# Role of Caspases in Immunotoxin-Induced Apoptosis of Cancer Cells†

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ABSTRACT: Immunotoxins composed of antibodies linked to plant or bacterial toxins are being evaluated in the treatment of cancer. It is known that the toxin moieties of immunotoxins, including *Pseudomonas* exotoxin A (PE), diphtheria toxin, and ricin, are capable of inducing apoptosis. Since the efficiency of induction of apoptosis and the apoptosis pathway may have direct effects on the therapeutic usefulness of immunotoxins, we have studied how B3(Fv)-PE38, a genetically engineered immunotoxin in which the Fv fragment of an antibody is fused to a mutated form of PE, induces apoptosis of the MCF-7 breast cancer cell line. We show for the first time that a PE-containing immunotoxin activates ICE/ced-3 proteases, now termed caspases, and causes characteristic cleavage of the "death substrate" poly(ADP)-ribose polymerase (PARP) to an 89 kDa fragment with a time course of cleavage comparable to that induced by TNFα. Also the fluorescent substrate, DEVD-AFC, is cleaved 2−4-fold more rapidly by lysates from B3(Fv)-PE38 treated MCF-7 cells than untreated control cells, suggesting that a CPP32-like caspase is involved in B3(Fv)-PE38-mediated apoptosis. B3(Fv)-PE38-induced PARP cleavage is inhibited by several protease inhibitors known to inhibit caspases (zVAD-fmk, zDEVD-fmk, zIETD-fmk) as well as by overexpression of Bcl-2 providing additional evidence for caspase involvement. zVAD-fmk, a broad spectrum inhibitor of most mammalian caspases, prevents the early morphological changes and loss of cell membrane integrity produced by B3(Fv)-PE38, but not its ability to inhibit protein synthesis, arrest cell growth, and subsequently kill cells. Despite inhibition of apoptosis, the immunotoxin is still capable of selective cell killing, which indicates that B3(Fv)-PE38 kills cells by two mechanisms: one requires caspase activation, and the other is due to the arrest of protein synthesis caused by inactivation of elongation factor 2. The fact that an immunotoxin can specifically kill tumor cells without the need of inducing apoptosis makes such agents especially valuable for the treatment of cancers that are protected against apoptosis, e.g., by overexpression of Bcl-2.

Immunotoxins are cytotoxic agents composed of an antibody and a toxin (*1*−5). The antibody directs the toxin to a target cell, and the toxin causes cell death. A variety of bacterial and plant toxins have been used to make immunotoxins (*1*−*5*). Our laboratory has focused on the use of *Pseudomonas* exotoxin A (PE),[1] which is a 66 kDa protein composed of three major structural and functional domains: an amino-terminal cell binding domain (domain Ia), a central translocation domain (domain II), and a carboxyl-terminal domain (domain III), which catalyzes the ADP-ribosylation and inactivation of elongation factor 2 (*6*). To target PE to cells, we have deleted the cell binding domain (domain Ia) and replaced it with the Fv fragment of an antibody or a growth factor. In B3(Fv)-PE38 (LMB-7) the Fv fragment recognizes the Lewis$^y$ antigen which is expressed on many epithelial cancers including colon and breast (*7*, *8*). A phase I clinical trial using B3(Fv)-PE38 (LMB-7) is ongoing (*9*). A phase I trial with a chemical conjugate of the B3 antibody and Lys-PE38 (LMB-1) in patients with solid tumors has been completed (*10*). In the latter trial several responses were observed.

The only known activity of the bacterial toxins PE and diphtheria toxin (DT) is their ability to ADP-ribosylate and inactivate elongation factor 2 (*11*, *12*). This leads to the arrest of protein synthesis and subsequently to cell death. Recently, it has been found that toxins such as DT, ricin toxin, cholera toxin, and PE are capable of inducing DNA fragmentation or apoptosis (*13*−*19*). It is not clear how these toxins induce apoptosis, but it is a toxin-specific mechanism and not simply a general reaction to inhibition of protein synthesis (*13*, *14*, *18*). Treatment of MCF-7 breast cancer cells with PE or an immunotoxin containing PE induced degradation of chromosomal DNA (*18*). Overexpression of Bcl-2 in MCF-7 cells decreased the sensitivity of these cells to PE and PE-derived immunotoxins 3−10-fold (*19*).

Recent analyses of the death pathways in various systems have begun to delineate the biochemical basis of apoptosis (*20*−22). Activation of ICE-like cysteine proteases, now termed caspases (*c*ysteine *asp*artate-specific prote*ases*) (*23*, *24*), and the cleavage of so-called "death substrates" such

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[1] Abbreviations: AFC, 7-amino-4-(trifluoromethyl)coumarin; B3(Fv)-PE38, single chain immunotoxin targeted to the B3 antigen; CHO, aldehyde; DT, diphtheria toxin; EF-2, elongation factor 2; fmk, fluoromethyl ketone; Fv, variable domains of an antibody; LMB-7, B3(Fv)-PE38; $IC_{50}$, the concentration that produces a 50% inhibition of protein synthesis; PARP, poly(ADP)-ribose polymerase; PE, *Pseudomonas* exotoxin; PE38, domains II and III of PE; TNFα, tumor necrosis factor α.

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as poly(ADP-ribose) polymerase (PARP) have proven to be two hallmarks of apoptotic death (*25*, *26*). There are presently 10 human homologues of the ced 3 cysteine protease first defined by genetic analysis of cell death in *Caenorhabditis elegans* (*24*, *27*). These enzymes exist as inactive proenzymes which become active when cleaved to subunits of 17−20 and 10−12 kDa. The caspase family members can be divided into three subgroups: ICE-like, ICH-1-like, and CPP32-like proteases, based on their sequence homology (*23*). Recognition sequences have been identified for three caspases: caspase-1 (ICE) recognizes the sequence YVAD or even better WEHD (*28*). Caspase-3 (CPP32) recognizes DEVD and therefore cleaves poly(ADP-ribose) polymerase (*29*). Caspase-6 (Mch2) recognizes VEID and cleaves lamin. It is uncertain whether each ICE family member has a specific substrate; it seems more likely that subfamily members can cleave the same substrate and have a similar recognition sequence. Caspases can activate each other and in some cases also appear to undergo autocatalytic cleavage (*24*). Caspase-3 (CPP32) has been reported to be a dominant death-associated protease in CD95 (APO1/Fas)- and TNFα-mediated apoptosis (*20*, *30*).

Here we describe for the first time the activation of a CPP32-like caspase in MCF-7 cells upon treatment with the immunotoxin B3(Fv)-PE38 as well as the consecutive characteristic cleavage of PARP to an 89 kDa fragment. We evaluate the influence of the peptide-based inhibitors zVAD-fmk, zDEVD-fmk, and zIETD-fmk on B3(Fv)-PE38-induced PARP cleavage and the effect of the inhibitor zVAD-fmk on the PE-induced inhibition of cellular protein synthesis. The role of zVAD-fmk, which is a universal inhibitor of nuclear apoptosis in mammalian cells, in preventing morphological changes and increasing cell viability upon B3(Fv)-PE38 exposure of cells is assessed in comparison to TNFα-induced cell damages. Our results indicate that the immunotoxin B3(Fv)-PE38 induces cell killing by two different mechanisms: inhibition of protein synthesis and induction of apoptosis through caspase activation.

