A cleavage-site-directed inhibitor of interleukin-1β-converting enzyme-like proteases inhibits apoptosis in primary cultures of rat hepatocytes

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Apoptosis induced in primary hepatocytes by transforming growth factor β_1 and staurosporine produces chromatin condensation, DNA cleavage as detected by *in situ* end-labelling, field inversion and conventional gel electrophoresis, and cell detachment. These effects were abolished by benzyloxycarbonylvalinylalanylaspartylfluoromethyl ketone, a cleavage-site-directed inhibitor of interleukin-1β-converting enzyme-like proteases, and this finding suggests that these enzymes are involved in liver apoptosis.

INTRODUCTION

Apoptosis is a form of cell death [1] which appears to be an essential mechanism in multicellular organisms for the removal of redundant, damaged and/or senescent cells [2]. In hepatocytes, several agents, including menadione [3], tumour necrosis factor α [4], Fas antibody [5], transforming growth factor β_1 (TGF-β_1) [6] and staurosporine [7], also induce apoptosis. We have been investigating the mechanisms of apoptosis in primary cultures of hepatocytes and have used TGF-β_1 and staurosporine as model compounds for inducing cell death. Although the mechanisms by which these compounds induce apoptosis are unclear, it is apparent that the initial events must involve different pathways. Thus TGF-β_1 binds co-operatively to type-I and type-II receptors and triggers a serine/threonine kinase cascade [8]. Little is known about this signalling pathway, although G_1-growth arrest induced by TGF-β_1 involves hypophosphorylation of retinoblastoma protein [9]. Thus apoptosis induced by TGF-β_1 may also involve an effect on the cell-cycle machinery. In contrast, staurosporine is generally regarded as a protein kinase C inhibitor, but in fact it is only poorly selective for this enzyme and inhibits a wide range of protein kinases [10]. Thus the only common link between TGF-β_1 and staurosporine is that they may both result in hypophosphorylation of some key intracellular proteins.

Generally, the mechanisms underlying the apoptotic process are poorly understood, but increasing evidence from studies with thymocytes [11–13] and HL-60 cells [14] show that protease inhibitors block both the nuclear and cytoplasmic changes of apoptosis. A protease involvement has also been suggested from genetic studies which have shown that the product of the *ced-3* gene is a mediator of cell death in the nematode *Caenorhabditis elegans* and has a high degree of homology with interleukin-1β-converting enzyme (ICE), a mammalian cysteine protease [15]. Other ICE-like proteases have also been identified including those encoded by the mouse gene *Nedd2* [16], its human homologue *ICH-1* [17], CPP32 [18], TX [19] and *Mch2* [20]. Mutants of *C. elegans* lacking ced-3 do not undergo programmed cell death (see Ellis et al. [21] for a review) and transfection/overexpression of ICE/ced-3-like proteases induces apoptosis in a variety of host cells (see Kumar [22] for a review).

However, recent studies have shown that thymocytes and macrophages from mice deficient in ICE undergo normal apoptosis, thereby suggesting that there is not an absolute requirement for ICE itself [23]. Also, Lazebnik et al. [24] have shown that, during apoptosis, poly (ADP-ribose) polymerase is proteolytically cleaved by an ICE-like protease which has been identified as being derived from CPP32 [25]. These results suggest that a key universal step in apoptosis involves one or more ICE/ICE-like protease(s) and it should be possible to identify the involvement of such protease(s) in both TGF-β_1 and staurosporine-induced apoptosis in primary rat hepatocytes. We have examined this possibility by using cleavage-site-directed inhibitors of ICE and ICE-related proteases, namely *N*-acetyltyrosinylvalinylalanylaspartylchloromethyl ketone (YVAD.CMK) and benzyloxycarbonylvalinylalanylaspartylfluoromethyl ketone (Z-VAD.FMK). Both these inhibitors block TGF-β_1 and staurosporine-induced apoptosis, suggesting that an ICE/ICE-like protease(s) is involved in the induction of apoptosis in rat hepatocytes.

