An ICE-like protease is a common mediator of apoptosis induced by diverse stimuli in human monocytic THP.1 cells

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Received 18 September 1995

Abstract **Apoptosis was induced in THP.1 cells, a human monocytic tumour cell line, by diverse stimuli including cycloheximide, thapsigargin, etoposide and staurosporine. Induction of apoptosis by all these stimuli, except etoposide, was enhanced in the presence of the trypsin-like protease inhibitor, Nα-tosyl-L-lysinyl chloromethyl ketone (TLCK). Induction of apoptosis, assessed by morphological, flow cytometric and biochemical criteria, including proteolysis of poly(ADP-ribose) polymerase and cleavage of DNA to large kilobasepair fragments, was completely abrogated when cells were pretreated with an ICE-like protease inhibitor, Z-Val-Ala-Asp·fluoromethylketone. This suggested that an ICE homologue was a common mediator of apoptosis in THP.1 cells.**

Key words: Apoptosis; ICE-like proteases; THP.1 cell; Z-Val-Ala-Asp·fluoromethylketone

1. Introduction

Apoptosis is a major form of cell death important in vertebrate development, tissue homeostasis and in many diseases [1,2]. It is recognized by distinct morphological changes, including cell shrinkage, nuclear condensation and fragmentation [3]. It has been proposed that all cells except blastomeres would die by apoptosis in the absence of survival signals [4]. The ability of cells to undergo apoptosis can be induced by diverse stimuli including chemicals, radiation and viruses [3,5]. These diverse stimuli trigger a range of different signalling events, including elevations in cytosolic Ca^{2+}, cAMP accumulation, alterations in protein kinase C and tyrosine kinase activities, oxidative stress, generation of ceramide or nitric oxide [6]. These different signals ellicit a common response by apparently converging on a single pathway(s) characterised by common biochemical and morphological changes of apoptosis [3,5].

Some features of the cell death program appear highly conserved from worm to man. During development, 131 cells of the nematode, *Caenorhabditis elegans*, die and display an apoptotic morphology [1,7]. Two genes *ced-3* and *ced-4* are required for these cells to die. Little is known of *ced-4* but *ced-3* encodes a protein, which has sequence homology to the mammalian cysteine protease, interleukin-1β converting enzyme (ICE) [8]. Recent studies have identified a family of *ced-3* related genes including *ich-1*$_L$, *nedd-2* and CPP32, whose overexpression induces cell death by apoptosis [8–12]. The *C.elegans* gene, *ced-9*, suppresses cell death induced by *ced-3* and *ced-4* and encodes a protein homologous to the mammalian protein Bcl-2 [13].

Biochemically, the characteristic most commonly associated with apoptosis is internucleosomal cleavage of DNA recognized as a DNA ladder on conventional agarose gel electrophoresis [14]. It appears that DNA is initially cleaved into large kilobase-pair fragments (200–300 and 30–50 kbp in length) [15,16] and it is from these that the DNA ladders are derived [16–18]. Increasing attention is now being paid to the role of proteolysis in apoptosis. In addition to a *ced-3*/ICE related protease, a possible role(s) for other proteases has been implicated in a number of different biological systems [19–21]. In a recent study, TLCK inhibited apoptosis induced by diverse stimuli suggesting a trypsin-like protease is a common effector of thymocyte apoptosis [22].

During the early stages of apoptosis a number of proteins including histones, lamins, DNA topoisomerase I and II, poly(ADP ribose)-polymerase (PARP) and U1 small ribonucleoprotein are degraded [19,23]. Although the proteolytic cleavage of PARP has been proposed as an early biochemical marker of apoptosis [24], we do not as yet know the biologically relevant substrate(s) of any protease involved in apoptosis. Much interest has focussed on ICE due to its structural similarity to *ced-3* [8]. ICE, identified and isolated from human monocytic THP.1 cells, cleaves proIL-1β between Asp^{116} and Ala^{117} to yield the active 17.5 kDa cytokine [25]. This protease has an unusual requirement for aspartic acid in the P1 position, a property possessed by only one other known protease, granzyme B [26]. In contrast, TLCK inhibits trypsin-like proteases, which require a basic amino acid in the P1 position [27]. Peptide substrates for ICE must also contain at least four amino acids in the P1–P4 positions [25]. A critical role in apoptosis for an ICE homologue(s) rather than ICE per se was suggested by the finding that thymocytes and macrophages from ICE-deficient mice undergo apoptosis normally [28], although it is not possible to exclude redundancy in the system.

