Bcl-2 and Caspase Inhibition Cooperate to Inhibit Tumor Necrosis Factor-α-induced Cell Death in a Bcl-2 Cleavage-independent Fashion*

(Received for publication, December 29, 1998, and in revised form, March 5, 1999)

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The ability of proteins of the Bcl-2 family to either induce or inhibit apoptosis is dependent on both cell type and the apoptotic stimulus. We have shown in the murine pro-B cell line FL5.12 that Bcl-2 is incapable of inhibiting tumor necrosis factor α (TNFα)-induced cell death and is cleaved during this process. One potential explanation for this observation is that caspase activation directly or indirectly inhibits Bcl-2 function. It has been suggested that caspase cleavage of Bcl-2 is responsible for its inability to block certain cell deaths. Consistent with Bcl-2 cleavage being a caspase-mediated event, this cleavage is inhibitable by 50 μM CBZ-Val-Ala-Asp-fluoromethylketone (zVAD-fmk). Furthermore, Bcl-2 can cooperate with the caspase inhibitor zVAD-fmk in a dose-dependent manner to block TNFα-induced cell death. Overexpression of Bcl-2 results in a 10-fold decrease in the amount of zVAD-fmk required to inhibit TNFα-induced apoptosis. However, cleavage-defective mutants (D31A and D34A) show no enhanced viability relative to wild-type Bcl-2 in response to TNFα-induced cell death and also show the same cooperativity with zVAD-fmk. These results suggest that Bcl-2 cleavage is not important for the inhibition of TNFα-induced cell death but do not preclude an involvement in a post-commitment phase of apoptosis.

Apoptosis, or programmed cell death, is an essential mechanism involved in development and homeostasis. Apoptotic signals can be induced by various stimuli, such as DNA damage, anticancer drugs, growth factor withdrawal, and members of the tumor necrosis factor receptor (TNFR) family of death receptors (reviewed in Refs. 1 and 2). Tumor necrosis factor-α (TNFα) is a pleiotropic cytokine that is primarily involved in inflammatory responses; however, it has also been shown to have the ability to induce cell death through one of the two isoforms of its receptor, p55 TNFR1 (9). In some cells, however, the addition of TNFα is not sufficient to trigger cell death unless protein synthesis is also inhibited by the addition of cycloheximide. This implies that de novo synthesis of survival genes may be regulated through TNFR1 signaling (4–6).

TNFR1 has no intrinsic intracellular signaling domains, so signaling is carried out by adapter molecules that recruit effector proteins to the receptor complex. The adapter protein TNF receptor-associated death domain protein binds directly to TNFR1 through a conserved motif referred to as the death domain contained in each molecule and also to either the adapter protein Fas-associated death domain protein or a member of the TNFR-associated factor family (primarily TNFR-associated factor 2) (3, 7, 8). It has been suggested that signaling through TNFR-associated factor 2 ultimately leads to activation of nuclear factor κB and cell survival, whereas Fas-associated death domain protein signaling leads to activation of a death signal (4–6, 9, 10). Fas-associated death domain protein has an additional domain that is required for cell death termed the death effector domain, which binds to the homologous domain in the protein procaspase-8 (FLICE/MACH1) (11, 12). Caspases are cysteine proteases that cleave after aspartic acid residues (reviewed in Refs. 13–16). All caspases are produced as inactive proenzymes and must be activated through cleavage to release their active subunits. It is believed that the binding of TNFα to TNFR1 causes receptor trimerization and that this allows three caspase-8 proenzymes to transactivate each other and induce a caspase cascade leading to cell death. This caspase cascade can occur via two mechanisms. In some cells, caspase-8 can directly activate caspase-3, whereas in others, caspase-8 signaling induces cytochrome c release from the mitochondria (17). Once released from the intermembrane space, cytochrome c participates with the cd4-4 homologue Apaf-1 in the activation of caspase-9 (18–20). Caspase-9 can then continue the cascade by activating downstream caspases, such as caspase-3 or caspase-7. The anti-apoptotic members of the Bcl-2 family function at the stage of cytochrome c release and caspase-9 activation (reviewed in Refs. 21–24).

