Caspase activity is required for commitment to Fas-mediated apoptosis

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Recognition of the widespread importance of apoptosis has been one of the most significant changes in the biomedical sciences in the past decade. The molecular processes controlling and executing cell death through apoptosis are, however, still poorly understood. The ICE (Interleukin-1 β Converting Enzyme) family—recently named the caspases for cysteine aspartate-specific proteases—plays a central role in apoptosis and may well constitute part of the conserved core mechanism of the process. Potentially, these proteases may be of great significance, both in the pathology associated with failure of apoptosis and also as targets for therapeutic intervention where apoptosis occurs inappropriately, e.g. in degenerative disease and AIDS. However, this is only likely if caspase activity is required before commitment to mammalian cell death. Here, we have used both peptide inhibitors and *crmA* transfection to inhibit these proteases in intact cells. Our experiments show that selective inhibition of some caspases protects human T cells (Jurkat and CEM-C7) from Fas-induced apoptosis, dramatically increasing their survival (up to 320-fold) in a colony-forming assay. This suggests that dysfunction of some, but not all, caspases could indeed play a crucial part in the development of some cancers and autoimmune disease, and also that these proteases could be appropriate molecular targets for preventing apoptosis in degenerative disease.

Keywords: apoptosis/caspase/CD95 (Fas/APO-1)/interleukin 1 β converting enzyme/MACH/FLICE

Introduction

Studies on a range of experimental systems have suggested that the ICE family of cysteine proteases, recently renamed the caspases (Alnemri *et al.*, 1996), play a central role in active cell death which has been conserved over a long period of evolution (reviewed by Thompson, 1995; also Martin and Green, 1995; Yuan, 1995; Hale *et al.*, 1996; Whyte, 1996). In *Caenorhabditis elegans*, loss of function of *ced-3*, which encodes a nematode caspase, prevents developmentally programmed cell death (Ellis and Horvitz, 1986), and in *Drosophila*, inhibition of caspases inhibits both developmental apoptosis (White *et al.*, 1996) and that induced artificially by transfection of the apoptosis inducer *reaper* (Pronk *et al.*, 1996).

Control of apoptosis in mammalian cells is, not surprisingly, more complex than in invertebrates (Wyllie *et al.*, 1980; Hale *et al.*, 1996). Inhibition of caspases with peptide inhibitors designed to mimic known target sequences has been shown to suppress apoptotic changes in several short-term vertebrate cell culture systems (Chow *et al.*, 1995; Enari *et al.*, 1995; Fearnhead *et al.*, 1995; Los *et al.*, 1995; Milligan *et al.*, 1995; Zhu *et al.*, 1995; Jacobson *et al.*, 1996; Schlegel *et al.*, 1996; Slee *et al.*, 1996) including the early formation of high-molecular weight DNA fragments (Zhu *et al.*, 1995). However, it is not yet known if the process can be completely prevented in this way, or if it is merely delayed, with the cells dying, either through necrosis or apoptosis, some days later. Indeed, analysis of the effect of the peptide inhibitor Z-VAD.fmk on apoptosis in Rat-1 fibroblasts has suggested that caspase activity is not required for plasma membrane blebbing, an early event in apoptosis, in this system (McCarthy *et al.*, 1997). In addition, previous studies on vertebrate or invertebrate cells have not employed colony-forming assays, and it is therefore crucial to determine whether mammalian cells protected from apoptosis by suppression of caspase activity can survive and subsequently form colonies.

In mammalian cells, apoptosis can be induced by a diverse range of stimuli, many of which are cell type-specific (Wyllie *et al.*, 1980; Martin and Green, 1995; Thompson, 1995; Hale *et al.*, 1996). Induction of apoptosis in T lymphocytes expressing the cell surface receptor Fas (Apo-1/CD95) (Trauth *et al.*, 1989; Yonehara *et al.*, 1989) is likely to be an important element in the physiological regulation of T-cell populations, particularly in the later stages of an immune response, and in T-cell-mediated cytotoxicity (Rouvier *et al.*, 1993; Nagata and Golstein, 1995). Defective Fas/Fas-ligand stimulation results in autoimmune disease (Watanabe-Fukunaga *et al.*, 1992; Adachi *et al.*, 1993; Nagata and Golstein, 1995). Several studies have indicated that caspases are required at some stage in the apoptosis programme initiated by anti-Fas antibody (Chow *et al.*, 1995; Enari *et al.*, 1995; Los *et al.*, 1995; Enari *et al.*, 1996; Jacobson *et al.*, 1996; Slee *et al.*, 1996), but their involvement in commitment to cell death has not previously been analysed.