MATERIALS AND METHODS

Materials

All chemicals unless stated otherwise were obtained from the Sigma Chemical Co., Poole, Dorset, U.K. Terminal deoxynucleotidyl transferase and most culture reagents were from Gibco, Paisley, Scotland, U.K. Anti-digoxigenin–fluorescein Fab fragments (anti-Dig), digoxigenin-11–2′-deoxyuridine 5′-triphosphate (Dig-11-dUTP) and rat tail collagen were obtained from Boehringer-Mannheim U.K., Lewes, East Sussex, U.K. Recombinant human TGF-β_1 was obtained from R&D Systems Europe, Abingdon, Oxon, U.K. and dissolved in 4 mM HCl containing 1 mg/ml BSA. Z-VAD.FMK (methyl ester) and YVAD.CMK were from Enzyme Systems Inc. Dublin, CA, U.S.A.

Cell cultures

Hepatocytes were isolated by *in vitro* collagenase perfusion of livers from male F344 rats (180–200 g) as described previously [26]. Cells (5×10^6/ml) were plated on to either collagen-coated

Abbreviations used: ICE, interleukin-1β-converting enzyme; Z-VAD.FMK, benzyloxycarbonylvalinylalanylaspartylfluoromethyl ketone; YVAD.CHO, *N*-acetyltyrosinylvalinylalanylaspartyl aldehyde; YVAD.CMK, *N*-acetyltyrosinylvalinylalanylaspartylchloromethyl ketone, FIGE, field-inversion gel electrophoresis; ISEL, *In situ* end-labelling; TGF-β, transforming growth factor β; TdT, terminal deoxynucleotidyl transferase; anti-Dig, anti-digoxigenin–fluorescein Fab fragments; Dig-11-dUTP, digoxigenin-11–2′-deoxyuridine 5′-triphosphate.

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glass coverslips in 6/12-well plates or plastic collagen-coated Petri dishes as described by Oberhammer et al. [6] at a cell density of 87500 cells per cm² in Williams E. medium, supplemented with 6 μM hydrocortisone, 1.4 nM insulin, 14 nM dexamethasone, 1% (v/v) foetal calf serum, 2 mM glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin and left for 4 h to attach to the substratum. The medium was then removed and the cells washed before incubation with new medium containing the various agents. Staurosporine and Z-VAD.FMK were dissolved in DMSO before addition to the medium (final DMSO concentration 0.2%, v/v) and appropriate solvent controls were used.

Morphological evaluations

At the indicated times the culture medium was removed and the cells fixed with 1% formaldehyde in PBS for 5–10 min. Chromatin was then stained with Hoechst 33258 (8 μg/ml), essentially as described by Oberhammer et al. [6]. With this method the hepatocyte nuclei are stained with the dye which gives a blue fluorescence under UV light. Fluorescent nuclei were visualized with an Olympus BH2 microscope and the cells scored as either (1) normal in which the chromatin was uncondensed with low blue fluorescence and uniformly spread over the whole nucleus or (2) apoptotic in which the nucleus contained masses of condensed chromatin with high blue fluorescence. Approx. 2000–3000 nuclei were counted for each treatment. Cell viability was assessed by dye exclusion with 10 μg/ml propidium iodide which was incubated with the cells for 10 min before washing with PBS, followed by formalin fixation and staining with Hoechst 33258.

Enucleation procedure

Nuclei were obtained from monolayer hepatocyte cultures on collagen-coated Petri dishes initially seeded with 9.8×10^6 (large 13.5 cm-diameter dishes) using a new method [26a] for lysing the cells. Briefly, after treatment, the culture medium was removed and the cells enucleated at 5 °C with a 1% citric acid buffer containing 0.1% Tween 20. The nuclei were then harvested by centrifugation at 1000 ***g*** for 5 min and washed and resuspended in PBS before centrifugation and resuspension in 0.8 ml of PBS.