Bcl-2 family members can localize to outer membranes of the mitochondria, endoplasmic reticulum, and nucleus (25, 26). It has been shown that Bcl-xL can sequester pro-caspase-9 at the mitochondria by forming a trimolecular complex with the human cd4-4 homologue Apaf-1 and pro-caspase-9 (27). Bcl-2 has also been shown to block the release of the apoptotic co-factor cytochrome c (28, 29). Caspase-9 knockout mice die perinatally due to enlarged cerebrums, but thymus development is normal (30). Also, Caspase-9−/− embryonic fibroblasts are resistant to UV and γ-irradiation, but thymocytes are sensitive to UV irradiation and Fas-induced cell death (31). These results imply that whereas caspase-9 is important in some cell deaths, other cell deaths can occur in its absence. It is therefore believed that there may be multiple pathways involved in cell death and that these pathways are either dependent or independent of mitochondrial events. It is also unclear how Bcl-2 activity is regulated. It has been suggested that caspases are capable of di-
rectly cleaving Bcl-2 and that this may be responsible, at least in part, for the inability of Bcl-2 to block IL-3 withdrawal- and Fas-induced cell death (32). It has also been suggested that phosphorylation of Bcl-2 in the 60-amino acid loop domain is an important negative regulatory mechanism for Bcl-2 function (33).

We have shown that Bcl-2 overexpression can block cell death induced by IL-3 withdrawal in the murine pro-B cell line FL5.12 but is unable to block cell death induced by TNFα in the presence of the protein synthesis inhibitor cycloheximide. One difference that we have observed is that Bcl-2 is cleaved directly in response to TNFα, but not in response to IL-3 withdrawal. This cleavage can be blocked by the addition of the caspase inhibitor zVAD-fmk, suggesting that this cleavage is a caspase-mediated event. Moreover, addition of zVAD-fmk (50 μM) could also block TNFα-induced apoptosis in Bcl-2-transfected cells, but not in vector control-transfected cells. Surprisingly, cells that overexpress Bcl-2 require a 10-fold lower concentration of zVAD-fmk to block TNFα-induced cell death. This result implies that there is cooperativity between Bcl-2 overexpression and caspase inhibition. One possible explanation of these results is that zVAD-fmk blocks caspase-mediated cleavage of Bcl-2 so that Bcl-2 can exert its anti-apoptotic effects. However, cleavage-defective Bcl-2 mutants had no effect on viability in response to TNFα. Moreover, these cleavage-defective mutants maintained the same level of cooperativity with zVAD-fmk. Our results demonstrate that direct cleavage of Bcl-2 by caspases does not play a role in all cell deaths and imply that cleavage may be a late event in death receptor-mediated cell death.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—The murine pro-B cell line FL5.12 was grown at 37 °C in 5% carbon dioxide in RPMI 1640 medium supplemented with 10% fetal bovine serum, 20 mM HEPES, WcHi-3B supernatant (a source of IL-3), penicillin/streptomycin, and 2 mM l-glutamine and in the presence of 1 mg/ml G418 for transfected cells. All cell culture materials were obtained from Life Technologies, Inc.

Cell transfections were carried out as described previously (33). Briefly, 1 × 10⁹ cells were spun for 5 min at 1100 rpm and resuspended in 0.4 ml of FL5.12 medium in an electroporation cuvette. Generally, 15 μg of DNA were used per transfection, and electroporation was carried out at 250 V and 960 microfarads as described. Cells were incubated at room temperature for 10 min and transferred to 10 ml of medium. After a 48-h incubation at 37 °C, cells were added to medium containing G418 to select for neomycin resistance.

Generation of Bcl-2 Cleavage Mutants—The untagged Bcl-2 construct was in the pSFFV-Neo vector (Bcl-2) and a hemagglutinin-tagged full-length Bcl-2 construct in pSFFV-Neo (HA-Bcl-2) have been described previously (33). The HA-Bcl-2 construct was used for site-directed mutagenesis using the Chameleon double-stranded DNA mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. The following mutagenesis primers were used to generate Bcl-2 cleavage-defective mutants D31A and D34A, 5′-PAGAGGGGCC-TACGAGT GGGCTGCGGGAGATGTGGGCG-3′, D31A and 5′-PAGAGGGGCC-TACGAGT GGGCTGCGGGAGATGTGGGCG-3′, D31A and D34A. Bcl-2 mutants were verified by sequencing and by Western blot analysis (see below).