Results

Peptide inhibitors of caspases inhibit Fas-induced markers of apoptosis in Jurkat T cells

Consistent with previous reports (Enari *et al.*, 1995; Schlegel *et al.*, 1995; Slee *et al.*, 1996), treatment with anti-Fas antibody induced apoptosis in the human T-cell line Jurkat, resulting in chromosomal condensation, degradation of DNA to oligonucleosomal fragments, and loss of plasma membrane integrity (Figure 1). Incubation

with Z-VAD.fmk [benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone], a peptide caspase inhibitor (Chow *et al.*, 1995; Jacobson *et al.*, 1996; Slee *et al.*, 1996) produced substantial inhibition both of loss of membrane integrity and of the appearance of apoptotic morphology and oligonucleosomal DNA fragments (Figure 1). Z-DEVD.fmk also inhibited these changes, although at a higher concentration than required for Z-VAD.fmk.

Z-DEVD.fmk, like Ac-DEVD-CHO (Nicholson *et al.*, 1995), is a selective inhibitor of the CPP32/Apopain/Yama/caspase-3 sub-family and its effects are likely to include inhibition of MACH/FLICE/caspase-8 which may play an important role in linking Fas-induced intracellular signals to the cysteine proteases (Boldin *et al.*, 1996; Muzio *et al.*, 1996). Z-YVAD.cmk, a selective inhibitor of the ICE/caspase-1 sub-family also inhibited apoptosis but was noticeably less effective than the other inhibitors (Figure 1).

One of the earliest intracellular events of apoptosis in a range of systems is the cleavage of the nuclear enzyme poly(ADP-ribose) polymerase (PARP) (Kaufmann *et al.*, 1993; Lazebnik *et al.*, 1994). Treatment of Jurkat cells with anti-Fas antibodies induced PARP cleavage, resulting in conversion of the 116 kDa polypeptide to the characteristic 85 kDa cleavage product, as indicated by Western blotting (Figure 2). All the caspase peptide inhibitors previously used markedly inhibited PARP cleavage, as did the aspartate-based protease inhibitor Boc-D.fmk (Mashima *et al.*, 1995). Inhibition by Z-YVAD was incomplete and some 85 kDa cleavage product was detectable.

Jurkat cells rescued from Fas-induced apoptosis by peptide inhibitors retain proliferative and colony-forming ability

In order to determine if a genuine rescue from the process of apoptosis was accomplished, we first examined the effect of Z-VAD.fmk on cellular capacity for proliferation and colony formation on induction of apoptosis. Jurkat cells treated with anti-Fas antibody in the presence of Z-VAD.fmk not only maintained high viability, but even continued to proliferate, though at a reduced rate relative to that of untreated cells (Figure 3). We therefore examined changes in cell survival as measured by the most stringent of criteria—the ability to form a colony from a single cell. Addition of anti-Fas antibody, as expected, produced a substantial reduction in colonies subsequently formed in soft agar. The presence of Z-VAD.fmk, however, produced a 26-fold (SE = 6, from three separate experiments) increase in the number of colony-forming cells surviving this treatment (Figure 3), indicating that caspases are required for commitment to Fas-mediated apoptosis in Jurkat cells. Lower concentrations of the inhibitor tested produced a lower level of protection (data not shown).

Since Z-VAD.fmk is a general inhibitor of caspases, we examined the effect of more selective inhibitors, i.e.