In situ end-labelling (ISEL) and flow-cytometric analysis of normal and apoptotic nuclei

ISEL and flow cytometry on isolated nuclei was carried out as described previously [27] using TdT and Dig-11-dUTP to end-label the strand breaks which were detected with an anti-Dig fluorescein labelled antibody. The nuclei were resuspended in PBS with 10 μg/ml propidium iodide and a FACScan flow cytometer (Becton Dickinson) was used to measure the red (propidium iodide) and green (fluorescein; anti-Dig) fluorescence of the labelled nuclei, using doublet discrimination. Data from 10^4 singlet nuclei were collected and displayed as univariate frequency histograms [i.e. number of nuclei/channel plotted against the anti-Dig antibody (green fluorescence)]. Typically, this gave two populations (see Figures 3 and 4) which from the propidium iodide fluorescence (results not shown) corresponded to diploid (2N) and tetraploid (4N) nuclei. LYSYS II software was used to gate around these populations to measure and determine the numbers of normal and apoptotic nuclei (see Figures 3 and 4).

Gel electrophoresis

DNA cleavage in isolated nuclei (1×10^6) was analysed as described previously [27] by conventional agarose-gel electrophoresis to detect internucleosomal cleavage and field-inversion gel electrophoresis (FIGE) to separate large fragments of DNA.

RESULTS

Z-VAD.FMK blocks chromatin condensation in apoptotic hepatocytes

Apoptosis was assessed in monolayer hepatocyte cultures after the chromatin had been stained with Hoechst 33258 and using fluorescence microscopy to count nuclei with classical apoptotic morphology. After 16 h of culture, TGF-β_1 (1 ng/ml) induced about 9% apoptosis (Figure 1A) and this was accompanied by a 10% decrease in the total number of cells on the slide. TGF-β_1 did not produce any necrosis as more than 99% of treated cells excluded propidium iodide (results not shown). Thus this loss of cells was due to the apoptotic cells rounding up and detaching from the substratum [28]. Z-VAD.FMK (1–5 μM)

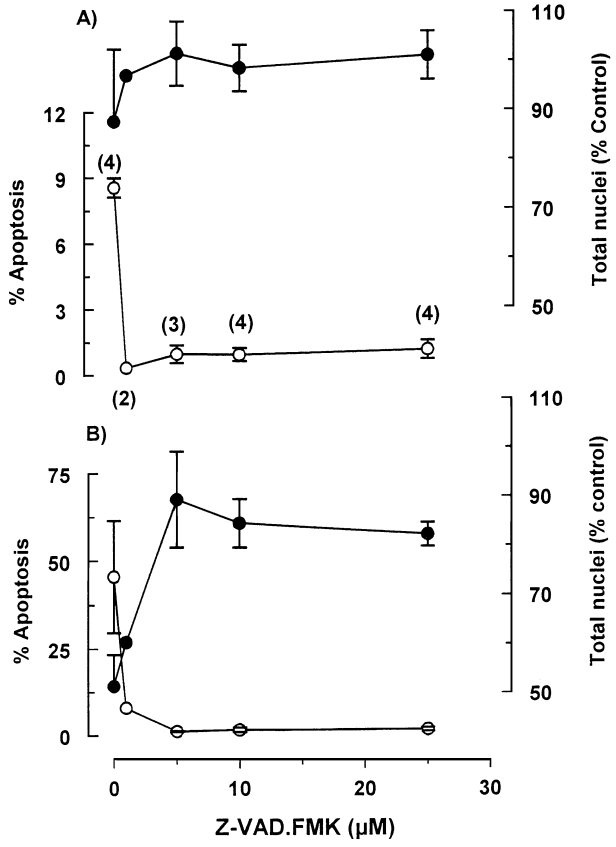

Figure 1 Effect of Z-VAD.FMK on TGF-β_1- and staurosporine-induced apoptosis in hepatocytes

