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Differential activity of bcl-2 and ICE enzyme family protease inhibitors on Fas and puromycin-induced apoptosis of glioma cells

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Abstract

Fas ligand is a potent inducer of apoptosis in human glioma cells by the Fas/Fas ligand pathway. With comparable efficiency, metalloprotease inhibitors including puromycin and bestatin induce apoptosis in glioma cells. To evaluate the involvement of potential components involved in Fas ligand- and metalloprotease inhibitor-induced apoptosis, we investigated the effect of anti human Fas antibody, soluble Fas ligand and puromycin on cultures of human malignant glioma cell lines (LN-18, LN-229, T98G). Stimulation with Fas ligand lead to apoptotic cell death within 16 h. Costimulation with the translational inhibitor cycloheximide and the transcription blocker actinomycin D did not reduce Fas ligand toxicity. In contrast, apoptosis induced by puromycin was blocked by cycloheximide and decreased by subtoxic doses of actinomycin D in all three gliomas. Whereas inhibition of caspase activity with the general inhibitor zVAD-fmk resulted in a complete block of Fas ligand-induced cell death, puromycin-mediated apoptosis was found to be unaffected by zVAD-fmk as well as by more specific inhibitors for caspase-1 (Interleukin-1 β converting enzyme) and caspase-3 (CPP32/Yama). Other prominent components involved in many apoptotic pathways as bcl-2 and reactive oxygen intermediates were also examined. Bcl-2 which protects glioma cells from Fas ligand-induced cell death, was shown to have only a small protective effect on puromycin-induced apoptosis. The tested radical scavengers did not reduce Fas- or puromycin-mediated killing of human glioma cells.

Keywords: Apoptosis; bcl-2; Fas; Human glioma; Caspase; Protease inhibitor

1. Introduction

Induction of apoptosis by Fas ligand (FasL) through binding to its cell surface receptor Fas has been shown to be an effective way to induce programmed cell death (PCD) in several different cell types. In the treatment of human gliomas, induction of apoptosis by the Fas/FasL system has been suggested as an effective way to eliminate tumor cells. Human malignant glioma cells are also forced into apoptosis by metalloprotease inhibitors including puromycin (PM) and bestatin. PM exerts multiple effects on very different components of the cellular metabolism and signal transduction machinery. Dependent on dosage, PM has been shown to inhibit protein synthesis [1,2], irreversibly block the activity of several enzymes

Abbreviations: ActD, actinomycin D; αFas Ab, anti-human Fas antibody; BPN, *N-tert*.-butyl-α-phenyl-nitrone; CHX, cycloheximide; FasL, Fas ligand; HSP, heat shock proteins; ICE, interleukin-1 β converting enzyme; NAC, *N*-acetyl-L-cystein; PCD, programmed cell death; PM, puromycin; ROI, reactive oxygen intermediates

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including aminopeptidases and acetylcholinesterases [3–6], induce c-myc RNA superinduction [7,8], inhibit protein import into mitochondria [9], or confer thermoresistance to selected types of cells [10,11].

The mechanism of action of PM is well established for some of these phenomenons but less well characterized for others. In protein synthesis for example, PM acts as an analogue of aminoacyl-*t*RNA leading to premature release of nascent polypeptide chains [12]. Inhibition of aminopeptidases has been shown to be competitive [13,14] while action on acetylcholinesterase activity has been found to be noncompetitive [15,16]. Superinduction refers to augmentation and stabilization of mRNA levels through the action of PM which at high dosage affects protein synthesis. Another mechanism of superinduction is operative when using PM at low levels not affecting protein synthesis [8]. Inhibition of protein import into mitochondria and induction of thermotolerance can both be caused by PM. The compound acts on completely distinct pathways involving either ATP-dependent steps inside the inner mitochondrial membrane or increased synthesis of stress proteins of the HSP family respectively [9,10]. To learn more about the mechanisms involved in induction of apoptosis in human malignant gliomas, we investigated the functional role of molecules known to regulate PCD for their influence on apoptosis of gliomas induced by low doses of PM in comparison to apoptosis induced by the Fas/FasL system.