## MATERIALS AND METHODS

*Reagents.* The protease inhibitors Cbz-Val-Ala-Asp(OMe)-fluoromethyl ketone (zVAD-fmk), Cbz-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-fluoromethyl ketone (zDEVD-fmk), Cbz-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (zIETD-fmk), and Cbz-Phe-Ala-fluoromethyl ketone (zFA-fmk) were purchased from Enzyme Systems Products (Dublin, CA), dissolved as stock solutions of 50 mM in DMSO, and stored at −20 °C. Working solutions were made in culture medium immediately before use. Polyclonal rabbit anti-PARP antibody, human recombinant TNFα, and Protease Inhibitor Cocktail Tablets (complete™) were obtained from Boehringer Mannheim (Indianapolis, IN), monoclonal anti-caspase-2 (ICH-1L) and anti-caspase-3 (CPP32) antibodies from Transduction Laboratories (Lexington, KY), polyclonal goat anti-ICEp20 and ICH-1L from Santa Cruz Biotechnology (Santa Cruz, CA), and peroxidase-labeled secondary antibodies from Jackson Immunoresearch Laboratories, Inc. (West Grove, PA). Protein gels (7.5% and 15%) and nitrocellulose membranes were obtained from Bio-Rad (Hercules, CA), ECL enhanced chemiluminescence reagents from Amersham Life Science, Inc. (Arlington Heights, IL), and etoposide and cycloheximide from Clontech (Palo Alto, CA).

*Cell Culture.* MCF-7 cells were cultured in RPMI 1640 medium containing 5% fetal bovine serum. MCF-7/Bcl-2 cells and MCF-7/pcDNA3 cells are MCF-7 cells stably transfected with a plasmid for overexpression of human Bcl-2 and the vector pcDNA3 alone, respectively (*19*). These cells were propagated in RPMI medium containing 5% fetal bovine serum and G418 (375 μg/mL).

*Production of Recombinant Immunotoxin B3(Fv)-PE38 (LMB-7).* The protein was produced from inclusion bodies, properly folded, and purified as previously described (*8*, *31*).

*Preparation of Whole Cell Lysates for Immunoblotting.* Cells were seeded at $2 \times 10^6$ cells/T175 flask in 35 mL of medium and allowed to grow overnight before the different substances were added. In case of experiments which included treatment with a peptide inhibitor, this inhibitor was added 1 h before the addition of B3(Fv)-PE38 or TNFα. After 24 h the medium was removed and spun, and the scraped cells were added to this pellet and spun as well. The pellet was washed twice with ice-cold PBS and resuspended in 100 μL of lysis buffer (20 mM HEPES, pH 7.9; 0.2% NP-40; 10% glycerol; 0.1 mM EDTA; 400 mM NaCl; 1 mM DTT). Twenty microliters of the protease inhibitor cocktail "complete mini" (Boehringer Mannheim) was added. Cells were lysed for 30 min, and subsequently the lysate was cleared by centrifugation for 10 min at 12000*g*. All manipulations were done at 4 °C. Total protein content of the lysate was assayed with the Coomassie Plus Protein Assay Reagent (Pierce, Rockford, IL). Typically 10−30 μg of protein was resolved by SDS−PAGE. Subsequently, proteins were transferred to nitrocellulose in a Bio-Rad transblot apparatus at 250 mA for 2 h. Ponceau red staining was performed to ensure that equal amounts of protein were loaded. Blots were washed once in TBS-T (0.1% Tween-20) and blocked with 10% nonfat dry milk/TBS-T (blotto) for 1 h at room temperature and then incubated overnight at 4 °C with the primary antibody diluted in blotto. Membranes were washed five times with TBS-T and incubated with HRPO-conjugated affinity purified secondary antibodies (dilution 1:10 000) in blotto for 1 h at room temperature. Membranes were then washed five times with TBS-T, and the reaction was developed using the ECL detection system. The primary antibodies were used at the following dilutions: anti-PARP 1:2000, anti-CPP32 1:1000, anti-ICH-1L (Transduction) 1:1000, anti-ICH-1 (Santa Cruz) 1:200, and anti-ICEp20 1:200.

*CPP32-like DEVDase Activity.* The ApoAlert CPP32 fluorescent assay kit was used according to the instructions of the manufacturer (Clontech, Palo Alto, CA). Briefly, MCF-7 cells were seeded at $2 \times 10^6$ cells/T175 flask in 35 mL of medium and allowed to grow overnight before B3(Fv)-PE38 was added at 30 ng/mL for 16 h or at 30 μg/mL for 1−5 h. Cells were harvested and lysed in 100 μL of the provided buffer and chilled on ice for 10 min. A 50 μL reaction buffer, containing 10 mM DTT, was added to 50 μL of cell lysate. DEVD-AFC was added at a final concentration of 50 μM, and the samples were incubated at 37 °C for 1 h. Lysates from untreated cells were used as control. In the case where the aldehyde inhibitor was used, DEVD-CHO was added to the B3(Fv)-PE38-treated cell lysates at a final concentration of 10 μM before the

fluorogenic substrate was added, and the sample was incubated at 37 °C for 30 min. Cleavage of the fluorogenic substrate was quantitated using a fluorometer (excitation, $\lambda$ 400 nm; emission, $\lambda$ 505 nm).

*Cytotoxicity and Cell Viability Assays.* Inhibition of protein synthesis of B3(Fv)-PE38, TNFα, and cycloheximide was determined by [$^3$H]leucine incorporation as described (*8*). Cell viability determinations with WST-1 assays were carried out according to the instructions of the manufacturer (Boehringer Mannheim). Because only viable cells are capable of proliferation, this assay can be used to determine not only cell growth/proliferation but also the percentage of viable cells in a population. Briefly, cells were seeded at 3 $\times$ $10^3$ cells/well in 200 $\mu$L of medium into 96-well plates and allowed to grow overnight before addition of the different substances. In case of treatment with a peptide inhibitor, the final DMSO concentration never exceeded 0.1%. After 48 h of culture the reagent WST-1, a tetrazolium salt, was added at 20 $\mu$L/well. Metabolically active cells were allowed to produce the formazan product for 4-h at 37 °C. Optical density was read at 450 nm. Viability was also analyzed at different time points by the Trypan blue dye exclusion assay (*32*). All of the cytotoxicity and cell viability assays were performed in triplicate.