Hepatocytes were cultured on collagen-coated glass coverslips as described in the Materials and methods section with each treatment carried out in duplicate. In (**A**) cells were incubated with 1 ng/ml TGF-β_1 and the Z-VAD.FMK was added 4 h later, and in (**B**) the cells were preincubated with Z-VAD.FMK for 30 min before the addition of 5 μM staurosporine. After 16 h the number of normal and apoptotic nuclei were determined as described in the Materials and methods section. A minimum of ten fields (approx. 200 cells/field) for each coverslip was examined and the average number of nuclei/field calculated. The results are shown where appropriate as the mean ± S.E.M. with the number of experiments in parentheses. Total nuclei (i.e. normal + apoptotic) were expressed as a percentage of the control, i.e. untreated hepatocytes. Apoptotic nuclei were expressed as a percentage of the total nuclei and in control cells this was 0.6 ± 0.3% ($n = 4$). Z-VAD.FMK on its own produced little or no effect. Solid and open symbols refer to total and apoptotic cells respectively.

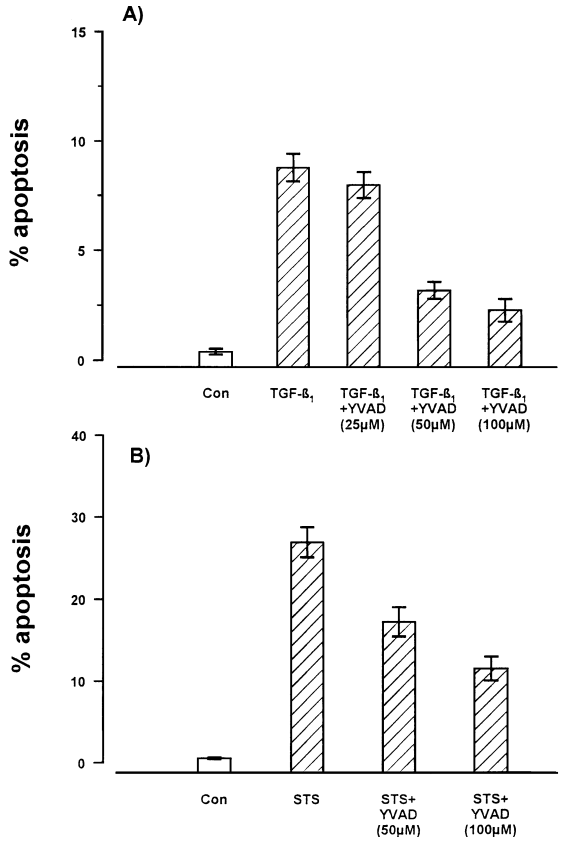

Figure 2 Effect of YVAD.CMK on TGF-β_1- and staurosporine-induced apoptosis in hepatocytes

Hepatocytes were cultured as described in Figure 1 and each treatment was carried out in duplicate. In (**A**) the cells were preincubated with and without YVAD.CMK (YVAD) and 1 ng/ml TGF-β_1 was added 30 min later; in (**B**) the cells were incubated with 5 μM staurosporine (STS) with and without YVAD.CMK which was added 30 min before the staurosporine. The cells were fixed and stained after 16 h as described in Figure 1 and the number of apoptotic nuclei counted and expressed as a percentage of the total nuclei. The results from one experiment are shown; essentially the same results were obtained from a separate hepatocyte preparation. The values given are means $\pm$ S.E.M from a minimum of ten fields and thus show the interfield variation.