Cell Death Assays—For TNFα-induced cell death assays, cells were counted, spun at 1100 rpm for 5 min, and resuspended at a concentration of 5 × 10⁵ cells/ml. Recombinant human TNFα (2 ng/ml; Calbiochem) and the protein synthesis inhibitor cycloheximide (CHX) (10 μg/ml) in phosphate-buffered saline; Sigma) were added for 6 h to induce cell death. To determine the dose response and time course used for TNFα-induced apoptosis to occur, cells were either incubated as described in the presence of 0–5 ng/ml TNFα and 10 μg/ml CHX (kept constant) for 6 h or incubated in the presence of 2 ng/ml TNFα and 10 ng/ml CHX for 0–9 h. In some experiments, the caspase inhibitor zVAD-fmk (Enzyme Systems Products, Livermore, CA) was added at the indicated concentrations. Cells were incubated at 37 °C for 6 h and analyzed for cell viability by a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) by propidium iodide (PI, Sigma) and annexin V-FITC (PharMingen, San Diego, CA) staining.

IL-3 withdrawal assays were carried out in a similar fashion. Cells were harvested, washed three times with RPMI 1640 medium, and resuspended at a final concentration of 5 × 10⁵ cells/ml in medium lacking IL-3. At the indicated time points, cell viability was ascertained by PI staining as described.

Western Blotting—Cells from TNFα or IL-3 withdrawal cell death experiments were harvested, washed once with 1× phosphate-buffered saline, and lysed in radioactive precipitation buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0) supplemented with protease inhibitors (170 μg/ml phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, and 2 μg/ml aprotinin). Protein concentrations were determined by the BCA method (Pierce). Lysates were boiled for 5 min and loaded onto a 15% SDS-polyacrylamide gel electrophoresis gel. Generally, 50 μg of total protein were loaded per lane. Gels were transferred to nitrocellulose by standard methods (33). The following antibodies were used to detect full-length Bcl-2 and cleavage products: (a) a hamster anti-human Bcl-2 monoclonal antibody (6C8, PharMingen), (b) a mouse anti-hamster immunoglobulin mixture (PharMingen), and (c) a goat anti-mouse immunoglobulin linked to horseradish peroxidase (Amersham Pharmacia Biotech). Protein bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech).

RESULTS

Bcl-2 Can Block Cell Death Induced by IL-3 Withdrawal but Cannot Block TNFα-induced Cell Death—We have shown that the murine pro-B cell line FL5.12 can be triggered to undergo programmed cell death in response to TNFα in the presence of the protein synthesis inhibitor CHX. As shown in Fig. 1A, FL5.12 cells transfected with a vector control (Neo) were 33 ± 19.1% viable as determined by PI and annexin V-FITC staining after a 6-h incubation with 2 ng/ml TNFα and 10 μg/ml cycloheximide. When FL5.12 cells overexpressing either Bcl-2 or HA-Bcl-2 are incubated with TNFα and CHX, there is no effect on viability (viability for Bcl-2 was 35.4 ± 10.9%; viability for HA-Bcl-2 was 35.5 ± 13.9%). The fact that Bcl-2 and HA-Bcl-2 cells die to the same extent implies that the addition of the HA tag to wild-type Bcl-2 does not confer any selective advantages for cell survival. The inability of Bcl-2 or HA-Bcl-2 to block TNFα-induced apoptosis was intrinsic to the signal and not the cell because these molecules were sufficient to block IL-3 withdrawal-induced cell death in the same cells (Fig. 1B).

To further characterize the inability of Bcl-2 to inhibit TNFα-induced apoptosis, dose-response (Fig. 1C) and time course (Fig. 1D) experiments were performed. As shown in Fig. 1C, Bcl-2 overexpression was unable to block TNFα-induced apoptosis at all doses of TNFα between 0.5 and 5 ng/ml in the presence of a constant concentration of CHX (10 μg/ml) after a 6-h incubation period. There was virtually no increase in apoptosis between the 2 and 5 ng/ml doses of TNFα, thus we chose the concentration of 2 ng/ml TNFα for subsequent experiments. Fig. 1D shows a time course of TNFα-induced apoptosis in control- and Bcl-2-transfected cells. Importantly, cycloheximide at the concentration used throughout these experiments did not induce apoptosis (closed symbols) and only began to display toxic effects after 24 h (data not shown). As indicated in the figure, a very limited amount of apoptosis occurs in these cells after 3 h, but by 6 h there is a dramatic increase in the number of apoptotic cells. There is little difference between the amount of apoptosis observed at 6 and 9 h, thus we chose 6-h treatments for subsequent experiments.