We have investigated the possible role of ICE or its homologues in apoptosis in THP.1 cells chosen because they are rich in ICE, Ich-1 and other ICE homologues [12]. In order to ensure that the biochemical events studied lie on a common pathway, apoptosis was induced by mechanistically diverse stimuli which act within their own private pathways [5]. Our results indicate that in THP.1 cells an ICE-like protease is a common mediator of apoptosis induced by diverse stimuli acting by different mechanisms.

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Abbreviations: ICE, interleukin-1β-converting enzyme; TLCK, Nα-tosyl-L-lysinyl chloromethyl ketone; Z-VAD.FMK, benzyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethylketone; YVAD.CHO, *N*-(*N*-acetyl-tyrosinyl-valinyl-alaninyl)-3-amino-4-oxobutanoic acid; PARP, poly(ADP-ribose) polymerase.

SSDI 0014-5793(95)01116-1

2. Materials and methods

2.1. Materials

Media and serum were purchased from Gibco (Paisley, UK). Pronase and TLCK were from Boehringer-Mannheim UK (Lewes, UK). Z-VAD.FMK was from Enzyme Systems Inc. (Dublin, CA, USA). *N*-Acetyl-tyrosinyl-valinyl-alaninyl-aspartic acid chloromethylketone (YVAD.CMK) was obtained from Bachem (Bubendorf, Switzerland). *N*-(*N*-acetyl-tyrosinyl-valinyl-alaninyl)-3-amino-4-oxobutanoic acid (Y-VAD.CHO) was kindly provided by Prof. L. Rubin (Eisai Laboratories, London, UK). All other chemicals were from Sigma Chemical Co. (Poole, UK).

2.2. Cell culture

THP.1 cells were obtained from Dr. K. Whaley, University of Leicester and were maintained as a suspension culture at 37°C in a 95% humidified atmosphere containing 5% CO_2 in RPMI 1640 medium (with glutamine), supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were split every third day and the density was kept at 2.5–5 × 10^5/ml. Logarithmically growing cultures of THP.1 cells were used for all experiments and were seeded at a density of 0.7 × 10^6/ml one day prior to the experiment.

2.3. Field inversion gel electrophoresis

Agarose plugs (100 μl, containing 0.5 × 10^6 cells) were prepared for field inversion gel electrophoresis as previously described [16]. To detect internucleosomal cleavage of DNA using conventional agarose gel electrophoresis, 1 × 10^6 cells were loaded per lane [29].

2.4. Apoptosis assessed by flow cytometry

We have previously described a flow cytometric method for the separation and quantitation of viable normal and apoptotic thymocytes [30]. The basis of this method appears to be the increased permeability of the apoptotic compared to the normal cells [31]. We validated that this method with minor modifications, as described, was also suitable for the separation and quantitation of normal and apoptotic THP.1 cells. Cells were initially treated with or without apoptosis inducing agents and then stained with 1.5 μg/ml Hoechst 33342 for 5 min and 0.05 μg/ml propidium iodide. Analysis was carried out using a Becton Dickinson FACS Vantage flow cytometer. After gating out non-viable cells (indicated by propidium iodide inclusion), the remaining viable cells were displayed as a cytogram of blue fluorescence versus forward light scatter.

2.5. PARP proteolysis

Cells (0.5 × 10^6) were prepared for SDS-PAGE as described [32]. Proteins were resolved on a 7% SDS-polyacrylamide gel, transferred onto nitrocellulose and Western blotting carried out using rabbit antiserum (318) to PARP (diluted 1:8000 in Tris-buffered saline, 0.1% Tween 20, pH 7.4). The antibody to PARP was a kind gift from Dr. G. Poirier (Quebec, Canada). Detection was achieved with a secondary antibody (goat anti-rabbit IgGs) conjugated to horseradish peroxidase (diluted 1:2000 in Tris-buffered saline, 0.1% Tween 20, pH 7.4) and an ECL detection kit (Amersham Life Science, UK).