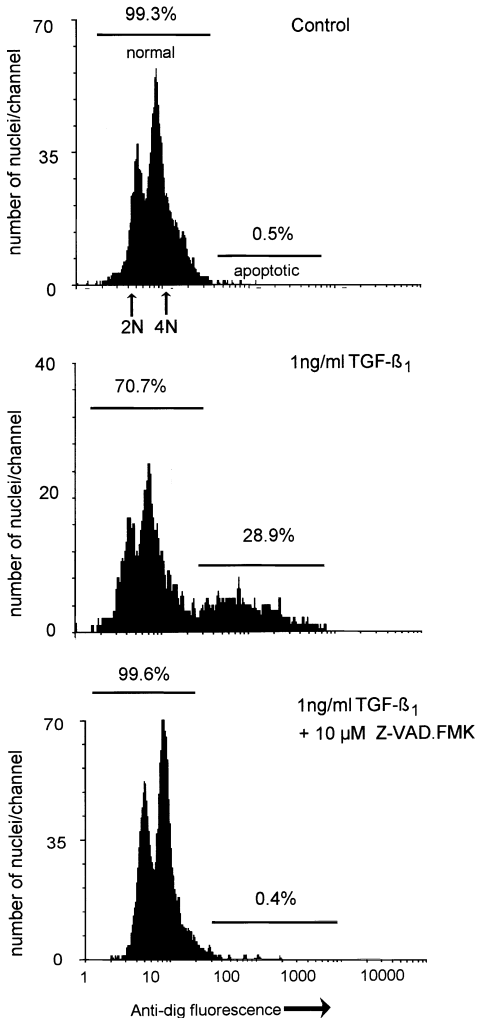

Figure 3 Effect of Z-VAD.FMK on ISEL of nuclei extracted from hepatocytes treated with TGF-β_1

Hepatocytes were prepared as described in the Materials and methods section and incubated for 16 h without additions (control), with 1 ng/ml TGF-β_1 or with 1 ng/ml TGF-β_1 plus 10 μM Z-VAD.FMK. The nuclei were extracted and end-labelled as in the Materials and methods section. A univariate frequency histogram is shown with anti-Dig antibody fluorescence (the amount of DNA cleavage) plotted as a logarithmic scale on the *x*-axis and the number of nuclei per channel plotted on the *y*-axis. For analytical purposes only the 2N and 4N nuclei were gated (i.e. higher ploidy nuclei and debris were excluded) and in the control this accounted for approx. 60% of the total number of nuclei (i.e 10^4) counted. The normal and apoptotic nuclei are given as percentage of the gated nuclei and are shown as horizontal bars. The position of the diploid (2N) and tetraploid (4N) nuclei are indicated with arrows.

totally blocked TGF-β_1-induced apoptosis and reversed the cell loss, demonstrating that this inhibitor was particularly potent at blocking the changes in the morphology of apoptotic nuclei (Figure 1A).

The experiments with staurosporine (Figure 1B) were more complicated as this compound produced an unusual nuclear morphology in that all the nuclei were much smaller (C. Couet and K. Cain, unpublished work) than those observed in the control and TGF-β_1-treated cells. However, the chromatin in approximately 50% of these nuclei had an apoptotic morphology which was condensed and aggregated at the nuclear membrane as hemilunar caps. Virtually all of the staurosporine-treated cells included propidium iodide (results not shown), suggesting that staurosporine was inducing both necrosis and apoptosis. This was reflected in the almost 50% loss of cells (Figure 1B) from the coverslip. However, Z-VAD.FMK (5 μM) completely inhibited the formation of nuclei with an apoptotic chromatin morphology and the number of cells remaining on the coverslip increased to 85% (Figure 1B), although the size of the nuclei remained small and the cells still included propidium iodide. Thus Z-VAD.FMK blocked the apoptotic morphology produced by staurosporine but it did not abrogate the necrotic effects.

YVAD.CMK, which on the basis of its tetrapeptide structure should be a more specific ICE inhibitor, also blocked the morphological changes in the nucleus induced by TGF-β_1 and staurosporine (Figure 2). However, YVAD.CMK was much less potent than Z-VAD.FMK as even 100 μM YVAD.CMK did not produce complete inhibition of apoptosis and this may indicate that ICE itself is not involved. Alternatively, this may reflect a permeability problem as Los et al. [29] found that YVAD.CHO inhibited apoptosis at 10 nM provided the cells were permeabilized briefly with hypotonic shock. Remaining experiments were therefore carried out with the more potent, Z-VAD.FMK.