Fig. 1. Caspase inhibitors prevent late stages of Fas-mediated apoptosis in Jurkat cells. All data refer to cells after 24 h treatment with 5 ng/ml anti-Fas antibody. Inhibitor concentrations for (B) and (C) were 200 μM Z-VAD.fmk, 200 μM Z-DEVD.fmk and 500 μM Z-YVAD.cmk. (A) Dose-response curves for inhibition of loss of cell viability (exclusion of nigrosin) by Z-VAD.fmk, Z-DEVD.fmk and Z-YVAD.cmk. Means and standard errors ($n = 4$), except where encompassed by the symbols, are shown. (B) Inhibition of development of nuclear apoptotic morphology (acridine orange staining) by Z-VAD.fmk, Z-DEVD.fmk and Z-YVAD.cmk. Standard errors are shown ($n = 3$). (C) Agarose gel electrophoresis of total cellular DNA. Lane M, 100 bp ladder DNA markers; lane 1, no inhibitor, no antibody; lane 2, no inhibitor, with anti-Fas antibody; lane 3, Z-VAD.fmk, no antibody; lane 4, Z-VAD.fmk, with anti-Fas antibody; lane 5, Z-DEVD.fmk, no antibody; lane 6, Z-DEVD.fmk, with anti-Fas antibody; lane 7, Z-YVAD.cmk, no antibody; lane 8, Z-YVAD.cmk, with anti-Fas antibody.

Z-DEVD.fmk, which preferentially inhibits the CPP32/caspase-3 sub-family (Nicholson *et al.*, 1995), and Z-YVAD.cmk, which preferentially inhibits ICE/caspase-1 sub-family proteases. Z-DEVD.fmk allowed cell proliferation in the presence of anti-Fas antibody to a noticeably greater extent than did Z-VAD.fmk. Consistent with this, Z-DEVD.fmk produced a dramatic increase [321-fold (SE = 47 from three separate experiments)] in the colony-forming cells surviving treatment with anti-Fas antibody (Figure 4).

The effect produced by Z-YVAD.cmk contrasted strongly with the other two inhibitors. Even at concentrations as high as 500 μ M (higher concentrations are precluded by significant toxicity), Z-YVAD.cmk failed to allow sustained proliferation in the presence of anti-Fas antibodies and produced no increase in colony-forming cells surviving treatment (Figure 5). No protection was seen in these assays with lower concentrations of Z-YVAD.cmk (data not shown).

Fig. 2. Caspase inhibitors prevent PARP cleavage in Jurkat cells treated with 5 ng/ml anti-Fas antibody for 24 h. Lane 1, 0.4% DMSO control; lane 2, anti-Fas antibody alone; lane 3, anti-Fas antibody with Boc-D.fmk (50 μ M); lane 4, anti-Fas antibody with Z-VAD.fmk (200 μ M); lane 5, anti-Fas antibody with Z-DEVD.fmk (200 μ M); lane 6, anti-Fas antibody with Z-YVAD.cmk (500 μ M).

Fig. 3. Z-VAD.fmk allows anti-Fas treated Jurkat cells to proliferate and form colonies. Cells were incubated with or without Z-VAD.fmk (200 μ M) (A) in the absence, or (B) in the presence of anti-Fas antibody (5 ng/ml). Cell density was determined after staining with nigrosin. Means and standard errors (except where encompassed by the symbols) are shown ($n = 4$). (C) Cells were assayed for colony-forming ability 24 h after apoptosis induction in the presence or absence of Z-VAD.fmk (200 μ M). Means and standard errors of three independent experiments each containing three replicates are shown. Plates are illustrated below the corresponding columns of the histogram.

CrmA transient transfection also produces increased colony formation after anti-Fas treatment

Transfection with vectors expressing the gene for CrmA (Ray *et al.*, 1992), a cowpox virus serpin, or micro-injection of this protein, has provided a complementary approach to caspase inhibition, which circumvents potential difficulties associated with differences in cell permeability to the peptide inhibitors (Ray *et al.*, 1992; Gagliardini *et al.*, 1994; Los *et al.*, 1995; Martin and Green, 1995; Tewari and Dixit, 1995; Yuan, 1995; Whyte, 1996; Jacobson *et al.*, 1996). Because of the inhibition of cell growth in Jurkat cells stably transfected with *crmA*, noted by ourselves and others (Enari *et al.*, 1995), we used a transient expression system to analyse the effect of CrmA on the production of colonies after treatment with anti-Fas antibodies. This approach also eliminated any possible artefacts associated with selection of clones. Transient CrmA expression produced a significant increase in the number of colonies after Fas treatment (Figure 6).