2. Material and methods

2.1. Materials

The human glioma cell lines LN-18 and LN-229 were kindly provided by Dr. N. Tribolet (Lausanne, Switzerland). T98G human glioma cells were obtained from ATCC (Rockville, MD). Bcl-2 overexpressing cells were generated by transfecting human malignant glioma cell lines [17]. G418 was purchased from Gibco BRL (Basel, Switzerland). The reagents for in situ DNA end labeling were purchased from Boehringer Mannheim (Germany). PM, actinomycin D (ActD) and cycloheximide (CHX) were obtained from Fluka (Switzerland), anti-human Fas monoclonal antibody (αFas Ab) was from Kamiya (Thousand Oaks, CA). The caspase inhibitors acetyl-Tyr-Val-Ala-L-Asp-aldehyde (Ac-YVAD-cho), acetyl-Tyr-Val-Ala-L-Asp-chloromethylketone (Ac-YVAD-cmk), acetyl-Asp-Glu-Val-L-Asp-aldehyde (Ac-DEVD-cho), and benzyloxycarbonyl-Val-Ala-DL-Asp-fluoromethylketone (zVAD-fmk) were from Bachem (Switzerland), *N*-Acetyl-L-cysteine (NAC) and *N-tert*.-butyl-α-phenyl-nitrone (BPN) were from Sigma (St. Louis, MO), and Alamar Blue was purchased from Biosource (Lucerna-Chem, Switzerland). FasL and control supernatants were generated by Neuro-2a cells transfected with mouse FasL cDNA or with the vector alone, respectively [18]. Treatment of cells with FasL in combination with the radical scavengers NAC and BPN was performed using previously concentrated FasL.

2.2. Cell culture

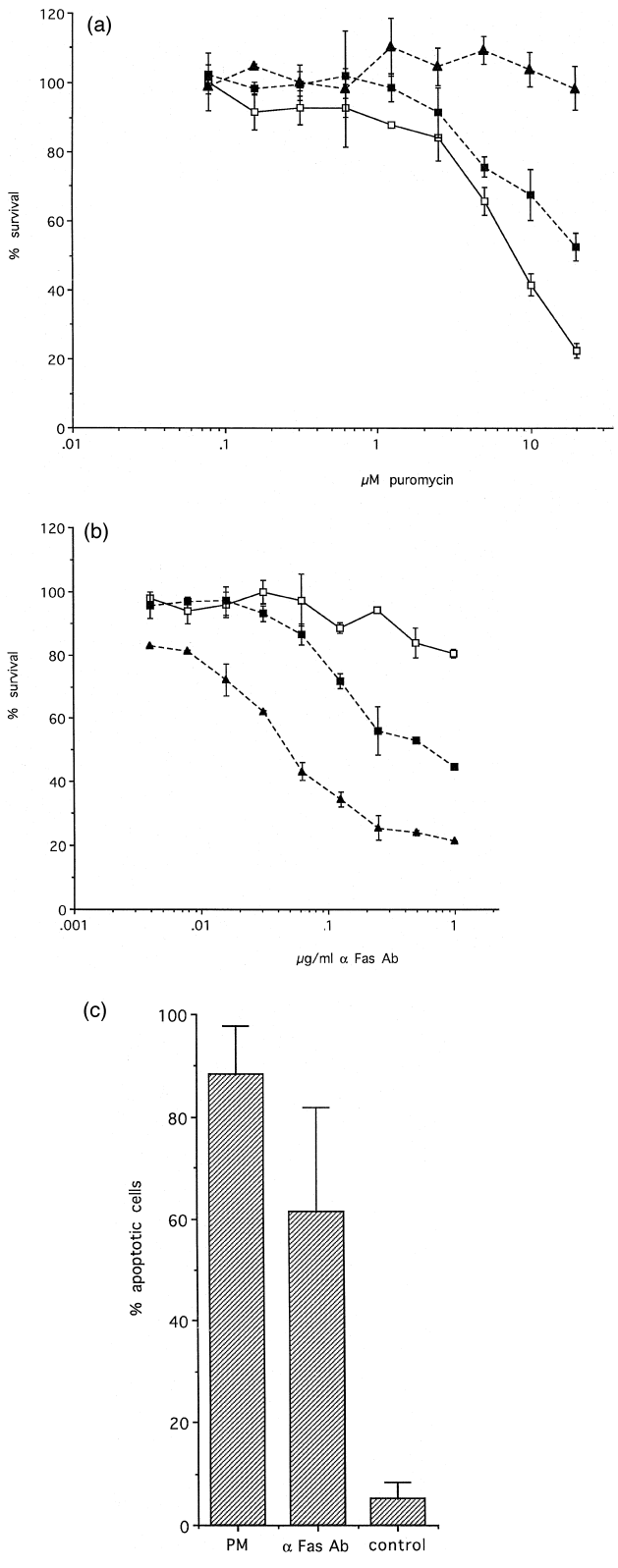
Cells were cultured in DMEM containing 10% FCS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin as described previously [17]. Cells transfected with bcl-2 were maintained in medium supplemented with G418 (500 μg/ml). For the treatment of cells with inhibitors, serum content in the culture medium was reduced to 0.5%.

