean fluorescence intensity of DEVD-G1D2-positive Bcl-xL-expressing cells was approximately one-half to two-thirds the amount seen in Neo cells (Table I). This is not due to a difference in the amount of procaspase-3 in Neo and Bcl-xL cells (Fig. 4). These results suggest that Bcl-xL cannot block the ability of TNF-α to induce the activation of effector caspases, but can affect the level of effector caspases activated in the cell. Therefore, despite its inability to block TNF-α-induced apoptosis, Bcl-xL can block a pathway that results in DEVDase activity. The most likely explanation is that Bcl-xL is blocking a mitochondrial pathway leading to DEVDase activity.

Bcl-xL Is Capable of Inhibiting Cytochrome c Release from Mitochondria while Having No Effect on Mitochondrial Depolarization—Type II signals from death receptors have been shown to result in the cleavage of BID, the release of cytochrome c, and subsequent activation of caspase-9 (31–33, 38, 39). We have shown that Bcl-xL had no effect on caspase-8 processing or BID cleavage, but could affect the level of DEVDase activity. Since the effect on DEVDase activity is not likely due to an effect on the ability of caspase-8 to cleave caspase-3, we reasoned that Bcl-xL was exerting its effects through inhibition of the Type II pathway. Therefore, we determined the effects of Bcl-xL and Z-VAD-fmk on cytochrome c release.

Neo cells were treated with TNF-α and CHX in the presence and absence of Z-VAD-fmk and separated into mitochondrial and cytosolic S100 fractions by cell fractionation and differential centrifugation. As shown in Fig. 5, untreated Neo cells retained the majority of their cytochrome c within the mitochondrial fraction, suggesting that fractionation did not result in the perturbation of the outer mitochondrial membrane. However, upon induction of cell death with TNF-α, cytochrome c was redistributed from the mitochondrial fraction to the S100 fraction, indicating that cytochrome c was released into the cytosol. Treatment of Neo cells with 50 μM Z-VAD-fmk did not prevent cytochrome c redistribution in response to TNF-α. This
is consistent with our findings regarding caspase-8 activation and BID cleavage. Therefore, Z-VAD-fmk is acting at a step that is distinct from cytochrome c release. In contrast, Bcl-xL cells were capable of preventing cytochrome c release even though >50% of these cells were apoptotic as determined by annexin V-FITC and PI staining (Fig. 5). These results suggest that inhibition of cytochrome c release by Bcl-xL is responsible for the decrease in effector caspase activity that was observed (Table I).

As a consequence of the electron transport chain, mitochondria establish a proton gradient across their inner membrane. This gradient, or $\Delta \Psi_m$, is used to synthesize ATP from ADP and P$_i$. A hallmark of apoptosis is the loss of this membrane potential (9, 10). However, loss of $\Delta \Psi_m$ may not be an initiating event in cell death, but rather a consequence. The release of cytochrome c has been shown to occur prior to the loss of $\Delta \Psi_m$ in
several cell death pathways, placing the loss of \( \Delta \Psi_m \) downstream of cytochrome \( c \) release in the death pathway (12, 14). Alternatively, others have shown that cytochrome \( c \) release is the result of opening of the PTP, leading to loss of \( \Delta \Psi_m \), mitochondrial swelling, and release of cytochrome \( c \) (18). These data place loss of \( \Delta \Psi_m \) upstream of cytochrome \( c \) release in the death pathway. Therefore, we examined the maintenance of \( \Delta \Psi_m \) in TNF-\( \alpha \)-induced apoptosis. \( \Delta \Psi_m \) was measured using the cationic and lipophilic dye tetramethylrhodamine ethyl ester, which accumulates in the mitochondrial matrix due to the proton gradient. As shown in Fig. 6, Neo cells had lost their membrane potential by 6 h during TNF-\( \alpha \)-induced apoptosis, and Z-VAD-fmk was unable to prevent this depolarization. Interestingly, Bcl-x\( \text{L} \) cells also depolarized after 6 h of TNF-\( \alpha \)-induced apoptosis, but the combination of Bcl-x\( \text{L} \) and Z-VAD-fmk blocked all depolarization. Taken together, these data suggest that during TNF-\( \alpha \)-induced cell death, Bcl-x\( \text{L} \) retains its ability to prevent cytochrome \( c \) release, but cannot prevent mitochondrial depolarization. Since the combination of Bcl-x\( \text{L} \) and Z-VAD-fmk is required to completely inhibit DEVDase activity and loss of \( \Delta \Psi_m \), these data suggest that activation of effector caspases can depolarize the mitochondria independent of cytochrome \( c \) release.