## RESULTS

*Immunotoxin B3(Fv)-PE38 Induces a Characteristic Cleavage of PARP to an 89 Da Fragment.* To determine whether the immunotoxin B3(Fv)-PE38 activates ICE/ced-3 proteases, now termed caspases, we investigated the cleavage of the DNA repair enzyme poly(ADP)-ribose polymerase (PARP), which is one of the first identified cellular substrates cleaved during apoptosis and a characteristic surrogate marker for apoptosis (*25*). Therefore, MCF-7 cells were treated with 30 ng/mL B3(Fv)-PE38 for various time periods. Then the cells were harvested, subjected to SDS−PAGE, and immunoblotted with an antibody to PARP. The concentration of B3(Fv)-PE38 used for treatment of MCF-7 cells was 30 ng/mL; this is approximately 30−40-fold higher than the $IC_{50}$ determined by analysis of protein synthesis inhibition in MCF-7 cells. For comparison purposes we treated cells with TNFα at 20 ng/mL, which induces programmed cell death and PARP cleavage in MCF-7 cells (*26*, *33*). As shown in Figure 1B, PARP (116 kDa) was significantly cleaved in MCF-7 cells after 16 h of exposure to B3(Fv)-PE38, which generated a characteristic PARP fragment of 89 kDa. The time course is similar to TNFα-induced cleavage of PARP (Figure 1B). The untreated control samples (Figure 1C) do not display PARP cleavage. Since 30 ng/mL B3(Fv)-PE38 and 20 ng/mL TNFα produced readily measurable effects on PARP cleavage, these concentrations were used for all subsequent experiments.

*Cycloheximide Does Not Induce PARP Cleavage in MCF-7 Cells.* Since PE-derived immunotoxins lead to protein synthesis inhibition, we wanted to know whether protein synthesis inhibition induces PARP cleavage by itself. To do this, we employed cycloheximide which inhibits protein synthesis by a different mechanism than PE, blocking the peptidyl transferase reaction on ribosomes. We treated MCF-7 cells for 24 h with cycloheximide at 1.2 or 2.4 $\mu$g/mL. These concentrations are 15- or 30-fold the $IC_{50}$ for

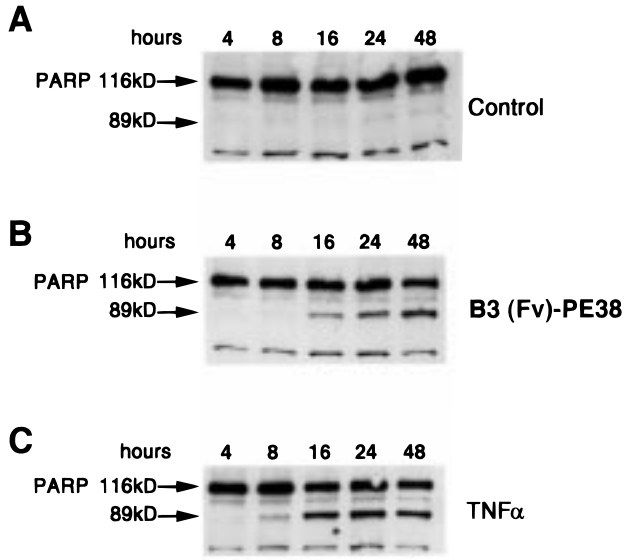


FIGURE 1: Immunotoxin B3(Fv)-PE38 induces a characteristic cleavage of PARP to an 89 kDa fragment in a time course similar to that of TNFα. Whole cell lysates of MCF-7 cells incubated with (panel A) vehicle alone, (panel B) B3(Fv)-PE38 (30 ng/mL), or (panel C) TNFα (20 ng/mL) for the indicated times were tested with the anti-PARP polyclonal antibody. 116 kDa is the size of the uncleaved PARP and 89 kDa the size of the cleaved fragment.

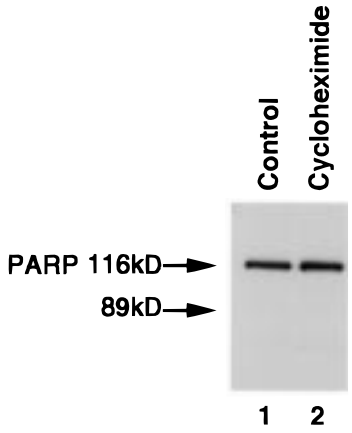


FIGURE 2: Cycloheximide does not induce PARP cleavage in MCF-7 cells. MCF-7 cells were exposed to cycloheximide at 1.2 $\mu$g/mL (lane 2) or the vehicle alone (lane 1) for 24 h after which cell lysates were prepared for immunoblotting with the anti-PARP polyclonal antibody. 116 kDa is the size of the uncleaved PARP and 89 kDa the size of the cleaved fragment.

[$^3$H]leucine incorporation (data not shown), thus comparable to the concentrations of immunotoxins that we used. Figure 2 shows that cycloheximide does not induce PARP cleavage at 1.2 or 2.4 $\mu$g/mL (data not shown for 2.4 $\mu$g/mL). Thus PARP cleavage is not a general response to inhibition of protein synthesis.

*Peptide Inhibitors zVAD-fmk, zDEVD-fmk, and zIETD-fmk Inhibit B3(Fv)-PE38- and TNFα-Induced PARP Cleavage.* To further assess the role of caspases in B3(Fv)-PE38-mediated apoptosis, we tested the influence of different cell permeable peptide-based fluoromethyl ketone inhibitors on PARP cleavage. zVAD-fmk is a broad spectrum inhibitor of most mammalian caspases (*34*, *35*). zDEVD-fmk is an inhibitor that is more selective for caspase-3 (CPP32) family proteases (caspases-3, -6, and -7) (*27*, *30*, *36*, *37*), and zIETD-fmk inhibits the first cleavage of the caspase-3 (CPP32) precursor to the p12 subunit and a 20 kDa protein