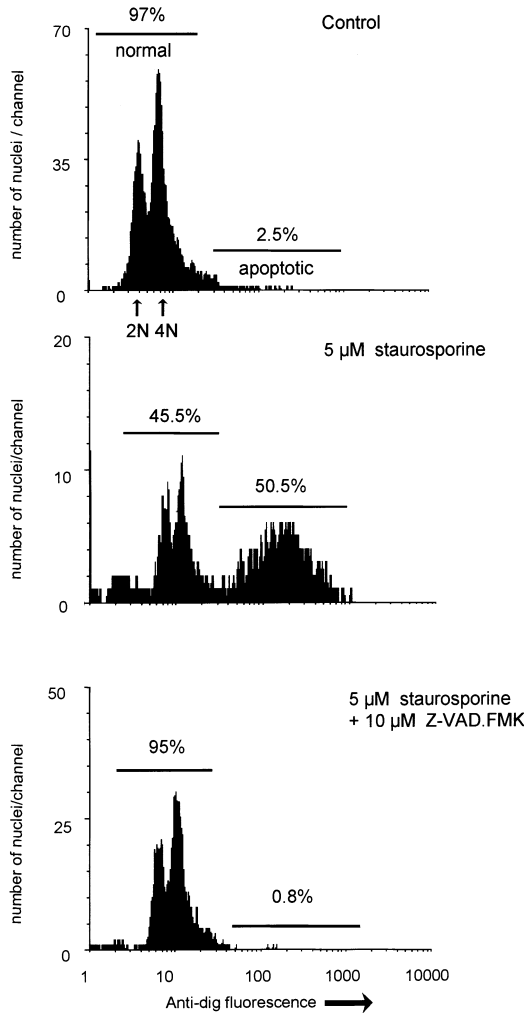

Figure 4 Effect of Z-VAD.FMK on ISEL of nuclei extracted from hepatocytes treated with staurosporine

Hepatocytes were prepared as described in the Materials and methods section and incubated for 16 h without additions (control), with 5 μM staurosporine or with 5 μM staurosporine plus 10 μM Z-VAD. FMK. The nuclei were extracted and end-labelled analysed as described in Figure 3.

