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Apoptosis in human monocytic THP.1 cells involves several distinct targets of N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK)

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Abstract

N-Tosyl-L-phenylalanyl chloromethyl ketone (TPCK), a chymotrypsin-like serine protease inhibitor, affected apoptosis in human monocytic THP.1 cells differently dependent on both the concentration used and the apoptotic stimulus. TPCK (50–75 μM) induced both biochemical and ultrastructural changes characteristic of apoptosis, including proteolysis of poly (ADP-ribose) polymerase (PARP) and lamins together with formation of large kilobase pair fragments of DNA, particularly of 30–50 and 200–300 kilobase pairs in length but without internucleosomal cleavage of DNA. The induction of apoptosis by TPCK also involved the processing of CPP32 and Mch 3 to their catalytically active subunits. Benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD.FMK), an ICE-like protease inhibitor, completely prevented all the biochemical and morphological changes induced by TPCK demonstrating the involvement of ICE-like proteases in the execution phase of apoptosis. Lower concentrations of TPCK (5–20 μM) prevented internucleosomal cleavage of DNA induced by other apoptotic stimuli. TPCK (10 μM) inhibited cell death induced by etoposide but potentiated that induced by cycloheximide demonstrating that it differentially affected apoptosis in THP.1 cells dependent on the stimulus used. These results are consistent with at least three distinct TPCK targets, one being important for cell survival, the second in facilitating internucleosomal cleavage of DNA and the third in the modulation of apoptosis induced by different apoptotic stimuli.

Keywords: apoptosis; proteases; THP.1 cells; N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK); CPP32; Mch 3

Abbreviations: PARP, poly (ADP-ribose) polymerase; ICE, interleukin-β converting enzyme; TPCK, N-tosyl-L-phenylalanyl chloromethyl ketone; Z-VAD.FMK, benzyloxycarbonyl-Val-Ala-Asp (OMe) fl uoromethyl ketone

Introduction

Apoptosis is a physiological form of cell death important in the control of cell populations and conserved from *Caenorhabditis elegans* to mammals (Arends and Wyllie, 1991; Ellis *et al*, 1991; Vaux, 1993). Abnormalities in this cell death programme may be important in several human diseases including cancer, autoimmune diseases and certain neurodegenerative disorders (Arends and Wyllie, 1991; Thompson, 1995; Vaux, 1993). Several features of the cell death programme are highly conserved (Ellis *et al*, 1991). *Ced-3*, one of the genes required for cells to die by apoptosis in *C. elegans*, encodes a protein, with sequence homology to the mammalian cysteine protease, interleukin-1β converting enzyme (ICE) (Yuan *et al*, 1993). In addition to ICE, nine related proteases have currently been identified including Nedd-2/Ich-1 (Kumar *et al*, 1994; Wang *et al*, 1994), CPP32/Apopain/Yama (Fernandes-Alnemri *et al*, 1994; Nicholson *et al*, 1995; Tewari *et al*, 1995), Mch2 (Fernandes-Alnemri *et al*, 1995a), Mch3/ICE-LAP3/CMH-1 (Duan *et al*, 1996a; Fernandes-Alnemri *et al*, 1995b), Lippke *et al*, 1996), ICE-LAP6/Mch 6 (Duan *et al*, 1996b; Srinivasula *et al*, 1996), TX/ICErel II/Ich2 (Faucheau *et al*, 1995; Munday *et al*, 1995; Kamens *et al*, 1995), ICEre1III (Munday *et al*, 1995) MACH/FLICE/Mch 5 (Boldin *et al*, 1996; Muzio *et al*, 1996; Fernandes-Alnemri *et al*, 1996) and Mch 4 (Fernandes-Alnemri *et al*, 1996). Overexpression of any of these proteases results in cell death. As thymocytes and macrophages from ICE-deficient mice undergo apoptosis normally, except possibly for Fas-induced apoptosis, other ICE homologue(s) may be important in apoptosis (Kuida *et al*, 1995; Li *et al*, 1995), although it is not possible to exclude functional redundancy in the system. The fact that more than one member of the ICE family may be expressed in the same cells (Wang *et al*, 1994) raises the possibility that these proteases may cooperate to regulate apoptosis.