2.3. Cell viability

Cell viability assays were performed in microtiterplates (Falcon 3072). Viability of the cells exposed to αFas Ab, FasL or PM was measured after 16 h by incubation further with Alamar Blue for 3 h. Extinction was read on a CytoFluor 2350 (Millipore). Staining of the adherent cells was done with crystal violet and absorption was measured on an ELISA reader at 540 nm.

2.4. In situ DNA end labeling

Cells were plated onto Labtek 8-well chamber slides and cultured in serum-reduced culture medium. Incorporation of biotinylated deoxyuridine triphosphate (50 μM) was performed using terminal transferase (0.25 U/ml) followed by a streptavidine-alkaline phosphatase detection system as described [17].



3. Results

To investigate the involvement of putative intracellular regulators of PCD in PM-induced apoptosis, we examined cellular activity, morphology and cell survival of the human malignant glioma cell lines LN-18, LN-229 and T98G after treatment with varying dosages of PM from 0.1 μM to 20 μM. Evaluation of the importance of protein synthesis, Bcl-2, caspases, or reactive oxygen intermediates (ROI) was done by pre- and coincubation of specific inhibitors in the case of protein synthesis, caspases and ROI, or by overexpression of bcl-2 in transfected glioma cell lines. For comparison, all the inhibitors used were tested in parallel on their influence on apoptosis induced by FasL and α Fas Ab, the apoptotic pathway being described to be regulated by caspase-1 (ICE) and bcl-2.

3.1. Effect of puromycin on human gliomas

Cultured human malignant glioma cell lines (LN-18, LN-229, T98G) were killed by PM in a dose-dependent manner (Fig. 1(A)). The concentration of PM