3. Results

3.1. Z-VAD.FMK inhibits apoptosis induced by diverse stimuli

Incubation of THP.1 cells for 4 h with etoposide (25 μM), thapsigargin (100 nM), cycloheximide (25 μM) or staurosporine (0.5 μM) resulted in the induction of apoptotic cells when examined by fluorescence microscopy. In order to obtain a quantitative assessment of the extent of apoptosis, treated cells were stained with Hoechst 33342 and propidium iodide and examined by flow cytometry. Two populations of viable cells, which exhibited either low (R1) or high (R2) blue fluorescence, were observed (Fig. 1). When these cells were sorted, those with high blue fluorescence (R2) displayed an apoptotic morphology, whereas almost all cells with low blue fluorescence (R1) exhibited a normal morphology (Fig. 1). These results confirmed the validity of this flow cytometric method for the separation and quantitation of normal and apoptotic THP.1 cells. Using this method, it was apparent that apoptosis was induced to varying degrees (8–32%) (Table 1) by all the stimuli used.

In thymocytes, TLCK is a potent inhibitor of apoptosis [22]. Therefore we investigated the effects of TLCK on apoptosis in THP.1 cells. TLCK (100 μM) alone did not induce apoptosis (Table 1) or affect cell viability or morphology. TLCK potentiated apoptosis induced by cycloheximide and thapsigargin but inhibited that induced by etoposide (Table 1). Staurosporine (0.5 μM) alone induced both apoptotic and necrotic cells. Larger cells, which included propidium iodide, were designated as necrotic and were evident as early as 2 h after treatment (data not shown), while apoptotic cells were not observed in significant numbers until 4 h. These results indicated that the necrotic cells induced by staurosporine did not arise from secondary necrosis of apoptotic cells. Cotreatment with TLCK potentiated the induction of apoptotic cells (Table 1) and also reduced the percentage of propidium iodide including cells induced by staurosporine.

To investigate the involvement of ICE like-protease activity in the induction of apoptosis, cells were incubated for 1 h with Z-VAD.FMK, an inhibitor of ICE-like proteases, prior to exposure to the apoptotic stimuli. Z-VAD.FMK (50 μM) caused an almost total inhibition of apoptosis induced by all the stimuli either alone or in the presence of TLCK (Table 1). However, Z-VAD.FMK had little or no effect on STS-induced necrotic cell death suggesting that the ICE-like protease activity was not involved in necrosis. Our data supported a significant role for an ICE-like protease as a common mediator of the induction of apoptosis by diverse stimuli.

3.2. Z-VAD.FMK inhibits the formation of large kilobasepair fragments of DNA induced by diverse stimuli

In order to ascertain at what stage of the apoptotic process Z-VAD.FMK was exerting its inhibitory effect, THP.1 cells