Caspase inhibitors also inhibit commitment to Fas-mediated death in CEM-C7 cells

Although the Jurkat cell line has been extensively used as an experimental system for the study of Fas-mediated apoptosis, it was important to confirm our observations independently in another cell line. Apoptosis, indicated by appearance of apoptotic morphology and breakdown of the cell membrane, was also induced in the human T-cell line CEM-C7 (Norman and Thompson, 1977) by anti-Fas antibodies, and this was inhibited by the peptide inhibitors Z-DEVD.fmk, Z-VAD.fmk and Z-YVAD.cmk (Figure 7). Apoptosis was confirmed by the appearance

Fig. 4. Z-DEVD.fmk allows anti-Fas treated Jurkat cells to proliferate and form colonies. Cells were incubated with or without Z-DEVD.fmk (200 μM) (A) in the absence, or (B) in the presence of anti-Fas antibody (5 ng/ml). Cell density was determined after staining with nigrosin. Means and standard errors (except where encompassed by the symbols) are shown ($n = 4$). (C) Cells were assayed for colony-forming ability 24 h after apoptosis induction in the presence or absence of Z-DEVD.fmk (200 μM). Means and standard errors of three independent experiments each containing three replicates are shown. Plates are illustrated below the corresponding columns of the histogram.

Fig. 5. Z-YVAD.cmk does not allow anti-Fas treated Jurkat cells to proliferate and form colonies. Cells were incubated with or without Z-YVAD.cmk (500 μM) (A) in the absence, or (B) in the presence of anti-Fas antibody (5 ng/ml). Cell density was determined after staining with nigrosin. Means and standard errors (except where encompassed by the symbols) are shown ($n = 4$). (C) Cells were assayed for colony-forming ability 24 h after apoptosis induction in the presence or absence of Z-YVAD.fmk (500 μM). Means and standard errors of three independent experiments each containing three replicates are shown. Plates are illustrated below the corresponding columns of the histogram.

of a strong oligonucleosomal DNA ladder on agarose gel electrophoresis (data not shown). The concentration dependence of the inhibition of CEM-C7 apoptosis was similar to that observed for Jurkat cells, except that Z-DEVD.fmk was less potent for CEM-C7s at concentrations below 50 μM (Figure 7A).

Western blotting with anti-PARP antibody demonstrated PARP cleavage and accumulation of the 85 kDa cleavage product after anti-Fas treatment of CEM-C7 cells (Figure 8). As for Jurkat cells, PARP cleavage was inhibited by

the peptide inhibitors Z-DEVD.fmk, Z-VAD.fmk and Boc-D.fmk. However, treatment with Z-YVAD.cmk—the weakest of the inhibitors in this system—produced only partial inhibition and the 85 kDa cleavage product was easily detected.

The effects of the peptide inhibitors on proliferating CEM-C7 cultures treated with anti-Fas antibody paralleled the effects on Jurkat cells. In the presence of anti-Fas antibody, Z-DEVD.fmk permitted rapid proliferation, Z-VAD.fmk permitted proliferation to a lesser extent, and

Fig. 6. Colony-forming ability of Jurkat cells transiently expressing *crmA* during treatment with anti-Fas antibody. Jurkat cells transiently transfected with a pcDNA3-*crmA* expression construct or the empty vector were allowed to recover for 24 h after electroporation, placed in fresh medium containing anti-Fas antibody (2 ng/ml) for 14 h, and then cloned in soft agar. Colonies were counted after 2 weeks and the number of colony-forming cells per culture was calculated. Means and standard errors from two independent experiments each containing three replicates are shown. Transfection efficiency as determined by staining for β -galactosidase activity was ~7%.

Z-YVAD.cmk produced a temporary protection of cell viability without apparent proliferation (Figure 9A). Z-DEVD.fmk once again produced a striking increase in colony-forming cells protected from apoptosis, Z-VAD.fmk produced a consistent though smaller effect, and Z-YVAD.cmk produced no protection of colony-forming cells (Figure 9B).