Bcl-2 Is Cleaved in Response to TNFα but Not IL-3 Withdrawal—One possible explanation of why Bcl-2 does not inhibit TNFα-induced apoptosis in FL5.12 cells is that it is inactivated by upstream caspases activated during death receptor signaling. This inactivation could occur through cleavage of Bcl-2 or activation of kinases and subsequent phosphorylation of Bcl-2. Therefore, we determined if Bcl-2 was cleaved in response to TNFα receptor ligation. As shown in Fig. 2A, Bcl-2 can be cleaved in response to TNFα at 6 h post-treatment. It has
recently been shown that Bcl-2 can be cleaved after amino acid 34, and this is consistent with the 21-kDa C-terminal cleavage product that was detected in response to TNFα treatment.

Cleavage of Bcl-2 was tested after IL-3 withdrawal but was not detected in Bcl-2-transfected cells up to 4 days after growth factor withdrawal (Fig. 2B).

Bcl-2 Cleavage Is Inhibited by the Caspase Inhibitor zVAD-fmk—To determine whether the cleavage of Bcl-2 in response to TNFα was a direct caspase-mediated event, we tested for Bcl-2 cleavage in response to TNFα in the presence or absence of 50 μM zVAD-fmk. As shown in Fig. 3, Bcl-2 is cleaved in response to TNFα as early as 6 h post-treatment, with an increase in the level of the indicated cleavage product occurring between 6 and 24 h. As expected, the addition of the caspase inhibitor zVAD-fmk efficiently blocked Bcl-2 cleavage in response to TNFα. This result suggests that the cleavage of Bcl-2 in response to TNFα is a direct caspase-mediated event.

Bcl-2 Cooperates with Caspase Inhibition to Block TNFα-induced Cell Death in a Dose-dependent Fashion—Because Bcl-2 cleavage was blocked by zVAD-fmk, we wished to determine the effects of the addition of this inhibitor to Bcl-2- and vector control-transfected cells on TNFα-induced apoptosis. Whereas zVAD-fmk (50 μM) did not lead to enhanced viability were incubated in the presence of 10 μg/ml CHX and the indicated concentrations of TNFα for 6 h. The percentage of viability was determined by flow cytometry staining with PI and annexin V-FITC. Mean ± standard deviations were derived from three independent experiments. D, Neo (squares), Bcl-2 (circles), or HA-Bcl-2 (diamonds) cells were incubated in the presence of 10 μg/ml CHX and in either the absence (closed symbols) or presence (open symbols) of 2 ng/ml TNFα. At the indicated time points, the percentage of viability was determined by flow cytometry staining with PI and annexin V-FITC. Mean ± standard deviations were derived from three independent experiments.
of control-transfected cells in response to TNFα, the addition of the same concentration of zVAD-fmk to cells overexpressing Bcl-2 restored viability to untreated levels (Fig. 4A). Importantly, whereas this concentration of zVAD-fmk was unable to block TNFα-induced apoptosis in control cells, this was not due to an inability of the caspase inhibitor to enter these cells, because caspase-3 activation was efficiently blocked in these cells. This result implies that there is some cooperation between overexpression of Bcl-2 and caspase inhibition.

To further characterize this finding of cooperativity between zVAD-fmk and Bcl-2, we performed a dose-response experiment to determine the magnitude of this cooperativity. As shown in Fig. 4B, TNFα-induced cell death could be blocked in Neo cells with the addition of 250–500 μM zVAD-fmk. However, there is a log difference between that amount and the amount that is necessary for inhibition of TNFα-induced cell death in Bcl-2-overexpressing cells. In Bcl-2-overexpressing cells, only 25–50 μM zVAD-fmk is required for complete inhibition of TNFα-induced apoptosis.