Table 1
Z-VAD.FMK inhibits apoptosis induced by various stimuli

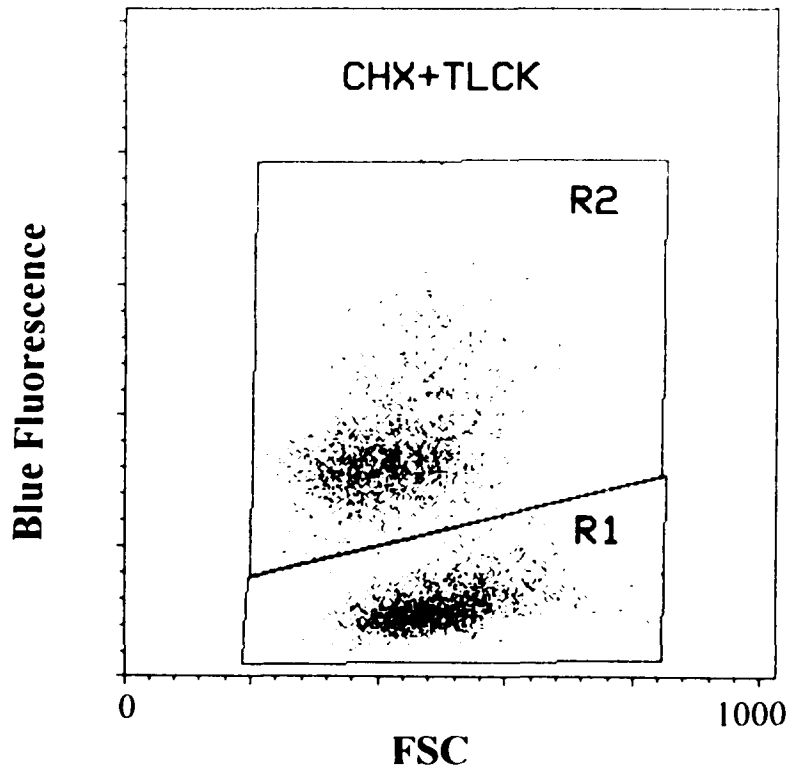
Stimulis	TLCK	Z-VAD.FMK	
		−	+
Control	−	2.3[a] ± 1.7	2.1 ± 1.5
Control	+	3.2 ± 0.8	2.3 ± 2.0
CHX	−	15.5 ± 0.5	5.4 ± 2.0
CHX	+	63.6 ± 3.7	1.5 ± 0.4
ETOP	−	32.0 ± 4.3	2.8 ± 1.20
ETOP	+	7.5 ± 2.9	1.8[b]
STS	−	18.1 ± 4.0[c]	3.2 ± 2.4
STS	+	47.2 ± 11.7	3.3 ± 0.4
THG	−	9.6 ± 1.3	4.0 ± 1.9
THG	+	59.3 ± 8.2	1.9 ± 0.7

THP.1 cells were incubated for 1 h either alone or in the presence of Z-VAD.FMK (50 μM). They were then further incubated for 4 h in the presence or absence of TLCK (100 μM) either alone or with cycloheximide (25 μM) (CHX), etoposide (25 μM) (ETOP), staurosporine (0.5 μM) (STS) or thapsigargin (100 nM) (THG).

[a] The percentage of apoptotic cells was assessed by flow cytometry. Results are expressed as mean ± S.E.M. of at least 3 separate experiments.

[b] As TLCK and Z-VAD.FMK alone inhibited etoposide-induced apoptosis, the combination was studied in one experiment only.

[c] With staurosporine alone, necrotic cells were also induced.