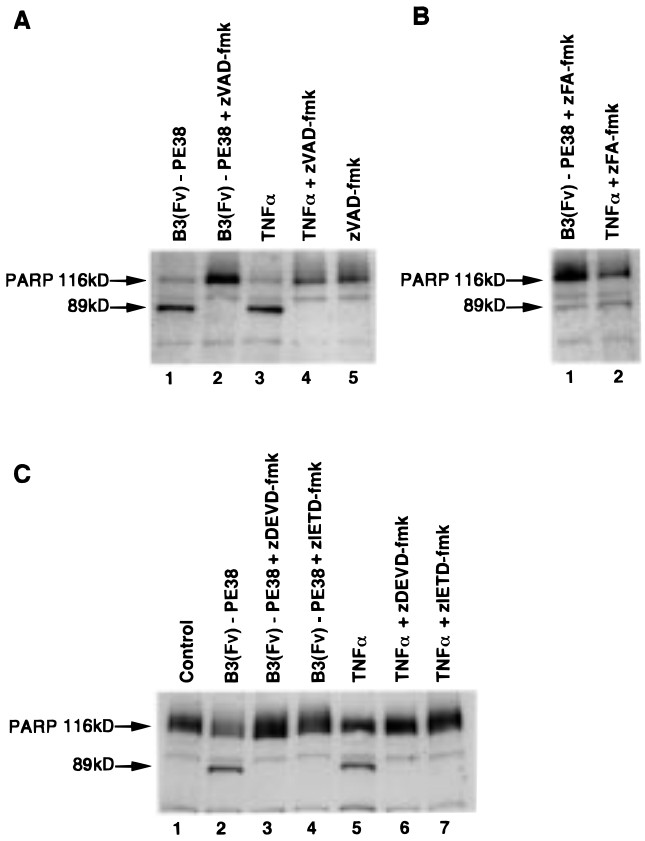


FIGURE 3: Peptide inhibitors zVAD-fmk, zDEVD-fmk, and zIETD-fmk inhibit B3(Fv)-PE38- and TNFα-induced PARP cleavage. MCF-7 cells were exposed to B3(Fv)-PE38 (30 ng/mL) or TNFα (20 ng/mL) for 24 h after which cell lysates were prepared for immunoblotting with the anti-PARP polyclonal antibody. The respective inhibitors were added at a final concentration of 50 $\mu$M 1 h prior the addition of B3(Fv)-PE38 or TNFα. Panel A: lane 1, B3(Fv)-PE38 alone; lane 2, B3(Fv)-PE38 with zVAD-fmk; lane 3, TNFα alone; lane 4, TNFα with zVAD-fmk; lane 5, zVAD-fmk alone. Panel B: lane 1, B3(Fv)-PE38 with control inhibitor zFA-fmk; lane 2, TNFα with control inhibitor zFA-fmk. Panel C: lane 1, vehicle alone; lane 2, B3(Fv)-PE38 alone; lane 3, B3(Fv)-PE38 with zDEVD-fmk; lane 4, B3(Fv)-PE38 with zIETD-fmk; lane 5, TNFα alone; lane 6, TNFα with zDEVD-fmk; lane 7, TNFα with zIETD-fmk. 116 kDa is the size of the uncleaved PARP and 89 kDa the size of the cleaved fragment.

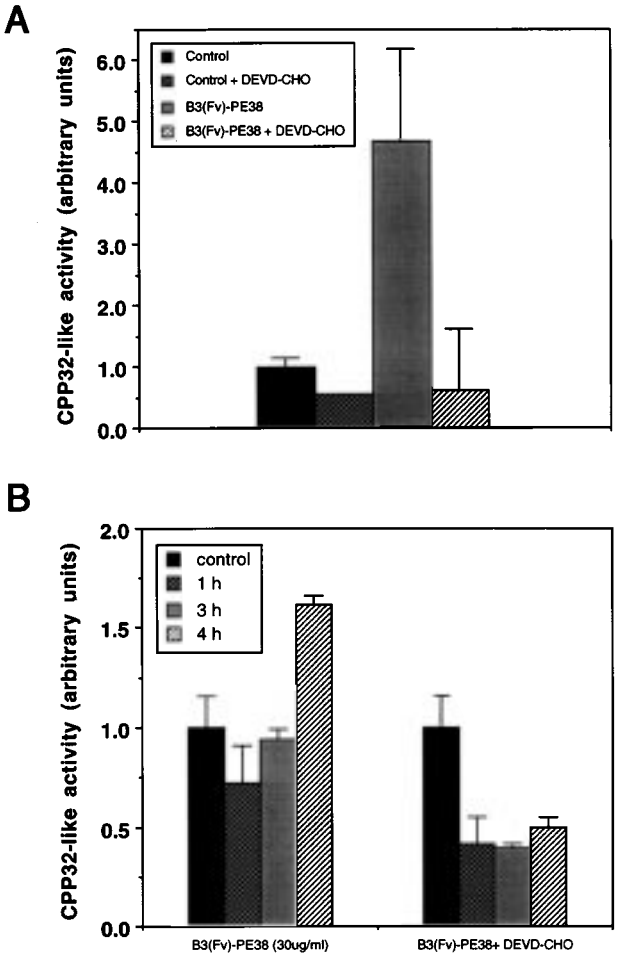


FIGURE 4: CPP32-like activity is induced by B3(Fv)-PE38 and inhibited by DEVD-CHO. Whole cell lysates of $1 \times 10^6$ MCF-7 cells, treated with B3(Fv)-PE38 or the vehicle alone, were incubated for 1 h at 37 °C in the presence of a fluorescent substrate DEVD-AFC (50 $\mu$M). In the case of the experiments with the inhibitor, DEVD-CHO (10 $\mu$M) was added for 30 min at 37 °C followed by the substrate. Cleavage of the fluorogenic substrate was quantitated using a fluorometer (excitation, $\lambda$ 400 nm; emission, $\lambda$ 505 nm). Panel A: cells were treated with B3(Fv)-PE38 at 30 ng/mL for 16 h before harvest. Panel B: cells were treated at 30 $\mu$g/mL B3(Fv)-PE38 for 1, 3, and 4 h, respectively. The data represent the mean ± SD of three experiments.

fragment (*37*). Panels A and C of Figure 3 show that B3(Fv)-PE38- as well as TNFα-induced PARP cleavage was completely blocked by treatment with all three peptide inhibitors used at a concentration of 50 $\mu$M each and added to the cells 1 h before exposure to B3(Fv)-PE38 or TNFα. Cells were exposed to the toxin or TNFα for 24 h. No inhibition of PARP cleavage was observed with the control inhibitor zFA-fmk, a cathepsin B inhibitor (Figure 3B), or vehicle alone (data not shown).

*CPP32-like Activity Is Induced by B3(Fv)-PE38 and Inhibited by DEVD-CHO.* The ability of the various inhibitors to block B3(Fv)-PE38-induced PARP cleavage suggested that a protease was induced or activated upon exposure to B3(Fv)-PE38. To measure protease activation, we utilized the fluorogenic substrate DEVD-AFC, which mimics the cleavage site at which CPP32 and CPP32-like caspases cleave PARP (*29, 36*). Cleavage of this substrate by whole cell extracts is thus indicative of *in vivo* proteolytic activation of these caspases in response to apoptotic signals. As shown in Figure 4A cleavage of DEVD-AFC with whole cell lysates from MCF-7 cells treated with B3(Fv)-PE38 (30 ng/mL) for 16 h increases about 4−5-fold when compared to the untreated control. Cleavage of DEVD-AFC by whole cell lysates from B3(Fv)-PE38 exposed cells was completely inhibited by preincubation of the cell extracts with the tetrapeptide aldehyde inhibitor DEVD-CHO at a final concentration of 10 $\mu$M. This indicates that B3(Fv)-PE38 exposure leads to the activation of a specific protease. Next we tried to define the earliest time point at which we could observe CPP32-like activity. Therefore, MCF-7 cells were treated with 3 or 30 $\mu$g/mL B3(Fv)-PE38 for 1, 2, 3, 4, and 5 h. The earliest activation of a CPP32-like enzyme was detected upon 4 h of treatment with B3(Fv)-PE38 (Figure 4B) and was a 1.5−2-fold increase compared to the untreated control. No difference was observed between 3 and 30 $\mu$g/mL of the immunotoxin (data not shown). We consistently observed a low level of DEVD-AFC cleavage in untreated MCF-7 cells, and DEVD-CHO-treated samples always showed values below the baseline level of the untreated control. These phenomena have been observed by others