Bcl-2 Cleavage-defective Mutants Do Not Block TNFα-induced Cell Death—If direct inhibition of Bcl-2 by caspase-mediated cleavage is the reason why Bcl-2 cannot block TNFα-induced cell death, then site-directed mutants that block Bcl-2 cleavage should have the same effect as the ectopic addition of zVAD-fmk. In other words, cleavage-defective mutants of Bcl-2 should provide increased protection against TNFα-induced cell death. It has recently been shown that mutations at amino acids 31 or 34 of the Bcl-2 sequence lead to a noncleavable Bcl-2 protein (32). We therefore generated two site-directed mutants with amino acid changes from aspartic acid to alanine at amino acids 31 and 34, respectively (D31A and D34A). Because the addition of the HA tag to Bcl-2 has been shown to have no effect on cell survival (see Figs. 1 and 4A), these site-directed mutants were generated from the HA-Bcl-2 DNA construct. We initially tested the ability of these mutants to functionally block Bcl-2 cleavage in response to TNFα. As shown in Fig. 5A, both site-directed mutants were defective in Bcl-2 cleavage, whereas wild-type Bcl-2 was cleaved into the expected 21-kDa C-terminal cleavage product. However, D31A and D34A cleavage-defective mutants were unable to block TNFα-induced cell death (Fig. 5B). Also, the cleavage-defective mutants cooperate with 50 μM zVAD-fmk to the same extent as wild-type Bcl-2. The Bcl-2 cleavage-defective mutants were tested for their response to IL-3 withdrawal, and, as in the case of TNFα, there was no effect on viability relative to wild-type Bcl-2 (Fig. 5C). This result also indicates that these site-directed mutations do not affect normal Bcl-2 function.

Bcl-2 Cleavage-defective Mutants Do Not Lower the Amount of zVAD-fmk Required for Complete Inhibition of TNFα-induced Cell Death—One possible explanation for our results with the Bcl-2 cleavage mutants is that Bcl-2 cleavage represents only one of several mechanisms that result in cooperativity between zVAD-fmk and Bcl-2; therefore, inhibition of cleavage alone is not sufficient to block TNFα-induced cell death. If this is the case, then it stands to reason that the concentration of zVAD-fmk required to cooperate with Bcl-2 cleavage mutants would be lower than that required for wild-type Bcl-2. Therefore, a zVAD-fmk dose-response experiment was performed again; however, this time, HA-Bcl-2 was compared with D31A and D34A mutants in a lower dose range of zVAD-fmk. Cleavage-defective mutants still required 25–50 μM zVAD-fmk to completely block TNFα-induced cell death, and no differences were observed relative to wild-type Bcl-2, even at lower concentrations of zVAD-fmk (Fig. 6). These results indicate that Bcl-2 cleavage plays no apparent role in the response

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2 B. W. Johnson and L. H. Boise, unpublished observation.
to TNFα-induced cell death in the murine pro-B cell line FL5.12. We believe that although Bcl-2 can be cleaved, this is a late event and occurs after the cell has been committed to die. Cleavage of Bcl-2 may amplify the apoptotic response, but cleavage does not appear to be an important regulatory mechanism involved in determining the ability of Bcl-2 to function.

**DISCUSSION**

We have studied the role of Bcl-2 in response to TNFα-induced cell death. Anti-apoptotic Bcl-2 family members have varying effects in response to different stimuli and different cell lines. It has been shown that Bcl-2 can block cytochrome c release from the mitochondria (25, 26). It has also been shown that Bcl-xL can form a ternary complex with Apaf-1 and procaspase-9 at the mitochondrial outer membrane (27). It is thus believed that Bcl-2/Bcl-xL may be exerting their effects by both sequestering pro-caspase 9 at the mitochondrial surface in an inactive conformation and simultaneously blocking cytochrome c release and subsequent pro-caspase-9 processing. However, it has recently been demonstrated that not all apoptotic mechanisms are dependent on mitochondria. For example, caspase-9 knockout mice are resistant to a number of apoptotic stimuli and die due to massive brain malformations, but thymocytes from these mice are sensitive to Fas-induced cell death (30, 31). It has also been shown that some cell lines are dependent on mitochondrial signals, whereas others are not, implying that this could be part of the reason for the varying anti-apoptotic responses of Bcl-2 in different cell lines (17).

In an attempt to decipher differences in the ability of Bcl-2 to block IL-3 withdrawal-induced cell death but not TNFα-induced cell death in the same cell line, we determined that Bcl-2 was cleaved in response to TNFα but not in response to IL-3.