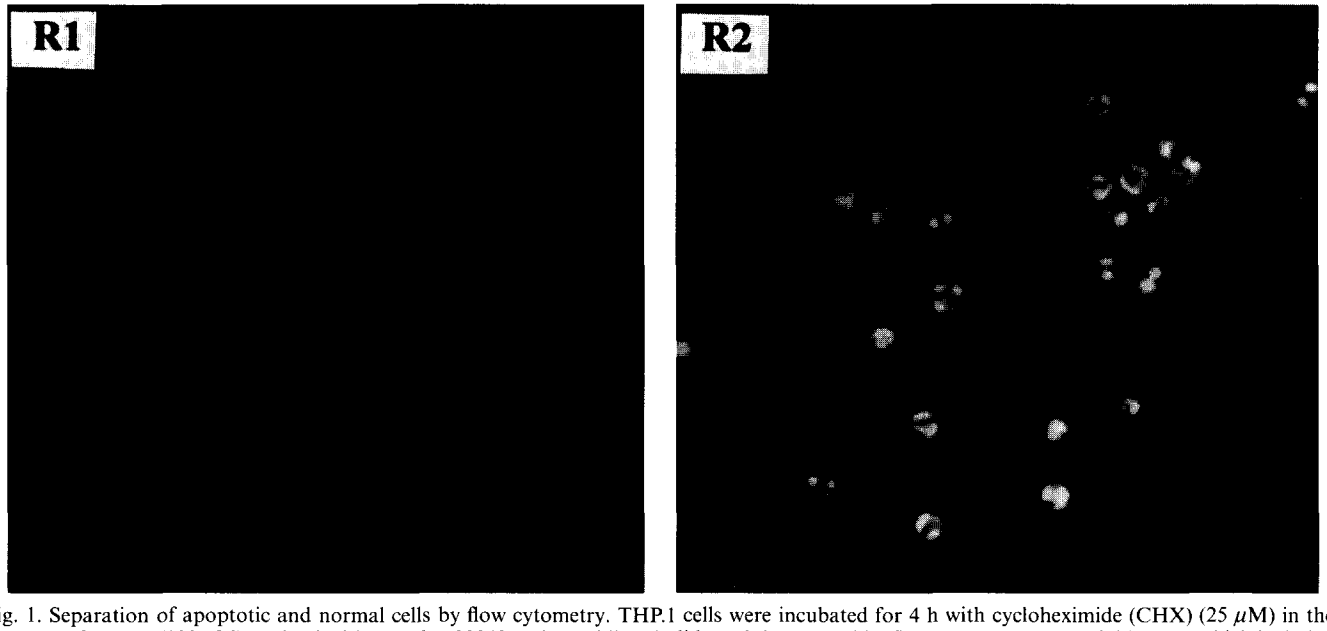


Fig. 1. Separation of apoptotic and normal cells by flow cytometry. THP.1 cells were incubated for 4 h with cycloheximide (CHX) (25 μM) in the presence of TLCK (100 μM), stained with Hoechst 33342 and propidium iodide and then sorted by flow cytometry. Non-viable cells, which included propidium iodide, were gated out and the remaining cells were displayed as a cytogram of blue fluorescence versus light forward light scatter (FSC). Two populations were sorted and examined by fluorescence microscopy. Cells exhibiting low blue fluorescence (R1) with morphology similar to untreated cells and those exhibiting high blue fluorescence (R2) with distinctive apoptotic morphology.

were exposed to diverse stimuli in the presence or absence of TLCK and Z-VAD.FMK and cleavage of DNA to large kilobasepair fragments was examined. Little or no DNA cleavage was observed in control cells and cells incubated with TLCK or Z-VAD.FMK alone (Fig. 2). However, substantial cleavage of DNA to large kilobasepair fragments was observed in cells exposed for 4 h to staurosporine, thapsigargin, cycloheximide and etoposide (Fig. 2). Coincubation of THP.1 cells for 4 h with