Discussion

Since Yuan *et al.* (1993) first noted the sequence similarity between *C.elegans* Ced-3 and ICE/caspase-1, considerable attention has been focused on the possible role of caspases in apoptosis. Loss of functional *ced-3* in the nematode abolished developmentally programmed cell death (Ellis and Horvitz, 1986) and, if the mammalian Ced-3 homologues, the caspases, were to prove similarly crucial to mammalian cell apoptosis, this could undoubtedly have far-reaching medical and biological consequences.

It is now clear that the mammalian caspase family has at least 10 members, each with a greater or lesser degree of sequence homology to Ced-3 (Alnemri *et al.* 1996; Hale *et al.*, 1996). However, the production by homologous recombination of mice genetically deficient in particular caspases has not so far produced the general failure of cell death seen in Ced-3-deficient nematodes. ICE/caspase-1-deficient mice, for example, are developmentally normal (Kuida *et al.*, 1995; Li *et al.*, 1995). Mice deficient in CPP32/caspase-3 live until 1–3 weeks after birth but show defective cell death in the brain (Kuida *et al.*, 1996). However, most cell death—including thymocyte apoptosis induced by a range of stimuli—occurs normally

Fig. 7. Caspase inhibitors prevent late stages of Fas-mediated apoptosis in CEM C7 cells. (A) Dose-response for inhibition of loss of cell viability (vital dye staining) by Z-VAD.fmk, Z-DEVD.fmk and Z-YVAD.cmk. Means and standard errors ($n = 4$), except where encompassed by the symbols, are shown. (B) Inhibition of development of nuclear apoptotic morphology (acridine orange staining) by Z-VAD.fmk (50 μ M), Z-DEVD.fmk (200 μ M) and Z-YVAD.cmk (600 μ M).

in these mice. These studies suggest that, although the mammalian caspase family may together fulfil an essential role in apoptosis analogous to Ced-3 in the nematode, individual caspases are usually not essential for apoptosis, possibly because of redundancy in the biochemical activities of the different family members.

Experimental inhibition of apoptosis by peptide caspase inhibitors or by CrmA therefore has the crucial advantage of inhibiting several caspases at the same time, presenting an opportunity to investigate the importance of this protease family, despite redundancy among its members. The striking protection against commitment to cell death produced by Z-DEVD.fmk and Z-VAD.fmk in particular strongly confirms the value of this approach.

Since these inhibitors each affect several of the 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 com-

Fig. 8. Caspase inhibitors prevent PARP cleavage in CEM C7 cells treated with 5 ng/ml anti-Fas antibody for 24 h. Lane 1, 0.4% DMSO control; lane 2, anti-Fas antibody alone; lane 3, anti-Fas antibody with Boc-D.fmk (50 μ M); lane 4, anti-Fas antibody with Z-VAD.fmk (50 μ M); lane 5, anti-Fas antibody with Z-DEVD.fmk (200 μ M); lane 6, anti-Fas antibody with Z-YVAD.cmk (600 μ M).

mitment to cell death in these cells. In addition, we cannot exclude the possibility that differences in inhibition by the peptide inhibitors are related to differences in cell uptake and/or to differences between fluoromethylketone and chloromethylketone inhibitors. Nevertheless, the failure of Z-YVAD.cmk to protect colony-forming ability or proliferation at any concentration in either Jurkat or CEM-C7 cells is consistent with the inability of anti-Fas-stimulated Jurkat cell extracts to cleave YVAD (Xiang *et al.*, 1996). These observations suggest that ICE/caspase-1 sub-family proteases are not required at this stage. The convincing protection produced by Z-DEVD.fmk in both cell lines, together with the cleavage of DEVD by anti-Fas-stimulated Jurkat cell extracts (Xiang *et al.*, 1996), indicates that one or more CPP32/caspase-3 sub-family proteases are required for commitment to Fas-induced apoptosis. However, the significant protection produced by CrmA even in a transient expression system, while not excluding involvement of CPP32/caspase-3, does suggest that a different member of the sub-family may be required, since CrmA inhibits CPP32/caspase-3 itself only very poorly (Nicholson *et al.*, 1995) and Fas-mediated apoptosis occurs normally in thymocytes from CPP32/caspase-3-deficient mice (Kiuda *et al.*, 1996). Although other explanations are possible, we note that the inhibition profile for protection of colony-forming ability, i.e. no protection by Z-YVAD.cmk but protection by Z-DEVD.fmk, by Z-VAD.fmk and by CrmA, is consistent with inhibition of MACH/FLICE/caspase-8 (Boldin *et al.*, 1996; Muzio *et al.*, 1996; F.Li *et al.*, personal communication). Since this protease is likely to be activated when bound through its death domain to the Fas signalling complex, i.e. at an early stage in the sequence of events induced by Fas engagement, it is reasonable to suggest that MACH/FLICE/caspase-8 activity may indeed be required for commitment to apoptosis in this system.