Fig. 1. (A) Cycloheximide (CHX) and actinomycin D (ActD) block and reduce PM-induced apoptosis respectively. LN-18 cells were cultured to 90% confluency and stimulated with varying concentrations of PM with or without CHX or ActD for 16 h. Cell survival was measured by crystal violet staining in reference to control cultures treated with CHX or ActD in the absence of PM. Error bars show standard deviation of mean values of triplicate measurements. – □ – PM, -- ▲ -- PM and CHX (10 μg/ml), -- ■ -- PM and ActD (0.5 μg/ml). (B) CHX and ActD enhance cytotoxicity of anti human Fas antibody (α Fas Ab) on glioma cells. Human glioma cells (T98G) were treated for 24 h with up to 1 μg/ml monoclonal α Fas Ab alone, or together with CHX or ActD. Comparable results were obtained with the other cell lines LN-18 and LN-229. Controls and error bars as in Fig. 1(A). – □ – α Fas Ab, -- ▲ -- α Fas Ab and CHX (10 μg/ml), -- ■ -- α Fas Ab and ActD (0.5 μg/ml). (C) In situ DNA end labeling (TUNEL) of PM- and α Fas Ab-stimulated human glioma cells confirms apoptotic cell death. TUNEL assay was performed 16 h after stimulation of LN-18 cells with 10 μM PM or 1 μg/ml α Fas Ab. The number of positively stained floating cells were compared to the total number of cells in the stimulated cell. Comparable results were obtained with LN-229 and T98G cells. Error bars show standard deviation of mean values of three experiments.

needed to kill 50% of the cells within 16 h (5–7 μM) was comparable to the amount needed to force other cell lines (JURKAT, LAN-1, COS-7) into apoptosis (data not shown). After 16 h of 10 μM PM treatment, up to 90% of the cells detached from the culture plate surface compared to 60% after treatment with 1 μg/ml αFas Ab and about 10% of the culture medium-treated cells. The correlation between loss of cell adherence and PCD was confirmed by in situ DNA end labeling (TUNEL). TUNEL staining was found to be positive in more than 90% of the detached cells Fig. 1(C). Furthermore, agarose gel electrophoresis of cellular DNA isolated after 16 h of PM incubation revealed the characteristic internucleosomal cleavage pattern.

3.2. Effect of actinomycin D and cycloheximide on puromycin- and Fas-mediated apoptosis

Addition of the transcriptional inhibitor ActD (0.5 μg/ml) and the translational blocker CHX (10 μg/ml) lead to lower killing rates in the case of ActD and to a complete block of induction of apoptosis upon costimulation of the cells with PM and CHX (Fig. 1(A)). Addition of increasing amounts of ActD and CHX lead to higher protection. Above critical levels of the inhibitors, blockage of the protein synthesis pathway caused toxic effects with decreased cell proliferation and cell survival. The effects of CHX and ActD were observed not only in LN-18 but also when using the LN-229 or T98G cells. For comparison, the same human glioma cell lines were coexposed to either ActD or CHX, together with varying concentrations (0.01–1 μg/ml) of monoclonal αFas Ab. In Fas-mediated apoptosis of the cell lines tested, an increase in cell killing of up to 40% in the presence of ActD (0.5 μg/ml) and of up to 70% in the presence of CHX (10 μg/ml) was observed at αFas Ab-concentrations of 0.3 μg/ml (Fig. 1(B)). Because of the high killing efficiency of the FasL supernatant on the cell lines tested, addition of ActD or CHX did not increase the amount of cell death after 16 h.