Apoptosis can be induced by withdrawal of growth factors or by exposure to diverse stimuli including chemicals, radiation and viruses (Arends and Wyllie, 1991; Cohen *et al*, 1992a; Raff, 1992). It was initially recognized by distinct morphological changes, including cell shrinkage, nuclear condensation and fragmentation (Arends and Wyllie, 1991). Biochemically, internucleosomal cleavage of DNA is the characteristic most commonly associated with apoptosis (Wyllie, 1980). Our studies and others suggest that DNA is initially cleaved into large kilobase pair fragments (200–300 and 30–50 kbp in length) and that these give rise to the nucleosomal fragments (Brown *et al*, 1993; Cohen *et al*, 1994; Oberhammer *et al*, 1993). During apoptosis a number of proteins including histones, lamins, DNA topoisomerase I and II, poly (ADP-ribose) polymerase (PARP), U1 small ribonucleoprotein and DNA-dependent protein kinase are degraded (Casciola-Rosen *et al*, 1994, 1995; Kaufmann,

1989; Kaufmann *et al*, 1993). Whilst ICE-like proteases are responsible for cleavage of some of these substrates, such as PARP, other proteases are also involved. Introduction of exogenous proteases into cells induces morphological and biochemical changes characteristic of apoptosis (Williams and Henkart, 1994) and various protease inhibitors block apoptosis in different cell types (Bruno *et al*, 1992; Chow *et al*, 1995; Ghibelli *et al*, 1995; Kaufmann *et al*, 1993; Sarin *et al*, 1993; Squier *et al*, 1994; Suffys *et al*, 1988; Zhivotovsky *et al*, 1995). Much interest has focused on ICE due to its structural similarity to *ced-3* (Yuan *et al*, 1993). Although ICE-like proteases have been implicated in the execution of apoptosis, the mechanism regulating the activity of these proteases is poorly understood. All known ICE-like proteases are expressed as inactive proforms which require cleavage at specific Asp residues to yield two subunits which combine to form the active protease (reviewed in Thornberry and Molineaux, 1995). Although some of these may autoprocess (Nicholson *et al*, 1995), CPP32 can be activated by the serine protease, granzyme B (Darmon *et al*, 1995). Thus CPP32 may be activated by another ICE-like protease and/or a serine protease with specificity for Asp in the P1 position.

Recently we demonstrated that apoptosis in THP.1 cells was inhibited by an ICE-like protease inhibitor, benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone (Z-VAD.FMK), which suggested that an ICE-like protease was a common mediator of apoptosis (Zhu *et al*, 1995). In these cells, N-tosyl-L-lysinyl chloromethyl ketone (TLCK) potentiated apoptosis induced by several stimuli whereas it inhibited apoptosis in thymocytes (Fearnhead *et al*, 1995a; Zhu *et al*, 1995), suggesting that a TLCK inhibitable protease may be a regulator of apoptosis. ICE and ICE-related proteases have an unusual requirement for aspartic acid in the P1 position (Thornberry *et al.,* 1992; Thornberry and Molineaux, 1995). TLCK requires a basic amino acid in the P1 position whereas the chymotrysin-like serine protease inhibitor used in this study, N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK), requires an aromatic amino acid in the P1 position (Powers and Harper, 1986). Thus in this study, we have investigated the ability of TPCK to modulate apoptosis in THP.1 cells, in order to investigate the possible relationship between chymotrypsin-like serine proteases and ICE-like proteases in the regulation of apoptosis.

Results

TPCK induces apoptosis assessed by flow cytometry

Incubation of THP.1 cells with TPCK, for 4 h caused a concentration-dependent induction of apoptosis (Table 1), as assessed by flow cytometry. TPCK (50–75 μM) induced apoptosis whereas lower concentrations (12.5–25 μM) did not. Z-VAD.FMK, an inhibitor of ICE-like proteases, inhibited TPCK-induced apoptosis (Table 1) supporting the involvement of ICE-like proteases in the induction of apoptosis. When treated cells were examined by fluorescence microscopy, an increase in the number of cells with high Hoechst 33342 blue staining together with condensed nuclei was apparent compared with control cells (data not shown).