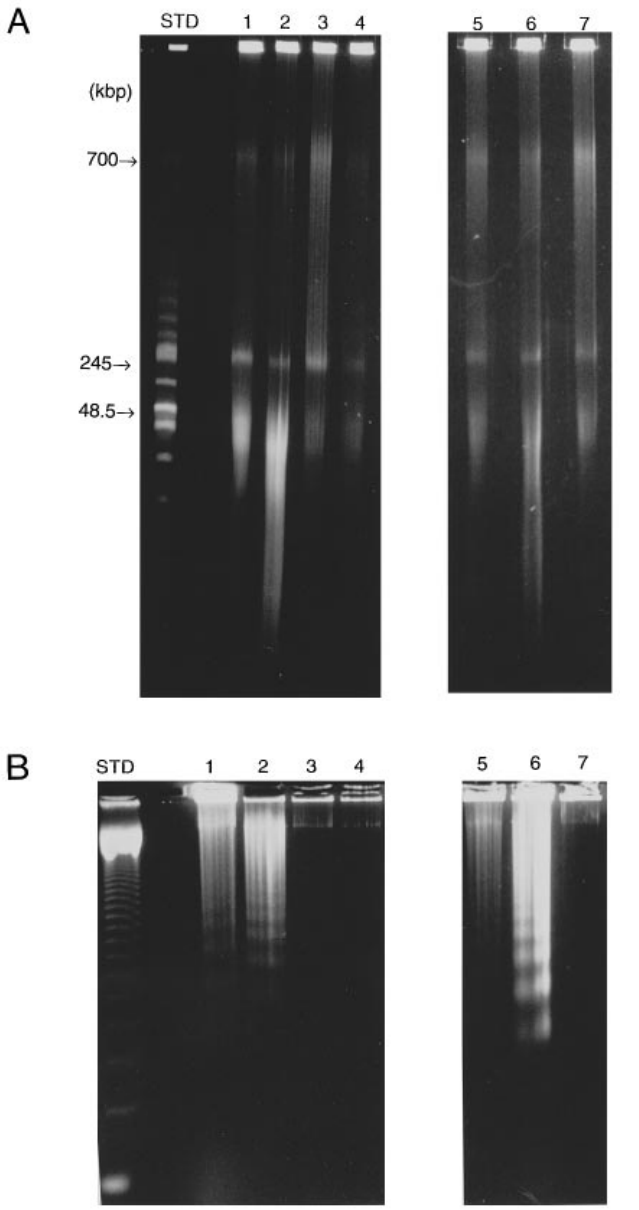

Figure 5 FIGE and conventional gel electrophoresises of nuclei from hepatocytes treated with TGF-β_1 and staurosporine with and without Z-VAD.FMK

Nuclei were extracted from hepatocytes as described in Figure 3 and DNA cleavage was analysed by FIGE (**A**) and conventional agarose-gel electrophoresis (**B**) as described in the Materials and methods section. In the FIGE gel, the STD lane refers to the kbp sizes indicated by arrows and in the conventional agarose gel, the STD lane refers to markers of 123 bp or multiples thereof. Lane 1, nuclei from DMSO solvent control hepatocytes; lanes 2 and 3, nuclei from hepatocytes treated with 5 μM staurosporine and 5 μM staurosporine + 10 μM Z-VAD.FMK respectively. Z-VAD.FMK (10 μM) alone is shown in lane 4 and the solvent control for the TGF-β_1 experiments in lane 5. The effects of 1 ng/ml TGF-β_1 and 1 ng/ml TGF-β_1 + 10 μM Z-VAD.FMK on DNA cleavage are shown in lanes 6 and 7 respectively.

Z-VAD.FMK blocks ISEL in apoptotic hepatocytes

The induction of apoptosis involves activation of endonucleases which generate double-strand breaks with 3′-OH ends which can be detected by end-labelling. Nuclei were extracted from the monolayer cultures and the DNA strand breaks were end-labelled [27] as shown in Figure 3. The frequency histograms showed two main peaks of low anti-Dig fluorescence, corresponding to the diploid (2N) and tetraploid (4N) nuclei. In the control, nearly all (i.e. 99.3 %) of the gated nuclei were in this region and were normal non-apoptotic nuclei. Only a small number of nuclei (0.5 %) had increased anti-Dig fluorescence (note the log scale) and were considered to be from apoptotic hepatocytes. The histogram for nuclei from hepatocytes treated with TGF-β_1 (1 ng/ml) showed that the number of normal nuclei had decreased to 70.7 % concomitant with an increase in the apoptotic fraction to 28.9 %. The apoptotic nuclei had a wide range of labelling intensities reflecting different levels of cleavage. However, the high level of DNA-strand breaks induced by TGF-β_1 was completely blocked by Z-VAD.FMK (10 μM) and 99.6 % of the nuclei were normal with only 0.4 % of the nuclei showing enhanced ISEL (Figure 3).

A slightly higher level of apoptosis (2.5 %) was observed when DMSO was used as a control solvent (Figure 4). Staurosporine (5 μM) caused a decrease in the number of normal nuclei to

45.5% with a concomitant increase in the number of apoptotic nuclei to 50.5%. This increase was completely blocked by Z-VAD.FMK and furthermore the number of normal nuclei increased to 95% which was nearly back to control levels.

Z-VAD.FMK abolishes internucleosomal cleavage and large-fragment formation

DNA from isolated nuclei was analysed by FIGE and conventional agarose gel electrophoresis (Figure 5). With the DMSO control, 30–50 kbp and 200–300 kbp fragments were detected (Figures 5A and 5B, lane 1) along with a small amount of 'DNA laddering'. Staurosporine (Figure 5, lane 2) caused an increase in the amount of 30–50 kbp fragments, and < 30 kbp fragments were detected. This was accompanied by increased 'DNA laddering' (Figures 5A and 5B, lane 2). Z-VAD.FMK totally blocked staurosporine-induced 'DNA laddering' and markedly inhibited the cleavage of the DNA into 30–50 kbp fragments (Figures 5A and 5B, lane 3). Virtually no DNA cleavage was observed with Z-VAD.FMK alone (Figures 5A and 5B, lane 4), suggesting that this inhibitor also blocked the endogenous apoptosis.