Whatever the precise identity of the protease or proteases involved, these observations have several important consequences for our understanding of apoptosis and disease. They indicate that genetic abolition of the relevant caspases could lead to inappropriate survival of cells signalled to die through Fas. Apoptosis induced through Fas is important in several areas of the immune system (Rouvier *et al.*, 1993; Nagata and Golstein, 1995) and recent work has shown that Fas normally acts as a tumour suppressor for B-cell lymphoma (Peng *et al.*, 1996). In addition, several groups have reported that other inducers of apoptosis act through stimulation of Fas/Fas-ligand interaction. These stimuli

A

B

Fig. 9. Z-VAD.fmk (50 μ M) and Z-DEVD.fmk (200 μ M), but not Z-YVAD.cmk (600 μ M) allow proliferation of CEM C7 cells (A) in the presence of anti-Fas antibody (5 ng/ml) (nigrosin staining) and (B) in a clonogenic assay (as described in the legend to Figure 5). Means and standard errors from two independent experiments each with three replicates are shown.

include engagement of the T-cell receptor/CD3 complex (Brunner *et al.*, 1995; Dhein *et al.*, 1995; Ju *et al.*, 1995), the cytotoxic anti-cancer agent doxorubicin (Friesen *et al.*, 1996) and engagement of Class II MHC on activated B cells (Truman *et al.*, 1997).

Cells deficient in the caspase activity needed for Fas-induced commitment to apoptosis could therefore be expected to form colonies and, after further genetic changes, either form cancers or, for cells carrying auto-reactive T-cell receptors, produce auto-immune disease.

Apoptosis in Rat-1 fibroblasts is not completely inhibited by inhibition of caspases (McCarthy *et al.*, 1997). This suggests that mammalian cell apoptosis is heterogeneous in this respect and the exact role of the proteases must be determined for different biological systems. In addition, even in Jurkat, one of the cell lines used in the present study, cell death induced by a different stimulus, Bax over-expression, is not blocked by Z-VAD.fmk or Boc-D.fmk (Xiang *et al.*, 1996), indicating that a requirement for caspase activity for commitment to cell death can differ for stimuli within the same cell. Nevertheless, we suggest that commitment to apoptosis

in other systems will also prove to be dependent on these proteases, offering the prospect of genuine rescue from apoptosis by specific protease inhibitors as a novel approach to therapy of degenerative disease.

Materials and methods

Materials

The protease inhibitors benzyloxycarbonyl-Val-Ala-Asp(OMe)fluoromethylketone (Z-VAD.fmk), benzyloxycarbonyl-Asp-Glu-Val-Asp(OMe)fluoromethylketone (Z-DEVD.fmk, mono-(OMe) modified) and *t*-butyloxycarbonyl-Asp(OMe)fluoromethylketone (Boc-D.fmk) were purchased from Enzyme Systems Products (Dublin, CA, USA). Benzyloxycarbonyl-Tyr-Val-Ala-Asp chloromethylketone (Z-YVAD. cmk) was purchased from Bachem (Switzerland). Anti-human Fas monoclonal antibody IPO-4 (IgM) was a gift from Dr David Mason (Oxford University).