Recently, cleavage of Bcl-2 has been reported in response to cell death induced by anti-Fas treatment, IL-3 withdrawal, viral infection, and anticancer treatment (32, 35). In these cases, transient transfection of the cleaved Bcl-2 C-terminal fragment was capable of increased cell death, whereas cleavage-defective mutants were more resistant to these cell deaths. It was therefore thought that Bcl-2 cleavage might be an important regulatory mechanism for Bcl-2 function. We have tested the effects of Bcl-2 cleavage in response to TNFα-induced cell death in the IL-3-dependent murine pro-B cell line FL5.12. In these cells, stable overexpression of Bcl-2 is sufficient to block cell death induced by IL-3.

**FIG. 5.** Cleavage-defective Bcl-2 mutants have no effect on viability in response to TNFα. A, HA-Bcl-2 or cleavage-defective HA-Bcl-2 mutants (D34A or D31A) were incubated for 6 h in the presence of TNFα and CHX. Whole cell lysates were prepared and analyzed by SDS-polyacrylamide gel electrophoresis and subsequent Western blotting with a monoclonal antibody toward Bcl-2 as described. The Bcl-2 cleavage product is indicated by an arrow. B, HA-Bcl-2 or cleavage-defective HA-Bcl-2 mutants (D34A or D31A) were incubated for 6 h in medium alone (Untreated), in the presence of TNFα and CHX (TNF/CHX), or additionally in the presence of 50 μM zVAD-fmk (TNF/CHX/zVAD-fmk). Cell viability was determined by flow cytometry double staining with PI and annexin V-FITC. Mean ± standard deviations were derived from three independent experiments.

**FIG. 6.** Bcl-2 cleavage-defective mutants do not lower the amount of zVAD-fmk required for complete inhibition of TNFα-induced cell death. HA-Bcl-2 or cleavage-defective mutants D34A or D31A were incubated for 6 h in the presence of TNFα and CHX and also in the presence of the indicated concentrations of zVAD-fmk. Mean ± standard deviations were derived from three independent experiments.
withdrawal, and Bcl-2 is not cleaved during the process. This differs from previously published data in which Bcl-2 was cleaved; however, these cells were only protected to a level of 50% viability after 5 days (32), whereas FL5.12 cells are protected to >80% viability for 8 days after the removal of IL-3. We believe that this difference may be due either to differences in Bcl-2 protein expression levels or to intrinsic differences in the two different cell lines that were used.

In contrast to IL-3 withdrawal, TNFα-induced apoptosis was unaffected by Bcl-2 overexpression and associated with Bcl-2 cleavage. This cleavage could be inhibited by the caspase inhibitor zVAD-fmk. Furthermore, Bcl-2 as well as HA-Bcl-2 can cooperate with 50 μM of the caspase inhibitor zVAD-fmk to block TNFα-induced cell death. This concentration of zVAD-fmk is capable of blocking caspase-3 activation in vector control-transfected cell lines, but these cells die in response to TNFα, implying that TNFα-induced cell death of FL5.12 cells can occur independently of caspase-3 activation. This result can be explained by the redundancy of effector caspases in that perhaps caspase-6 or caspase-7 is activated in these cells and can take the place of caspase-3 to mediate TNFα-induced cell death. Alternatively, caspase-9 activation by mitochondrial dysfunction and cytochrome c release may bypass the requirement for caspase-3 activation.

Interestingly, when a zVAD-fmk dose-response experiment was performed with Bcl-2 cells versus Neo control cells, overexpression of Bcl-2 lowered the amount of zVAD-fmk required for inhibition of TNFα-induced apoptosis 10-fold relative to control cells. This result is consistent with the hypothesis that caspase inhibition by zVAD-fmk could be regulating the ability of Bcl-2 to function in response to TNFα-induced cell death. Whereas the amount of Bcl-2 cleaved is not great, previous reports have suggested that the cleavage product could function as a dominant negative protein. Deletion analysis of Bcl-2 revealed that the loss of residues 6–31 created a dominant negative form (36). Furthermore, Cheng et al. (32) have demonstrated that overexpression of the caspase-3-cleaved product of Bcl-2 can kill cells.