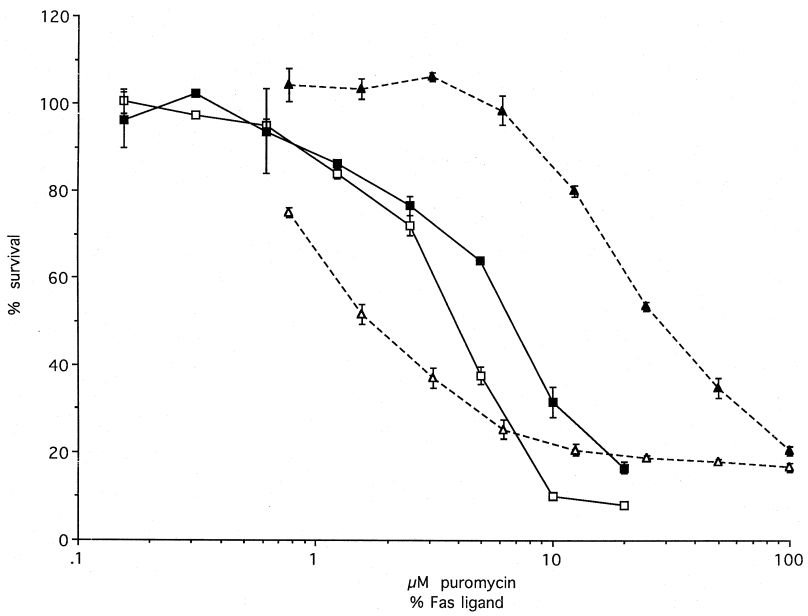


Fig. 2. Whereas bcl-2 overexpression confers resistance to Fas ligand (FasL)-induced apoptosis, bcl-2 does only minimally affect PM-induced apoptosis. LN-18 cells transfected with a bcl-2 overexpressing construct (LN-18 bcl-2) were compared to control transfected, neomycin resistant cells (LN-18 neo). Apoptosis was induced by 16 h-incubations with various concentrations of PM and FasL and assessed as described in legend to Fig. 1. – □ – PM-treatment of LN-18 neo; – ■ – and of LN-18 bcl-2 cells; — △ — treatment with FasL of LN-18 neo cells; — ▲ — and of LN-18 bcl-2 cells.

3.3. Influence of overexpression of bcl-2 on apoptosis induced by puromycin and Fas ligand

Overexpression of bcl-2 in all three human glioma cell lines (LN-18, LN-229, T98G) reduced the cytotoxicity of PM, although to a much lesser extent than apoptosis induced by FasL (Fig. 2). The dose response curve showed maximum protection by bcl-2 of 80% at FasL supernatant concentrations of 7%. The respective value for PM treated cells was 20% protection after addition of 10 μM PM (Fig. 2). Likewise bcl-2 was able to interfere with staurosporine-induced apoptosis (data not shown).

3.4. Involvement of caspases in puromycin- and Fas ligand-induced apoptosis

Involvement of caspases in PM- and FasL-induced apoptosis was tested by pre- and coincubation of the cells with specific tetrapeptide inhibitors of caspase-1 (ICE: peptide inhibitors Ac-YVAD-cho and Ac-YVAD-cmk) and of caspase-3 proteases (CPP32/Yama: inhibitor Ac-DEVD-cho), and with the more general caspase inhibitor zVAD-fmk. PM-induced apoptosis was neither inhibited by the three specific inhibitors for caspase-1 and caspase-3 nor by the general caspase inhibitor (Fig. 3(A)). To ensure that the concentrations of the caspase inhibitors were sufficient to protect the cells from apoptosis induced by proper stimuli, LN-18 and LN-229 cells were treated with up to 500 μM of the caspase inhibitors 1 h before and during exposure to FasL. Addition of > 10 μM of zVAD-fmk lead to a complete block of FasL-induced apoptosis after 16 h. Incubation with the tetrapeptide-inhibitors specific for caspase-1 (Ac-AVAD-cmk) and for caspase-3 (Ac-DEVD-cho) increased cell survival by about 10% and 20% respectively compared to Neo supernatant-treated cells (Fig. 3(B)).

3.5. Reactive oxygen intermediates in puromycin- and Fas ligand-induced apoptosis

ROI have been discussed as mediators of apoptotic cell death induced by a number of cytotoxic agents. Dependent on the cells examined and the apoptotic stimulus used, radical scavengers were shown to inhibit the cell death pathway. In the human glioma