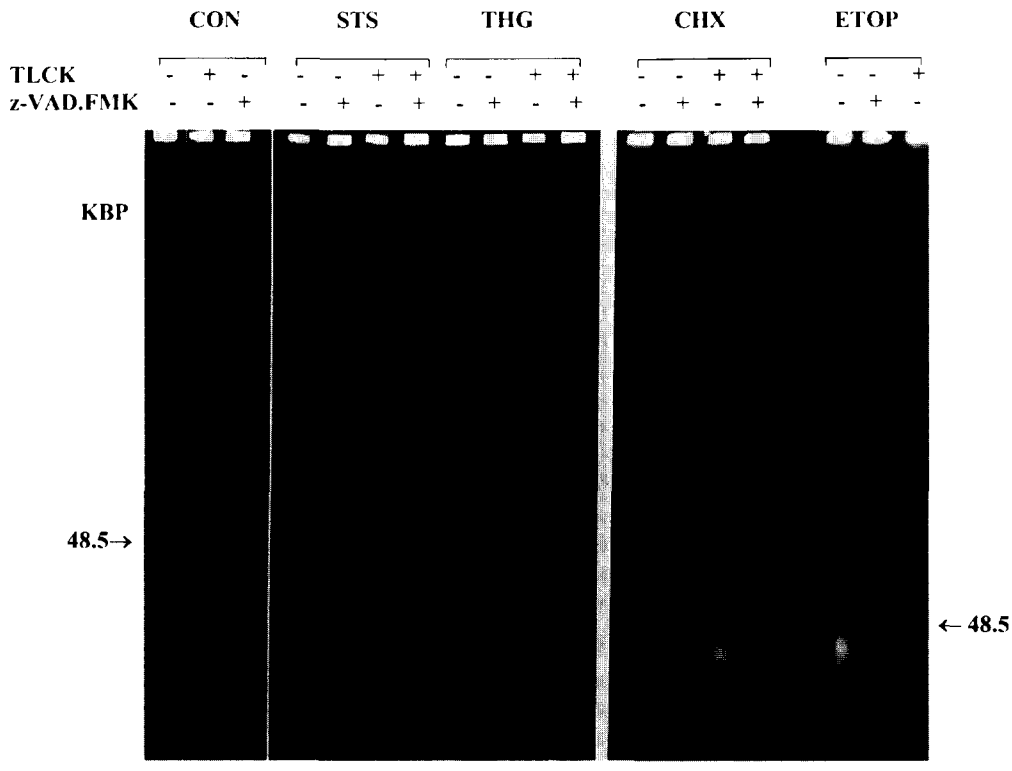


Fig. 2. Z-VAD.FMK inhibits formation of large kilobase-pair fragments of DNA induced by apoptotic stimuli. THP.1 cells were incubated either alone or with Z-VAD.FMK (50 μM) for 1 h and then further incubated for 4 h either with the apoptotic stimulus alone or in the presence of TLCK (100 μM). Cells were incubated with staurosporine (0.5 μM) (STS), thapsigargin (100 nM) (THG), cycloheximide (25 μM) (CHX) or etoposide (25 μM) (ETOP). They were then analysed for formation of large kilobasepair fragments by field inversion gel electrophoresis. The arrow on the left side of the figure indicates the 48.5 kilobase-pair standard for the control, staurosporine and thapsigargin experiments. The arrow on the right is the same standard for the experiments with cycloheximide and etoposide.

thapsigargin or cycloheximide in the presence of TLCK, resulted in increased formation of large kilobasepair fragments, particularly of 30–50 kbp (Fig. 2). This increase in DNA cleavage correlated well with the increased apoptosis of both these agents by TLCK (Table 1). TLCK inhibited etoposide-induced formation of large fragments of DNA (Fig. 2) also in agreement with its inhibition of etoposide-induced apoptosis (Table 1). Z-VAD.FMK almost completely inhibited the formation of large kilobasepair induced by all four stimuli (Fig. 2). Similarly Z-VAD.FMK almost completely inhibited the enhanced formation of large kilobasepair fragments induced by thapsigargin or cycloheximide in the presence of TLCK (Fig. 2), in good agreement with its ability to inhibit apoptosis induced by these treatments (Table 1). These stimuli also induced internucleosomal cleavage of DNA, which was inhibited by Z-VAD.FMK (data not shown). These results suggested that an ICE-like protease(s) acts at a stage prior to the formation of large kilobasepair fragments of DNA.

In order to test if ICE or an ICE homologue(s) was involved as a common mediator of apoptosis, cells were pretreated for 1 h with two other more specific inhibitors of ICE and then exposed to the apoptotic stimuli. YVAD.CHO (10–100 μM) did not inhibit the induction of apoptosis induced by thapsigargin, cycloheximide, etoposide or staurosporine as assessed by either flow cytometry or agarose gel electrophoresis (data not shown). YVAD.CMK (100 μM) had a differential effect, partially inhibiting DNA fragmentation induced by thapsigargin, cycloheximide, etoposide and staurosporine but did not inhibit the formation of apoptosis assessed by flow cytometry (data not shown).

3.3. *PARP proteolysis accompanies apoptosis and was inhibited by Z-VAD.FMK*

Proteolysis of intact PARP (116 kDa) to an 85 kDa fragment has been described as an early marker of apoptosis in a number of different cellular systems [24]. In order to obtain some insight on the possible involvement of proteolysis in the induction of apoptosis in THP.1 cells, we examined whether proteolysis of PARP accompanied apoptosis and whether it was sensitive to an inhibitor of ICE-like proteases. In control cells or cells treated with either Z-VAD.FMK or TLCK for 4 h, there was little or no degradation of intact PARP (116 kDa) (Fig. 3, lanes 1, 2 and 5, respectively). A small amount of PARP proteolysis was observed in cells exposed for 4 h to cycloheximide, staurosporine or thapsigargin (data not shown) commensurate with their induction of apoptosis. Co-treatment of cells with TLCK followed by cycloheximide or thapsigargin, which resulted in a potentiation of apoptosis (Table 1), resulted in an almost total loss of intact PARP (116 kDa) accompanied by the appearance of a number of proteolytic products, particularly an 85 kDa fragment (Fig. 3, lanes 3 and 6). Pretreatment for 1 h with Z-VAD.FMK (50 μM), which totally inhibited the inducton of apoptosis (Table 1) prevented the loss of intact PARP and the appearance of the 85 kDa fragment (Fig. 3, lanes 4 and 7).