Loss of \( \Delta \Psi_m \) associated with apoptosis has been suggested to proceed through the PTP. The PTP is made up of proteins found on the inner and outer membranes of the mitochondria and is thought to irreversibly open upon induction of apoptosis, resulting in depolarization. To determine whether the depolarization seen during TNF-\( \alpha \)-induced apoptosis is a result of the PTP opening, a known inhibitor of the PTP, cyclosporin A, was used to prevent permeability transition (24, 25). As shown in Fig. 6, cyclosporin A was unable to prevent depolarization in both Neo and Bcl-x\( \text{L} \) cells in response to TNF-\( \alpha \). These data suggest that the loss of \( \Delta \Psi_m \) observed during TNF-\( \alpha \)-induced apoptosis is not due to opening of the PTP, but rather to a novel pathway that leads to mitochondrial depolarization.

**DISCUSSION**

We have previously demonstrated that Bcl-2 can cooperate with the caspase inhibitor Z-VAD-fmk to inhibit TNF-\( \alpha \)-induced cell death in the murine pro-B cell line FL5.12 (23). In this report, we have extended these findings to Bcl-x\( \text{L} \) and have attempted to determine the molecular basis for this cooperativity. To determine where Bcl-x\( \text{L} \) and Z-VAD-fmk are acting to inhibit cell death, we tested their ability to inhibit signal transduction pathways during TNF-\( \alpha \)-induced apoptosis. Similar to previous findings (3), we have found that inhibition of receptor complex formation by FADD-DN and inhibition of caspase-8 by CrmA were both sufficient to inhibit TNF-\( \alpha \)-induced apoptosis. Bcl-x\( \text{L} \) had no apparent effect on procaspase-8 processing (Fig. 2D) or caspase-8 activity (Fig. 2D). However, Z-VAD-fmk exhibited a partial effect on both caspase-8 processing and BID cleavage, albeit insufficient to block apoptosis.

The Type II pathway involves the caspase-8-mediated cleavage of BID, which leads to cytochrome \( c \) release. Consistent with previous findings (31–33), we have shown that BID cleavage was not inhibited by Bcl-x\( \text{L} \), although Bcl-x\( \text{L} \) was capable of inhibiting subsequent cytochrome \( c \) redistribution. Although Z-VAD-fmk was capable of partially inhibiting BID cleavage, cytochrome \( c \) release was not blocked. In contrast, we showed...
that Bcl-xL acted at the level of the mitochondria by inhibiting cytochrome c release, even though it was not sufficient to block TNF-α-induced apoptosis. Surprisingly, Bcl-xL overexpression was not sufficient to inhibit loss of ΔΨm. Our results suggest that multiple pathways leading to mitochondrial dysfunction exist in dying cells (discussed below).

We also observed differences in the ability of Z-VAD-fmk and Bcl-xL to inhibit initiator and effector caspases. Although 50 μM Z-VAD-fmk had a partial effect on caspase-8 activity, it had no effect on caspase-3 processing or DEVDase activity. In contrast, Bcl-xL had no effect on caspase-8, but could partially inhibit DEVDase activity. Bcl-xL could accomplish this via two mechanisms. First, Bcl-xL could partially inhibit caspase-3 activation induced through cytochrome c release. Alternatively, Bcl-xL could inhibit caspase-7 activation induced by cytochrome c release. The latter possibility is consistent with several findings. Although both caspase-3 and caspase-7 can be targets of caspase-8 in vitro, only caspase-3 has been shown to be a target in vivo (40–42). Furthermore, MCF-7, a caspase-3-deficient cell line, can be converted from a Type II cell to a Type I cell by transfection of procaspase-3 (6). This would also imply that caspase-8 cannot activate caspase-7 directly. This could also offer an explanation as to why Bcl-xL may alter the pattern that caspase-8 cannot activate caspase-7 directly. This could also offer a solution to why Bcl-xL may alter the pattern of BID cleavage products. The p13 and p11 forms may be generated by effector caspases. If Bcl-xL is inhibiting effector caspase activity, then it may result in a different cleavage pattern. Although the mechanism by which Bcl-xL inhibits DEVDase activity remains to be determined, the data suggest that TNF-α signals through both Type I and II pathways (Fig. 7). Taken together with the effects of Z-VAD-fmk on caspase-8 and caspase-3, the data are most consistent with a model of cell death whereby TNFR1 activates caspase-8, which can initiate two caspase-dependent pathways of cell death. Z-VAD-fmk can partially inhibit caspase-8, but does not affect downstream caspases. Therefore, this partial inhibition may be the primary effect and may be sufficient to inhibit the direct activation of caspase-3. This would be consistent with a conversion from signaling via both pathways to signaling exclusively through the mitochondria. Bcl-xL can cooperate with this conversion to a Type II signal by blocking cytochrome c release. This combination would block both pathways to effector caspase activation and subsequent cell death.