Cell culture and treatments

The human T-leukaemic cell lines Jurkat (from Dr Jannie Borst, Netherlands Cancer Institute) and CEM-C7 (from Professor G.Melnikovych, Kansas) were cloned and subsequently maintained in RPMI 1640 medium (Sigma) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Hyclone), 200 µg/ml gentamicin and 2 mM glutamine (Sigma) at 37°C in a humidified incubator with 5% CO₂. All experiments were carried out using cells in logarithmic growth phase. To induce apoptosis, 2 × 10⁵ cells/ml (200 µl/well) were seeded into 96-well plates and incubated with 5 ng/ml anti-human Fas antibody for 24 h. The caspase inhibitors were dissolved in DMSO with the final DMSO concentration in cultures <0.4% (v/v). Control cells received 0.4% DMSO which had no effect on cell proliferation or viability. Cells were preincubated with the caspase inhibitors for 1 h before apoptosis induction. Cell density and viability were determined by 0.2% nigrosin staining.

Analysis of apoptosis

Cells were examined for nuclear apoptotic morphology using acridine orange staining and fluorescence microscopy. Following incubation, cells were harvested by centrifugation, resuspended in RPMI, mixed with an equal volume of 50 µg/ml acridine orange and mounted on a microscope slide with coverslip. A total of 200 cells/replicate sample were counted and the number of apoptotic cells expressed as a percentage. DNA fragmentation was examined as described previously (Smith *et al.*, 1989).

Western blot analysis

Sample preparation: 10⁶ cells were treated with 5 ng/ml anti-Fas antibody, alone or in combination with caspase inhibitors for 24 h, washed in ice-cold PBS and lysed in sample buffer (50 mM Tris-Cl, pH 6.8, 2% SDS, 10% glycerol, 0.1% bromophenol blue and 100 mM DTT) at 100°C for 10 min, and fractionated by 8% SDS-PAGE. Proteins were transferred to nitrocellulose membranes (Hybond-ECL, Amersham) by electroblotting. Following blocking in TTBS (10 mM Tris-Cl, pH 7.5, 100 mM NaCl and 0.1% Tween 20) containing 1% BSA, the PARP protein was detected using a 1:10 000 dilution of a monoclonal mouse anti-PARP antibody (C2-10; Kaufmann *et al.*, 1993; Enzyme Systems Products, Dublin, CA). Goat anti-mouse antibodies conjugated with horseradish peroxidase (Dako, Denmark) were used to visualize immunoreactive proteins at 1:500 dilution using enhanced chemiluminescence (Amersham).

Clonogenic assay

Following 24 h treatment with 5 ng/ml anti-Fas antibody, alone or in combination with Z-VAD.fmk, Z-DEVD.fmk or Z-YVAD.cmk the ability of cells to form colonies in soft agar was determined. An equal proportion of each culture was diluted in a total volume of 5 ml Iscoves medium (Sigma) containing 20% (v/v) fetal calf serum, 10% (v/v) Jurkat or CEM-C7 conditioned medium as appropriate, and 0.3% (w/v) noble agar (Difco) and plated in 60-mm dishes. Once set, dishes were overlaid with 2.5 ml Iscoves medium containing supplements and incubated for 2 weeks at 37°C in 5% CO₂ before counting colonies.

Transient transfection

crmA cDNA (Dr David Pickup, Duke University) was subcloned into the mammalian expression vector pcDNA3 (Invitrogen). The resulting expression construct, or pcDNA3 itself as a control, was introduced into Jurkat cells by electroporation (Gene Pulser, Bio-Rad). 8 × 10⁶ cells in

400 µl RPMI medium without serum were electroporated at room temperature with a total of 40 µg DNA at 310 V, 960 µF in 0.4 cm cuvettes (Bio-Rad). In order to follow transfection efficiency, pcDNA3-*crmA* or the empty vector were co-transfected with the pSV β-Gal plasmid (Promega) at a ratio of 1:1. At 24 h post-transfection, half the cells were stained for β-galactosidase activity (MacGregor, 1992). The remaining cells were treated with 2 ng/ml anti-Fas for 14 h. Clonogenic survival was determined by cloning in soft agar as described above.

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