We first wanted to show that we had in fact generated Bcl-2 cleavage-defective mutants (D31A and D34A). However, inhibition of Bcl-2 cleavage had no effect on viability in response to TNFα. Also, these mutants displayed the same level of cooperativity with 50 μM zVAD-fmk. Because overexpression of Bcl-2 lowered the amount of zVAD-fmk required for cooperativity 10-fold compared with control cells, we thought that perhaps cleavage-defective mutants of Bcl-2 would lower the amount of zVAD-fmk required for cooperativity relative to wild-type Bcl-2. However, when lower doses of zVAD-fmk were tested, this was not found to be the case. Cleavage-defective Bcl-2 mutants showed the same level of cooperativity with zVAD-fmk as did wild-type Bcl-2. These combined results imply that Bcl-2 cleavage may play a role in some cell deaths, but it has no apparent role in the response to TNFα. We believe that caspase-mediated cleavage of Bcl-2 is a late event in the apoptotic signal transduction cascade triggered by TNFα and that this cleavage is not an important regulatory mechanism involved in determining Bcl-2 function. Consistent with this hypothesis, it has recently been shown that Bcl-2 can be cleaved in vitro by recombinant caspase-1, -3, -5, -7, and -8 (35). Of the caspases tested, caspase-3 was most efficient in inducing Bcl-2 cleavage, whereas the upstream caspase, caspase-8, was the least efficient in cleaving Bcl-2, suggesting that Bcl-2 cleavage could be a late apoptotic event.

Phosphorylation of Bcl-2 and Bcl-xL in the 60-amino acid loop domain has also been suggested to play a role in regulating the ability of these proteins to exert their anti-apoptotic effects (37, 38). Chang et al. (33) have shown that Bcl-2 and Bcl-xL loop-deleted mutants display enhanced viability in response to IL-3 withdrawal and IgM-induced cell death, and these results correlated with a decrease in Bcl-2 phosphorylation. However, recent evidence suggests that Bcl-2 phosphorylation induced by microtubule-disrupting agents such as paclitaxel and an intact loop domain are required for inhibition of cell death induced by such agents (39). Whereas we cannot completely rule out the possibility of caspase-activated kinases inducing Bcl-2 phosphorylation, preliminary data suggest that the addition of zVAD-fmk does not lead to decreased Bcl-2 phosphorylation, as would be predicted from direct caspase-activated kinase activity toward Bcl-2. For this reason, we do not believe that inhibition of Bcl-2 phosphorylation by inhibition of a caspase-activated kinase is the explanation for the cooperativity that is seen in response to TNFα-induced cell death.

It has recently been shown that different cell lines can respond differently to cell death induced by anti-Fas treatment (17). One class of cells, termed type I cells, activates caspase-8 and caspase-3 efficiently in a manner independent of mitochondrial signals. For this reason, these cells are not rescued by Bcl-2 expression, which is able to block mitochondrial death signals. Type II cells, on the other hand, do not strongly activate caspase-8 and must use a signaling pathway that is dependent on mitochondrial death signals and is thus inhibited by Bcl-2 expression. FL5.12 cells do not behave like either of these two types of cells in response to TNFα; inhibition of caspase-3 activation by the addition of zVAD-fmk is insufficient to block TNFα-induced cell death, as is inhibition of mitochondrial dysfunction by overexpression of Bcl-2. We favor a model in our system in which type I and type II signals are generated and inhibition of both pathways is required to sufficiently block cell death. The addition of zVAD-fmk can block caspase-3 activation, and Bcl-2 can block mitochondrial signals. Our data also suggest that FL5.12 cells can die in the absence of caspase-3 activation by disruption of the mitochondria and, presumably, caspase-9 activation. We cannot rule out the possibility that caspase-9 activation will lead to the activation of a redundant effector caspase such as caspase-6 or caspase-7 that can cause apoptosis independent of caspase-3.

Whereas Bcl-2 cleavage does not appear to be important in the pre-commitment phase of TNFα-induced apoptosis, it may play a role in a post-commitment phase. It has been suggested that activation of caspase-3 can feed back upon the mitochondria and amplify the apoptotic response (40). Whereas the potential mitochondrial target of caspase-3 has been elusive, it may be that Bcl-2 is such a substrate. This cleavage of Bcl-2 may be able to amplify the apoptotic response. Further investigation into the pathways involved in TNFα-induced cell death is required to understand where the signaling pathways may diverge and how this may apply to the regulation of various cell deaths that occur in vivo.

Acknowledgments—We thank Brian Chang for generously providing us with the HA-Bcl-2 cDNA construct. We also thank Craig Thompson and Enrique Cepero for critical review of this manuscript.

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