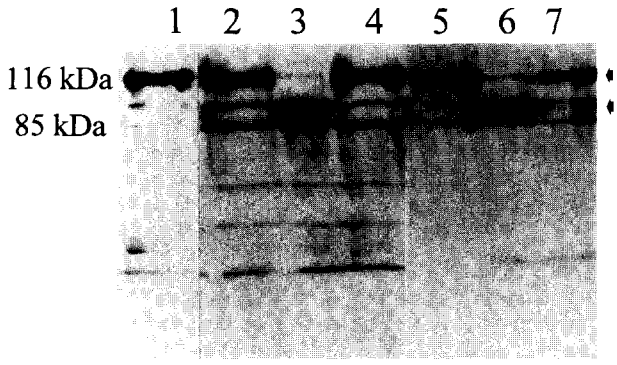


Fig. 3. Z-VAD.FMK inhibits PARP proteolysis induced by apoptotic stimuli. THP.1 cells were incubated for 4 h alone (lane 1). Cells were also incubated for 4 h with TLCK (100 μM) either alone (lane 5) or in the presence of cycloheximide (25 μM) (lane 3) or thapsigargin (100 nM) (lane 6). Cells were preincubated for 1 h with Z-VAD.FMK (50 μM) followed by an incubation for 4 h either alone (lane 2) or with TLCK (100 μM) and cycloheximide (25 μM) (lane 4) or TLCK (100 μM) and thapsigargin (100 nM) (lane 7). Proteolysis of PARP was induced by the apoptotic stimuli and inhibited by Z-VAD.FMK. The arrows indicate intact PARP (116 kDa) and the 85 kDa fragment.

Etoposide alone induced proteolysis of intact PARP which was almost completely inhibited by either TLCK or Z-VAD.FMK (data not shown). These data demonstrate that proteolysis of PARP correlated with the induction of apoptosis in THP.1 cells, i.e. treatments which enhanced apoptosis resulted in a greater proteolysis whereas treatments which inhibited apoptosis inhibited proteolysis. The inhibition of proteolysis of PARP by Z-VAD.FMK provided indirect evidence that Z-VAD.FMK was inhibiting a protease.

4. Discussion

Apoptosis was induced in THP.1 cells by a number of stimuli acting by diverse mechanisms (Table 1). This was consistent with the proposal that such stimuli activate private signalling pathways which ultimately converge on a common apoptotic pathway(s) characterized by a series of common biochemical and morphological changes. The induction of apoptosis by all these stimuli except etoposide was enhanced by TLCK and these effects were totally abrogated by Z-VAD.FMK. Z-VAD-FMK has also been shown to inhibit Fas-mediated apoptosis in T lymphocytes [33]. Our data strongly support the involvement in THP.1 cells of a Z-VAD.FMK inhibitable target, most probably an ICE-like protease, within a common apoptotic pathway (Fig. 4). This target acts at an early stage of the apoptotic process as Z-VAD.FMK inhibited the morphological and biochemical changes of apoptosis including formation of large kilobasepair fragments of DNA (Fig. 2) and internucleosomal cleavage (data not shown) as well as the induction of apoptotic cells (Table 1). A similar inhibition of dexamethasone-, etoposide- and thapsigargin-induced apoptosis was observed in thymocyte apoptosis. Our data demonstrating that Z-VAD.FMK but not YVAD.CHO and YVAD.CMK inhibited apoptosis may have been due to a differential cell permeability of the inhibitors or to the possible involvement of an ICE homologue(s) other than ICE as a common mediator of apoptosis. This is based on the observations of the absolute requirement of peptide substrates of ICE for an aspartate in the P1 position and also that they must contain at least four amino acids on the N-terminal side of the cleavage site, as removal of the amino acid in the P4 position results in a very marked loss of activity [25]. Thus Z-VAD.FMK, with an aspartate in the P1 position but no amino acid in the P4 position, should inhibit ICE-like proteases but not ICE per se. Whilst our data suggests the involvement in the induction of apoptosis of an ICE homologue rather than ICE itself, we cannot exclude a possible role for both ICE and an ICE homologue assuming that both activities are blocked by Z-VAD.FMK.