It is well established that the concentration of Z-VAD-fmk used in this study is sufficient to block anti-CD95 antibody-induced cell death in other cell lines (4, 32), and we have previously shown that Z-VAD-fmk alone is sufficient to inhibit TNF-α-induced death in Neo cells when used at concentrations 5–10-fold higher than those used in the present study (23). Therefore, the concentration of Z-VAD-fmk as well as the stimulus utilized to induce death may determine the level of caspase-8 inhibition. This is consistent with several previous findings. Biochemical studies with recombinant caspases suggest that caspase-8 is more sensitive to Z-VAD-fmk than caspase-3 (43, 44). Furthermore, a recent report suggests that antibody-mediated activation of a death receptor results in weaker DISC formation than ligand activation (45). Thus, although Z-VAD-fmk may block anti-CD95 antibody-induced caspase-8 activation, it may only weaken TNF-α-induced signaling. Consistent with this possibility, we have observed that the concentration of Z-VAD-fmk used in these studies can inhibit anti-CD95 antibody-induced apoptosis in CD95-transfected FL5.12 cells as well as in established human Type I cells.2

Previous studies have shown that death receptors can lead to BID cleavage and use the mitochondria to amplify apoptotic signals by releasing cytochrome c. However, whether cytochrome c release is due to the action of BID or mitochondrial depolarization remains controversial. Temporal studies have demonstrated that loss of ΔΨm follows cytochrome c release, suggesting that loss of ΔΨm is a late event in mitochondrial dysfunction (12, 14, 46, 47). Conversely, others have demonstrated that opening of the PTP leads to mitochondrial depolarization and subsequently cytochrome c release (16–18). Regardless of the mechanism of cytochrome c release, our data suggest that the PTP is not involved in TNF-α-induced apoptosis. The fact that cyclosporin A, an inhibitor of the PTP, was unable to prevent depolarization suggests that the PTP is not the causative agent of mitochondrial dysfunction. These data are consistent with a pathway independent of the PTP that leads to mitochondrial depolarization.

We propose that mitochondria can be located in at least two different positions within the death pathway and that release of cytochrome c and mitochondrial depolarization are separate and independent events that are determined by where the mitochondria are in the death pathway (Fig. 7). First, as is the case with the Type II signal, mitochondria are initiators of the caspase cascade by releasing cytochrome c, which results in the

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activation of effector caspases. Once effector caspases are activated, the mitochondria can become targets of the apoptotic program. This caspase-mediated depolarization does not require the release of cytochrome c or the PTP and is capable of overcoming the protective effects of Bcl-xL.

One potential target for effector caspases on the mitochondria could be Bcl-2 or Bcl-xL, since both of these proteins can be cleaved by caspase-3 (23, 48, 49). However, this is an unlikely explanation, as both Bcl-xL and Bcl-2 maintain their ability to prevent cytochrome c release. Rather, it seems more likely that effector caspases, once activated, target the mitochondria in a similar fashion as they target the genome or the cytoskeleton (Fig. 7). Once effector caspases are activated, the decision of the cell to die has been determined. Thus, depolarizing the mitochondria would block the majority of ATP production in a Bcl-xL-independent fashion and assure the demise of the cell.

Mitochondria would block the majority of ATP production in a similar fashion as they target the genome or the cytoskeleton or the mitochondria in a Bcl-xL-dependent manner (50). The mechanism that leads to this caspase-dependent mitochondrial depolarization remains to be determined.

Previous reports have suggested that CD95 signaling pathways could differ in various cell lines. Type I cells utilize a direct caspase cascade, whereas Type II cells must amplify a caspase-8 signal through the release of cytochrome c (6). The data presented here are most consistent with a death receptor that can signal through both Type I and II pathways. It remains to be determined whether this is a characteristic of all Type I cells or whether FL1.5.12 represents a third class of cells that are both Type I and II. Alternatively, the previous reports were performed with anti-CD95 antibody as the death stimulus (6, 45), whereas we have used TNF-α. Thus, the difference may be receptor- and not cell-specific. Further evaluation of the ability of caspase inhibitors to cooperate with overexpression of Bcl-xL to inhibit death receptor-induced apoptosis in established Type I cells is required to address these possibilities.

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