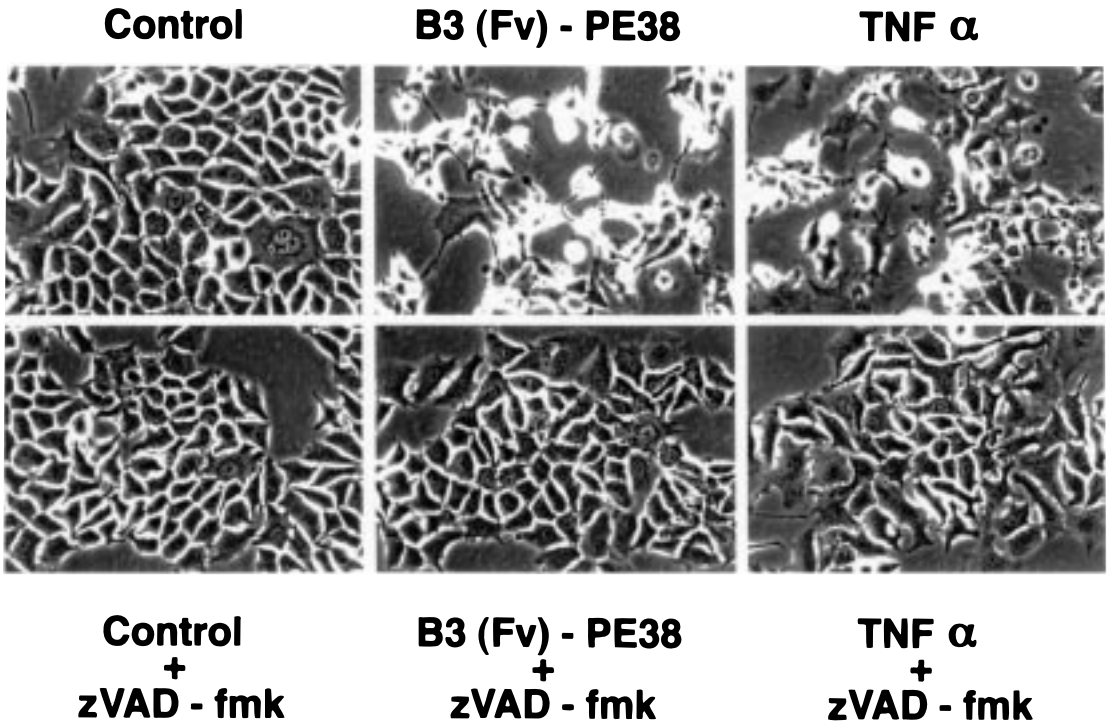


FIGURE 5: Peptide inhibitor zVAD-fmk prevents major morphological changes of MCF-7 cells after exposure to B3(Fv)-PE38 and TNFα. MCF-7 cells were seeded at a density of $4 \times 10^4$/mL in a 35 mm dish 1 day before exposure to B3(Fv)-PE38 or TNFα. zVAD-fmk was added 1 h prior the addition of B3(Fv)-PE38 (30 ng/mL) or TNFα (20 ng/mL) at a final concentration of 50 $\mu$M. Cells were exposed to the toxin or TNFα for 16 h before the photographs were taken in the medium. The final magnification is approximately 160-fold (figure reproduced at 75% of original size).

and may be a consequence of the low percentage of apoptotic cells in the normal cell population (*38*). Taken together, these results clearly show the activation of a CPP32-like protease in MCF-7 cells upon treatment with B3(Fv)-PE38 with the earliest detectable activation 4 h after exposure.

*Caspase-1 and Caspase-2 Cleavage Cannot Be Detected in B3(Fv)-PE38-Mediated Apoptosis.* Because our previous experiments indicated that CPP32 or a CPP32-like protease is the major caspase involved in B3(Fv)-PE38-mediated apoptosis, we examined CPP32 cleavage in immunoblotting experiments. However, using the well-known anti-CPP32 monoclonal antibody from Transduction Laboratories (*37, 39, 40*), we were unable to detect CPP32 in either cleaved or uncleaved form in MCF-7 cells. Up to 200 $\mu$g of protein was loaded on the SDS−PAGE gel without displaying a reactive band, although we needed much smaller protein concentrations to detect cleaved and uncleaved reactive bands in lysates of HL60 and HUT102 cells. Multiple species of CPP32, which vary with the cell line studied, have been described by others and are a possible explanation for this observation (*40*). Another explanation might be low abundance of the protein. Additionally, we used antibodies against the two other members of the three major caspase groups: caspase-1 (ICE) and caspase-2 (ICH-1). The uncleaved precursors of both caspases could be detected, but we could not unambiguously identify any cleavage products after B3(Fv)-PE38 treatment of MCF-7 cells (data not shown). Thus, it appears from the evidence presented above that a CPP32-like caspase is the major caspase activated upon stimulus with the immunotoxin B3(Fv)-PE38; assessing its precise identity however requires further evaluation. In MCF-7 cells CPP32-like protease activation occurs without detectable activation of caspases-1 and-2 upon exposure to B3(Fv)-PE38. Activation of caspases-1 and -2 can not be excluded, because the rate or amount of activation might be very low and therefore below the detection limit.

*Influence of Anti-Apoptotic Treatment on Morphology and Viability of MCF-7 Cells Exposed to B3(Fv)-PE38.* If protease activation has an important role in the death of cells caused by B3(Fv)-PE38, inhibitors of the proteases should prevent the morphological changes of the cells as well as have an effect on cell growth and viability. For these experiments we used the inhibitor zVAD-fmk, since it is considered a universal inhibitor of apoptosis (*34, 35*). Figure 5 shows the morphology of MCF-7 cells exposed to the toxin or TNFα for 16 h. The lower panel shows the cells to which zVAD-fmk was added at 50 $\mu$M 1 h before the other substances and demonstrates the beneficial effect of the inhibitor which prevents the severe morphological effects of B3(Fv)-PE38 and TNFα (Figure 5). The inhibitor alone has no influence on cell morphology (Figure 5). Since the phenomenon of apoptosis is also defined at the morphological level and is characterized by chromatin condensation and margination along the inner nuclear membrane, we examined the nuclear morphology of MCF-7 cells after staining with the DNA-binding dye propidium iodide by laser-scanning confocal microscopy. Untreated cells and cells pretreated with zVAD-fmk prior to toxin exposure reveal normal nuclei, whereas toxin-treated cells show condensation of chromatin and margination of the inner nuclear membrane (data not shown).

Another commonly used method to assess apoptosis is the demonstration of DNA degradation, although not all cell types show the typical DNA laddering. With MCF-7 cells a smear of degraded DNA in the low molecular weight range was visualized on an agarose gel with DNA samples from