Proteolysis of PARP (116 kDa) with the concomittant generation of an 85 kDa fragment has been proposed as a marker of early apoptosis [24]. In a cell-free model of apoptosis, this cleavage was carried out by an ICE-like protease (prICE) [34]. We therefore used PARP degradation as a marker for ICE-like proteolysis. Our results demonstrated that PARP proteolysis accompanied apoptosis in THP.1 cells (Fig. 3). This proteolysis was markedly enhanced by apoptotic stimuli in the presence of TLCK and this enhanced proteolysis was completely inhibited by Z-VAD.FMK (Fig. 3). This data is consistent with the involvement of an ICE-like protease in PARP proteolysis. However the significance of PARP proteolysis in apoptosis is unclear as a PARP knockout mouse developed normally [35]. This suggested that apoptosis probably does not have an absolute requirement for proteolysis of PARP but rather that it may be a consequence of cell death.

TLCK caused a marked enhancement of apoptosis induced by three of the stimuli used to induce apoptosis but inhibited apoptosis induced by etoposide, a DNA topoisomerase II inhibitor (Table 1). The reason why TLCK inhibits apoptosis induced by etoposide is unclear but may be related to an early step in the damage induced by etoposide. Similarly in HL-60 cells, TLCK inhibited apoptosis induced by camptothecin, a DNA topoisomerase I inhibitor, but potentiated that induced by cycloheximide [36]. The present results were in marked contrast to our earlier study with thymocytes when apoptosis induced by diverse stimuli was inhibited by TLCK [22]. These contrasting results suggest that the TLCK target(s) is not part of the effector mechanism but rather that it has an important function either positively or negatively regulating apoptosis depending on the cell type. The same signal may have opposite

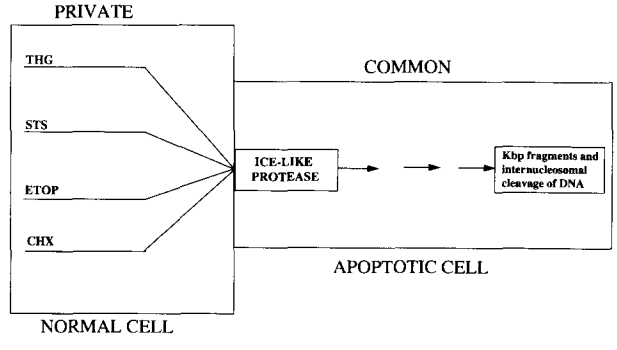


Fig. 4. Model of apoptosis in THP.1 cells. Diverse stimuli including thapsigargin (THG), staurosporine (STS), etoposide (ETOP) and cycloheximide (CHX) act on inidvidual private signalling pathways in normal THP.1 cells. Diverse agents induce a series of common biochemical and morphological changes characteristic of apoptotic cells culminating in the cleavage of DNA to large kilobasepair (kbp) (200–300 and 30–50 kbp) and nucleosomal fragments. Z-VAD.FMK, an ICE-like protease inhibitor, inhibits all the biochemical and morphological features of apoptosis induced by all these stimuli.

effects on different cell types and even on the same cell type at different stages of development [6,7].

In summary, our data support a model demonstrating a role in THP.1 cells for an ICE-like protease acting as a common mediator of apoptosis induced by various stimuli (Fig. 4). Our data lend further support to a common cell death program conserved between species.

Acknowledgements: We thank Mrs. R. Morgan for help with the preparation of the manuscript and Mr. R. Snowden for excellent technical assistance with the flow cytometry.

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