TGF-β_1 produced a small increase in the 'large fragment' formation and a marked increase in internucleosomal cleavage compared with the corresponding control (Figures 5A and 5B, compare lanes 6 and 5). Z-VAD.FMK completely blocked this internucleosomal cleavage and reduced the cleavage of DNA into 'large fragments' back to control levels (Figures 5A and 5B, lane 7).

DISCUSSION

Apoptosis was induced in monolayer cultures of hepatocytes by TGF-β_1 and staurosporine as assessed by chromatin condensation (Figures 1 and 2), ISEL (Figures 3 and 4), the formation of 'large fragments' and mono-/oligo-nucleosomes (Figure 5) and by rounding up and detachment/loss of the cells into the culture medium (Figure 1). All of these effects were blocked by Z-VAD.FMK and it is clear that, irrespective of the initial signalling events both TGF-β_1 and staurosporine initiate apoptosis via an enzyme or process that is extremely sensitive to Z-VAD.FMK and clearly essential for apopotosis.

As yet we have not identified the Z-VAD.FMK target and therefore cannot rule out the possibility that Z-VAD.FMK inhibits an enzyme or protein that is not ICE-related. However, there are compelling reasons to suggest that Z-VAD.FMK targets an ICE/ICE-like protease. First, the design and specificity of cleavage-site directed peptide inhibitors, such as Z-VAD.FMK, is based on the fact that peptide-substrate-specificity studies with ICE [30] have shown an almost absolute requirement for aspartic acid in the P_1 position. For substrates of ICE, four amino acids are required to the left of the cleavage site and liberal substitution can be accommodated in the P_2 position; valine is preferred in P_3 and hydrophobic amino acids particularly tyrosine are required for P_4. However, in the case of CPP32 the preferred substrate is DEVD [25], suggesting that the substrate specificity of ICE-like proteases is determined by the P_2, P_3 and P_4 amino acids. It is the resolution of these specific substrate-binding sites that has enabled the development of cleavage-site-directed inhibitors of ICE and CPP32 [25,30,31]. The most potent ICE inhibitor is the reversible competitive inhibitor YVAD.CHO the specificity of which has been demonstrated by the fact that an affinity ligand, Ac-Tyr-Val-(biotin)-Lys-Asp-$CH_2OC(O)$-[2,6-$(CH_3)_2$]Ph, based on this substrate specificity inhibits more than 99.99% of the ICE activity (which constitutes less than 0.001% of the total protein) of THP.1-cell cytosol without labelling any other proteins [32]. Other peptide inhibitors with less than four amino acids are even more potent ICE inhibitors [33] but have less specificity [31] for ICE and perhaps can be regarded as inhibitors of both ICE and ICE-like proteases. Benzyloxycarbonyl-valinylalanylaspartyl-$CH_2O(CO)$[2,6-(Cl_2)]Ph is the most active [31] and has been shown [34] to block IL-1β production in murine macrophages. Z-VAD.FMK also blocks the apoptotic nuclei promoting activity in Jurkat T-cells after Fas antigen ligation [35]. These studies strongly suggest that Z-VAD analogues are capable of blocking ICE or ICE-like proteases in intact cells.

Other studies in this laboratory have also shown that Z-VAD.FMK blocks apoptosis induced in thymocytes and THP.1 cells by a diverse range of stimuli [36,37]. These studies suggest that ICE or ICE-like proteases are common mediators of apoptosis and that Z-VAD.FMK is an extremely powerful inhibitor and therefore useful for investigating the biochemical role of these enzymes in the mechanisms of cell death.

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Received 7 September 1995/11 October 1995; accepted 17 October 1995