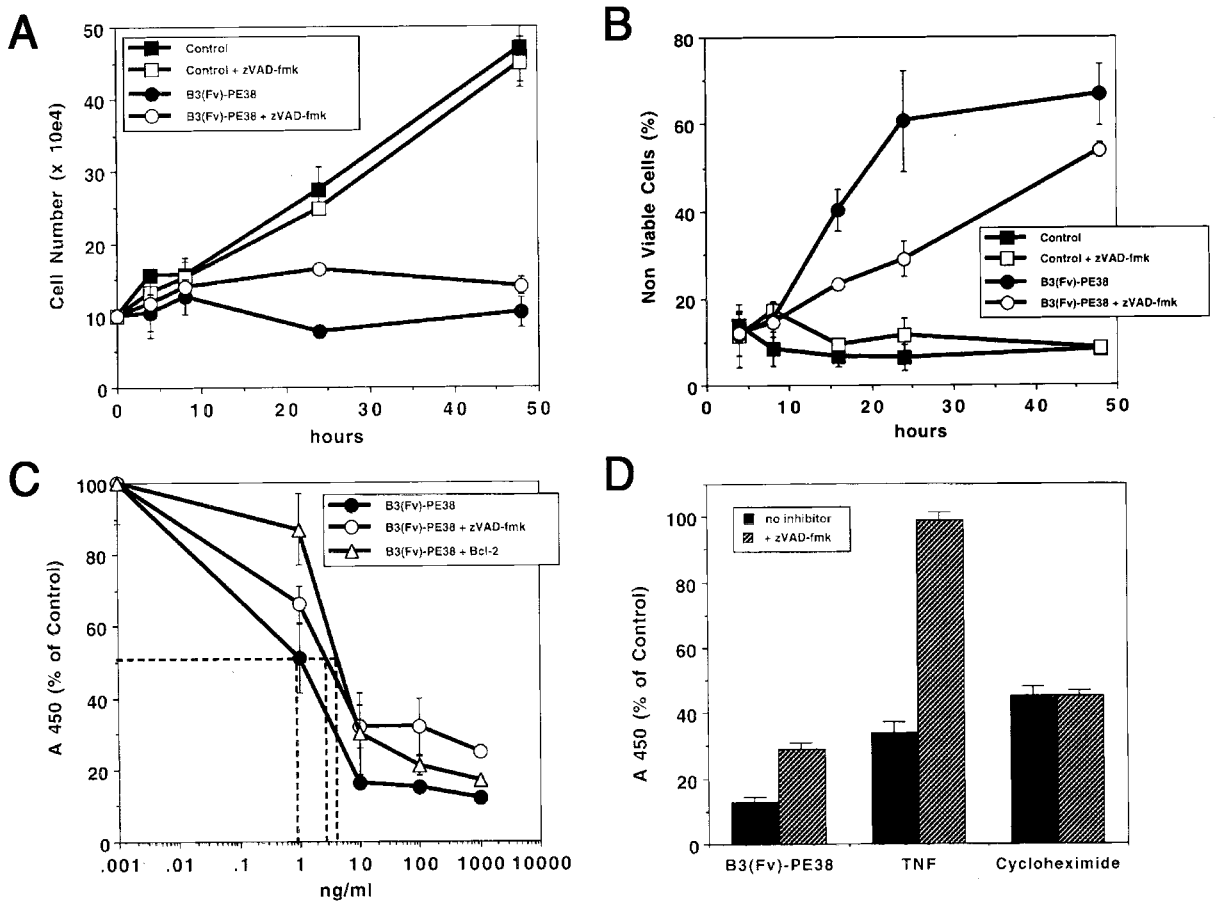


FIGURE 6: Influence of zVAD-fmk on the viability of B3(Fv)-PE38-treated MCF-7 cells. Panel A: The total number of cells was counted after trypan blue staining. Cells were treated either with or without B3(Fv)-PE38 and with or without pretreatment with zVAD-fmk (50 $\mu$M). The data represent the mean ± SD of three independent wells. Panel B: The percentage of nonviable cells in the presence or absence of B3(Fv)-PE38 (30 ng/mL) with or without pretreatment with zVAD-fmk (50 $\mu$M) was determined. Nonviability was assessed when the blue dye had penetrated the cell. The data represent the mean ± SD of three independent wells. Panel C: MCF-7 or MCF-7/Bcl-2 cells were seeded in 96-well plates 1 day before exposure to B3(Fv)-PE38. zVAD-fmk was added 1 h prior to the addition of B3(Fv)-PE38 at 50 $\mu$M. Cells were treated for 48 h before the tetrazolium salt WST-1 was added for 4 h. Formazan production was measued at OD 450 nm. The data represent the mean ± SD of three independent wells. Panel D: MCF-7 cells were seeded in 96-well plates 1 day before exposure to B3(Fv)-PE38 (100 ng/mL), TNFα (100 ng/mL), or cycloheximide (100 ng/mL). zVAD-fmk was added 1 h prior to the addition of B3(Fv)-PE38 at 50 $\mu$M. Cells were incubated for 48 h before the tetrazolium salt WST-1 was added for 4 h. Formazan production was measured at OD 450 nm. The data represent the mean ± SD of six independent wells.

cells treated with B3(Fv)-PE38 or TNFα; this degraded DNA is prevented by pretreatment of the cells with zVAD-fmk before exposure to B3(Fv)-PE38 or TNFα (data not shown).

We also analyzed cell viability by trypan blue exclusion. The results showed that zVAD-fmk enhanced the viability of B3(Fv)-PE38-treated MCF-7 cells 2−3-fold after 16 and 24 h of exposure to the toxin, but the beneficial effect of the apoptosis inhibitor decreased with time (Figure 6B). After 48 h of exposure to B3(Fv)-PE38 only very few of the cells treated with zVAD-fmk or not treated with the inhibitor were viable (Figure 6B). Thus the effects of the antiapoptotic treatment on trypan blue exclusion were transient. Calculating the total cell number from the trypan blue experiment showed that pretreatment of B3(Fv)-PE38-exposed cells with zVAD-fmk did not influence cell numbers significantly (Figure 6A), indicating that the B3(Fv)-PE38-mediated inhibition of protein synthesis does impair cell proliferation but in the presence of zVAD-fmk does not lead to a rapid loss of membrane exclusion of trypan blue. We next studied the effect of zVAD-fmk on cell viability using an assay in which viable cells metabolize a tetrazolium salt to formazan. The metabolic activity of the cells is considered a measure of cell viability and proliferation and is determined by adding a tetrazolium salt to the cells for 4 h after cells have been treated with B3(Fv)-PE38, TNFα, or cycloheximide with and without zVAD-fmk for 48 h. The formazan produced by viable cells is quantified spectrophotometrically. Panels C and D of Figure 6 show that MCF-7 cells become 2−4-fold resistant to B3(Fv)-PE38 with the inhibitor zVAD-fmk in this cell viability assay ($p < 0.0001$; paired $t$-test, $n = 6$; Figure 6D). In contrast, cells become fully resistant to TNFα upon treatment with zVAD-fmk. Cycloheximide-induced loss of viability is totally unaffected by the apoptosis inhibitor (Figure 6D). These experiments demonstrate that antiapoptotic treatment renders MCF-7 cells slightly, but significantly, more resistant toward immunotoxin treatment as assessed by different measurements of cell viability.

*Influence of zVAD-fmk on Protein Synthesis Inhibition in B3(Fv)-PE38-Treated MCF-7 Cells.* The earliest known action of PE and immunotoxins containing PE is inactivation of EF-2 due to ADP-ribosylation. This modification leads to protein synthesis inhibition which can be assayed by decreased [$^3$H]leucine incorporation (*11, 12*). The effect of B3(Fv)-PE38 on [$^3$H]leucine incorporation is shown in Figure

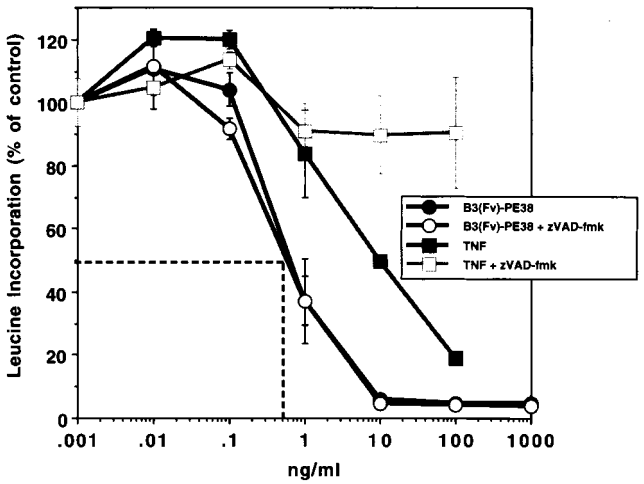


FIGURE 7: Influence of zVAD-fmk on protein synthesis inhibition in B3(Fv)-PE38- and TNFα-treated MCF-7 cells. MCF-7 cells were seeded in 96-well plates 1 day before exposure to B3(Fv)-PE38 or TNFα. zVAD-fmk was added 1 h prior to the addition of B3(Fv)-PE38 or TNFα at 50 $\mu$M. After 24 h [$^3$H]leucine was added for 2 h, then cells were harvested, and the [$^3$H]leucine incorporation was determined. Mean values of triplicates ± SD are given.