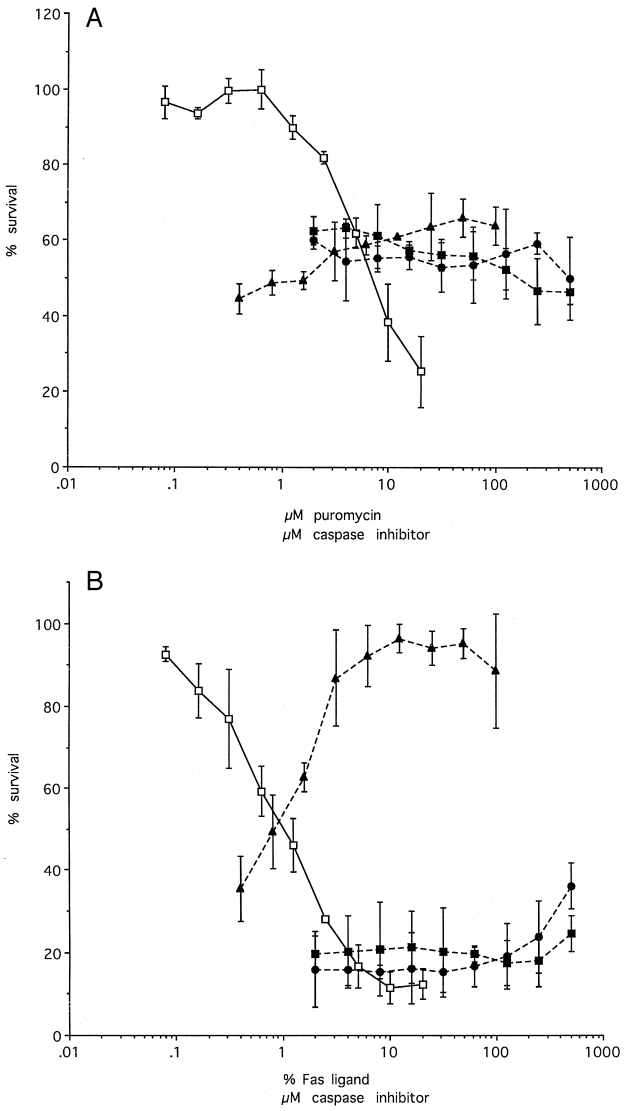


Fig. 3. (A) Caspase inhibitors have no effect on PM-induced cell death. LN-18 cells were preincubated for 1 h with the indicated amounts of the general caspase inhibitor zVAD-fmk, the caspase-1 tetrapeptide inhibitor Ac-YVAD-cmk, and the caspase-3 inhibitor Ac-DEVD-cho. After preincubation, cells were stimulated for 16 h with 5 μM PM in presence of the varying amounts of the inhibitors. For comparison, a dose response curve to PM is shown (– □ –). Cell survival was determined as in Fig. 1. – □ – PM, – ▲ – 5 μM PM and zVAD-fmk, – ■ – 5 μM PM and Ac-YVAD-cmk, – ● – 5 μM PM and Ac-DEVD-cho. (B) Inhibitors of caspase-1 and caspase-3 and a general caspase inhibitor reduce and block FasL-induced cell death. LN-18 cells were treated as in Fig. 3(A). – □ – FasL, – ▲ – 5% FasL and zVAD-fmk, – ■ – 5% FasL and Ac-YVAD-cmk, – ● – 5% FasL and Ac-DEVD-cho.

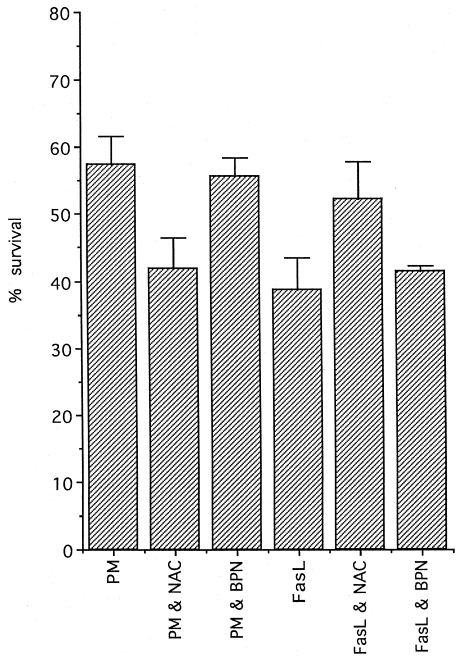


Fig. 4. Radical Scavengers fail to inhibit PM-induced apoptosis. After preincubation with NAC (5 mM) or BPN (20 μM) for 15 min, LN-18 cells were stimulated with 5 μM PM or with concentrated and rediluted FasL at a final concentration of 50%. Determination of cell survival was done as described in Fig. 1.

cells tested, addition of the radical scavengers NAC and BPN did not influence apoptosis induced by PM or FasL (Fig. 4).