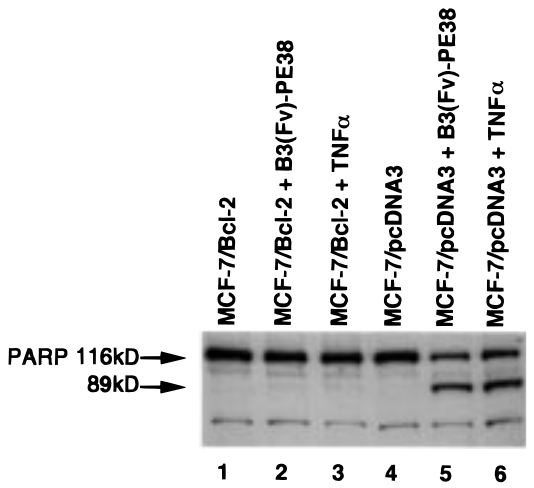


FIGURE 8: Bcl-2 inhibits B3(Fv)-PE38-induced PARP cleavage. MCF-7 stable transfectants with the human Bcl-2 gene or the vector pcDNA3 alone were exposed to B3(Fv)-PE38 (30 ng/mL) or TNFα (20 ng/mL) for 24 h after which cell lysates were prepared for immunoblotting with the anti-PARP polyclonal antibody. 116 kDa is the size of the uncleaved PARP and 89 kDa the size of the cleaved fragment.

7; the $IC_{50}$ is 0.7 ng/mL. Pretreatment of cells with zVAD-fmk had no effect on the ability of B3(Fv)-PE38 to inhibit protein synthesis, whereas it had a profound effect on TNFα ($p < 0.0028$ at 10 ng/mL; paired *t*-test, $n = 6$) (Figure 7). This indicates that inhibition of protein synthesis by TNFα is secondary to caspase activation which is blocked by zVAD-fmk, whereas the effect of B3(Fv)-PE38 on protein synthesis proceeds caspase activation and may in some way be responsible for caspase activation.

*Bcl-2 Overexpression Inhibits B3(Fv)-PE38-Induced PARP Cleavage.* Overexpression of Bcl-2 renders MCF-7 cells 3−10-fold more resistant to B3(Fv)-PE38 treatment (*19*). Using the ability of viable cells to produce formazan from a tetrazolium salt as measurement, Figure 6C shows a 3−4-fold increase in viability of B3(Fv)-PE38-treated MCF-7 cells overexpressing Bcl-2 compared to normal MCF-7 cells. Others have demonstrated that the anti-apoptotic protein Bcl-2 and its close homologue Bcl-$x_L$ can prevent CD95- as well as staurosporine-induced processing and activation of caspase-3 (CPP32), thus establishing a biochemical connection between Bcl-2 family proteins and ICE/ced-3 family proteins (caspases) (*35*, *41*, *42*). Therefore, we examined the influence of Bcl-2 overexpression on PARP cleavage in B3(Fv)-PE38-treated MCF-7 cells, since PARP is considered the major substrate for caspase-3 (CPP32) (*26*, *36*). As shown by the immunoblotting experiment in Figure 8, Bcl-2 overexpression inhibits PARP cleavage completely in MCF-7 cells treated with B3(Fv)-PE38 or TNFα for 24 h. Cells transfected with the vector pcDNA3 alone reacted with the expected cleavage of the substrate. We conclude that Bcl-2 acts by inhibiting the B3(Fv)-PE38-induced caspase activation which eventually leads to PARP cleavage.

## DISCUSSION

We have investigated the role of caspases in cell death produced by exposure to the immunotoxin B3(Fv)-PE38 and showed that there is PARP cleavage and increase in the activity of an enzyme with CPP32-like activity. Furthermore, caspase inhibitors reverse the effects of B3(Fv)-PE38 on cell morphology and transiently on cell viability.

It is well known that PE-containing immunotoxins induce cell killing by arresting protein synthesis due to inactivation of EF-2 (*11*, *12*). It has been shown by us and others that exposure of cells to toxins such as diphtheria toxin (DT) and *Pseudomonas* exotoxin A (PE) can cause apoptosis, though it has never been shown how these toxins induce apoptosis (*5*, *13*−*15*). It is not targeting by the Fv which triggers apoptosis, because PE alone causes PARP cleavage (our unpublished observation). Therefore, these studies are of general relevance to toxin action, since the mechanism of killing is identical whether an immunotoxin or the toxin itself is used. Immunotoxins with different scFvs, against different target antigens, can cause apoptosis as well (A. Keppler-Hafkemeyer and I. Pastan, unpublished observation, 1998).

*B3(Fv)-PE38 Induces PARP Cleavage.* Since the nuclear repair enzyme PARP is cleaved by caspases during apoptosis induced by TNF-RI and CD95 receptor triggering (*26*) as well as by a variety of other agents (*27*), we chose this substrate for our initial evaluation of caspase involvement upon immunotoxin exposure. We found that B3(Fv)-PE38 cleaves PARP in MCF-7 cells after 8−16 h of exposure. This time course is similar to TNFα-induced cleavage of PARP in MCF-7 breast cancer cells (*26*, *33*). In most experiments 30 ng/mL was purposely used in order to be in a saturating range of protein synthesis inhibition in order to clearly evaluate apoptotic mechanisms. Nevertheless, Figure 6C demonstrates that apoptosis is produced at 1 ng/mL, and this can be inhibited by the caspase inhibitor zVAD-fmk or by Bcl-2 overexpression.

*An Active Role for a CPP32-like Protease.* The presence of PARP cleavage in cells undergoing B3(Fv)-PE38-induced apoptosis suggests an active role for a CPP32-like protease (*29*, *36*, *43*−*45*). Cleavage of the fluorescent substrate DEVD-AFC by cell lysates from B3(Fv)-PE38-treated MCF-7 cells with 4−5-fold higher activity compared to the untreated control supports this view. Nevertheless, we were unable to detect the presence of CPP32 itself by immunoblotting. However, it is known that CPP32 is present in cells

as multiple active species, indicating differences in post-translational modifications as a possible explanation for the lack of detection. Another reason might be a low abundance of the protein (*40*). Further evidence of caspase involvement of immunotoxin-induced apoptosis is provided through studies with three cell-permeable fluoromethyl ketone inhibitors: zVAD-fmk, zDEVD-fmk, and zIETD-fmk. These inhibitors each affect several caspases, and there are likely to be further members of the family not yet identified. It is thus far impossible to identify unequivocally the enzyme, or enzymes, required for commitment to cell death under certain conditions, but the number of possibilities can be decreased. We found that all three inhibitors blocked B3(Fv)-PE38-induced PARP cleavage completely. zVAD-fmk potently inhibits several caspases so we cannot conclude which family members are critical for B3(Fv)-PE38-induced cell death. Because we cannot detect caspase-1 (ICE) or caspase-2 (ICH-1) activation by immunoblotting, these two caspases are unlikely to be involved. zDEVD-fmk and the DEVD-aldehyde, which efficiently blocks DEVD-AFC cleavage in our experimental system, are in addition to caspase-3 (CPP32) potent inhibitors of several caspases including caspase-7 (MCH-3), which is highly related to CPP32 (*43*). Our experiments suggest that CPP32 or a caspase related to this subfamily is predominantly involved in immunotoxin-induced apoptosis. The exact nature of this caspase remains to be determined, as well as the possible involvement of additional caspases in B3(Fv)-PE38-mediated apoptotic events.

*Effect of zVAD-fmk on Cell Morphology and Viability.* In order to learn more about the consequences of caspase activation in B3(Fv)-PE38-mediated cytotoxicity, the influence of zVAD-fmk was examined on cell morphology and cell viability. We show here that this broad spectrum caspase inhibitor almost completely blocks morphological changes induced by B3(Fv)-PE38 and TNFα after a period of 16 h of treatment. Measuring the cells capability to exclude the dye trypan blue reveals that zVAD-fmk makes cells exposed to the immunotoxin at least 2-fold less permeable for the dye for up to 24 h. A longer exposure of B3(Fv)-PE38 decreases the beneficial influence of the apoptosis inhibitor gradually. This is because apoptosis is blocked but protein synthesis inhibition is not. When cell viability was measured in an MTT-like assay, a similar result was obtained for B3(Fv)-PE38, namely, a 2−4-fold decrease in sensitivity to the toxin after zVAD-fmk pretreatment. In this assay TNFα-exposed cells become completely resistant after zVAD-fmk pretreatment, whereas the cycloheximide-induced reduction of cell viability is totally unaltered by the apoptosis inhibitor.

*zVAD-fmk Reduces Cells Sensitivity to the Toxin: Comparison with TNFα.* Our finding that caspases are involved in B3(Fv)-PE38-induced apoptosis underlines the critical role played by this family of proteolytic enzymes, as they appear to mediate almost every known form of apoptosis (*27*). Nevertheless, our data indicate that immunotoxin-induced apoptosis plays a much smaller role in cytotoxicity compared to TNFα, which is totally dependent on apoptotic mechanisms for cell killing. Overall, cells become only 2−3-fold more resistant toward immunotoxins upon treatment with the broad spectrum apoptosis inhibitor zVAD-fmk. However, this slight but significant reduction in sensitivity may be important at toxin concentrations that are not high enough or where the duration of exposure is not long enough to achieve sufficient inhibition of protein synthesis for direct killing of cells. Therefore, a 2−3-fold difference may have relevance in the treatment of cancer patients.

*Existence of a Common Pathway for Caspase Activation for PE, DT, and TNF.* The existence of a common pathway for DT, PE, and TNF-mediated apoptosis has been previously suggested on the basis of studies of synergistic cytotoxic effects of toxins and TNF (*14*, *18*). Our studies that show a similar time course of PARP cleavage induced by TNFα and B3(Fv)-PE38 and the prevention of PARP cleavage with the same three peptide-based inhibitors support this view.

*Influence of Bcl-2.* We have shown previously that Bcl-2 overexpression in MCF-7 cells renders cells 3−10-fold more resistant to B3(Fv)-PE38 but completely resistant toward TNFα as measured by viability assays (*19*). Here, we show that Bcl-2 overexpression completely blocks B3(Fv)-PE38- as well as TNFα-induced PARP cleavage. Others have demonstrated that Bcl-2 strongly suppresses CD95- and staurosporine-induced PARP cleavage in Jurkat cells (*35*, *42*). Bcl-2 and Bcl-x were shown to inhibit CD95- and TNFα-induced apoptosis in MCF-7 cells when analyzed by MTT assay (*46*). Taken together, these results support the view that Bcl-2 acts upstream of a CPP32-like protease (*42*).

*zVAD-fmk Does Not Alter the Toxin's Ability To Inhibit Protein Synthesis.* Although pretreatment with zVAD-fmk prevents B3(Fv)-PE38-induced PARP cleavage and renders cells 2−3-fold more resistant to the toxin, the ability of the immunotoxin to inhibit incorporation of [$^3$H]leucine into MCF-7 cells (the standard assay to quantitate PE-mediated toxicity) is unaffected. This explains that inhibition of apoptosis does not render cells fully resistant to immunotoxin-mediated cell death. A similar effect was previously observed with CAS antisense or the overexpression of Bcl-2 (*18*, *19*). Taken together, these observations support our suggestion that PE-induced caspase activation is a pathway downstream of protein synthesis inhibition but upstream of the "execution phase" of apoptosis. However, protein synthesis inhibition alone is not sufficient to induce apoptosis; for example, cycloheximide, which induces protein synthesis inhibition by a different mechanism of action, does not induce B3(Fv)-PE38-mediated PARP cleavage in MCF-7 cells. However, it has been reported that cycloheximide-mediated protein synthesis inhibition can induce apoptosis under certain experimental conditions, and also the delay or the inhibition of apoptosis has been reported (*47*−*50*). It appears that protein synthesis inhibition is not important per se, but the means by which protein synthesis is inhibited is important; e.g., cycloheximide inactivates ribosomes while toxins PE and DT inactivate elongation factor EF-2.

*Possible Implications of the Fact That Immunotoxins Mediate Apoptosis.* It is obvious that PE-containing immunotoxins can kill target cells by two mechanisms: (i) direct inhibition of protein synthesis and (ii) induction of apoptosis. The distinction of these death pathways and quantitation to what extent each mechanism contributes to the elimination of cancer cells by immunotoxins may be important for therapeutic applications of immunotoxins, especially because certain cancer types may be more resistant to apoptotic mechanisms than others. Our results clearly indicate that tumors exhibiting anti-apoptotic programs, for example

through overexpression of Bc1-2, can still be killed by immunotoxin therapy. On the other hand, these two signaling pathways offer the interesting possibility to protect organs endangered due to certain side effects by targeted delivery of caspase inhibitors, once more specific caspase inhibitors will be available and thereby widening the treatment window.

Further evaluation is needed to define how PE-containing immunotoxins lead to caspase activation, a process obviously located downstream of the mechanism of ADP-ribosylation of EF-2. In our experimental system we observed that 4 h of exposure to saturating levels of B3(Fv)-PE38 is the minimum time interval required for the detection of caspase activation. This 4 h time interval provides adequate time for the immunotoxin to reach the cytosol and begin to arrest protein synthesis (*51*). Future experiments will try to identify the mechanism by which EF-2 inactivation leads to caspase activation.

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