4. Discussion

When treating glioma cell lines with PM the cells undergo apoptosis. We find PM-induced apoptosis to be inhibited by up to 30% by ActD and to be completely blocked by costimulation with CHX. Whether the different efficiencies of ActD and CHX in inhibiting PM-induced cell death really represent a clue to the mode of action of PM in this process remains to be shown. Nonetheless, protein synthesis as a process including both, mRNA transcription and protein translation is an obligatory part of PM-induced apoptosis. Our results are in agreement with recent data on neurons, there apoptosis induced by PM was also inhibited by CHX [19].

Although high protein levels of the antiapoptotic proto-oncogene bcl-2 confer resistance to different inducers of PCD including FasL or αFas Ab, TNF-α, growth factor deprivation, γ-irradiation, multiple chemotherapeutic drugs, as well as many others [17,20,21], we did not find significant reduction of PM-triggered killing of transfected cells overexpressing bcl-2. This finding might reflect an apoptotic pathway not involving bcl-2 or the need of additional regulatory factors besides bcl-2 to prevent apoptosis [22]. Sufficient expression of bcl-2 to reduce apoptosis was assured by the reduced cell death of the bcl-2 overexpressing glioma cell lines upon exposure to FasL. In contrast to our results, in other cell types bcl-2 has been found to be ineffective in preventing Fas-mediated cell death [23]. Future experiments will have to show whether bcl-2 interferes more efficiently with PM-cytotoxicity in other cell types and which additional cell type-specific components are responsible for the differential protection by bcl-2 overexpression from apoptosis induced by PM or FasL.

Enzymes of the caspase family were shown to be key molecules involved in many apoptotic pathways. The inhibition of caspases greatly reduces the induction of apoptosis by FasL and other stimuli including staurosporine, cycloheximide, thapsigargin and etoposide [24,25]. This is confirmed in the study presented here. The addition of tetrapeptide-inhibitors specific for caspase-1 (ICE) and caspase-3 (CPP32/Yama) produced a marked decrease of apoptosis induced by the Fas/FasL system. By blocking all known caspases with the general inhibitor zVAD-fmk, FasL-induced apoptosis is completely abolished. Induction of apoptosis by PM is found unaffected by the specific inhibitors for caspase-1 and caspase-3 and by the general caspase inhibitor. This finding is in accordance with other ways of inducing PCD, for example with the recently shown induction of cell death by overexpression of bax, also being independent of caspase activity [26].

In some systems of PCD, the elimination of reactive oxygen intermediates leads to inhibition of the death program [20]. Depending on the cell types used, Fas-mediated apoptosis has been shown to be reduced [27] or unaffected by radical scavengers [28]. Pre- and coincubation of the glioma cells with the radical scavengers NAC and BPN showed no effect on the rate of cell killing by PM or FasL.

Taken together, these findings suggest a pathway of cell death induced by PM, which is shared in part

with other apoptotic pathways namely the requirement of protein synthesis. Whereas the influence of bcl-2 was less pronounced, other key regulators like caspases did not modulate PM-induced apoptosis. Thus, PM-induced apoptosis follows not yet identified apoptotic pathways which may be caused by blocking the action of puromycin-specific components like aminopeptidases or inhibition of transcriptional processes. Further investigations will have to prove whether the signal pathways in PM-induced and other apoptotic processes are completely distinct or whether they converge downstream of the caspases.

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