Table 1 TPCK induces apoptosis as assessed by flow cytometry

TPCK (μM)	Z-VAD.FMK	%Apoptosis
0	–	1.9 ± 1.7
12.5	–	2.8 ± 0.3
25	–	3.2 ± 2.5
50	–	10.7 ± 1.9
75	–	25.0 ± 2.5
75	+	0.4 ± 0.1

THP.1 cells were incubated for 4 h with the indicated concentration of TPCK either in the presence (+) or absence (–) of Z-VAD.FMK (20 μM). Apoptosis was then assessed by the Hoechst 33342/propidium iodide method as described in Materials and Methods. Results are the mean ± s.e.m. of at least three experiments.

TPCK induces an intermediate apoptotic morphology

As TPCK has been described as an inhibitor of apoptosis in several cellular systems (Ghibelli *et al*, 1995; Suffys *et al*, 1988; Ruggiero *et al*, 1987; Neamati *et al*, 1995; Weaver *et al*, 1993), we examined the ultrastructure of these cells in order to see if they were apoptotic. Untreated THP.1 cells were large (12–16 μm) and irregular in outline with many irregular microvilli and a multi-lobed nucleus (Figure 1A). The diffuse perinuclear and perinucleolar heterochromatin was weakly differentiated from the euchromatin but the nucleous was clearly visible in most sections. TPCK (2–6 μM), which did not induce apoptosis, had no detectable effect on the ultrastructure of THP.1 cells (data not shown). However TPCK (50–75 μM) resulted in cytoplasmic condensation accompanied by pronounced dilation of the endoplasmic reticulum, the resulting vacuoles showed signs of fusion with the cell membrane, usually at one pole of the cell (Figure 1B and D). The membrane of these cells was largely devoid of microvilli and the cytoplasm contained small (0.5–1.5 μm diam.) accumulations of fine granular material. Partial nucleolar disintegration was indicated by the dispersal of the dense fibrillar component which, like numerous small clumps of partially condensed chromatin, was distributed throughout the nucleoplasm (Figure 1B). This ultrastructure was compared with that of apoptotic THP.1 cells, obtained following treatment with cycloheximide and TLCK (Zhu *et al*, 1995), which exhibited the characteristic features of apoptosis including cell shrinkage with loss of microvilli, chromatin condensation and margination (Figure 1C). The degree of chromatin condensation in the cells treated with TPCK (Figure 1B and D) was intermediate between control cells (Figure 1A) and apoptotic cells (Figure 1C). Z-VAD.FMK completely inhibited TPCK-induced apoptosis as assessed by electron microscopy (Figure 1E) supporting the involvement of an ICE-like protease(s) in the development of the ultrastructural changes of apoptosis.

TPCK induces formation of large kilobase pair fragments of DNA

TPCK caused a concentration-dependent induction of large kilobase pair DNA fragments ranging from 30–50, 200–300 and 700 kbp (Figure 2). No fragments were observed at low concentrations of TPCK (25 μM), whereas they were clearly

seen at higher concentrations (50–75 μM) (Figure 2). These higher concentrations did not induce internucleosomal cleavage of DNA (data not shown). Thus formation of large kilobase pair fragments of DNA was observed at concentrations of TPCK (50–75 μM), which induced changes detected by both flow cytometry (Table 1) and electron microscopy (Figure 1b). Z-VAD.FMK completely prevented TPCK-induced formation of large kilobase pair fragments of DNA (Figure 2 compare lanes 4 and 5) supporting the involvement of an ICE-like protease(s) in TPCK-induced DNA fragmentation. Z-VAD.FMK alone did not induce formation of large kilobase pair fragments of DNA (Figure 2 lane 6). These results demonstrating that TPCK induced early biochemical and ultrastructural features of apoptosis (Figures 1 and 2) suggested that TPCK is an inducer of apoptosis and that a TPCK-inhibitable target, possibly a protease, may be involved in the survival of THP.1 cells.

TPCK induces proteolysis of PARP and lamins

To study further the biochemical features of apoptosis associated with the intermediate apoptotic morphology

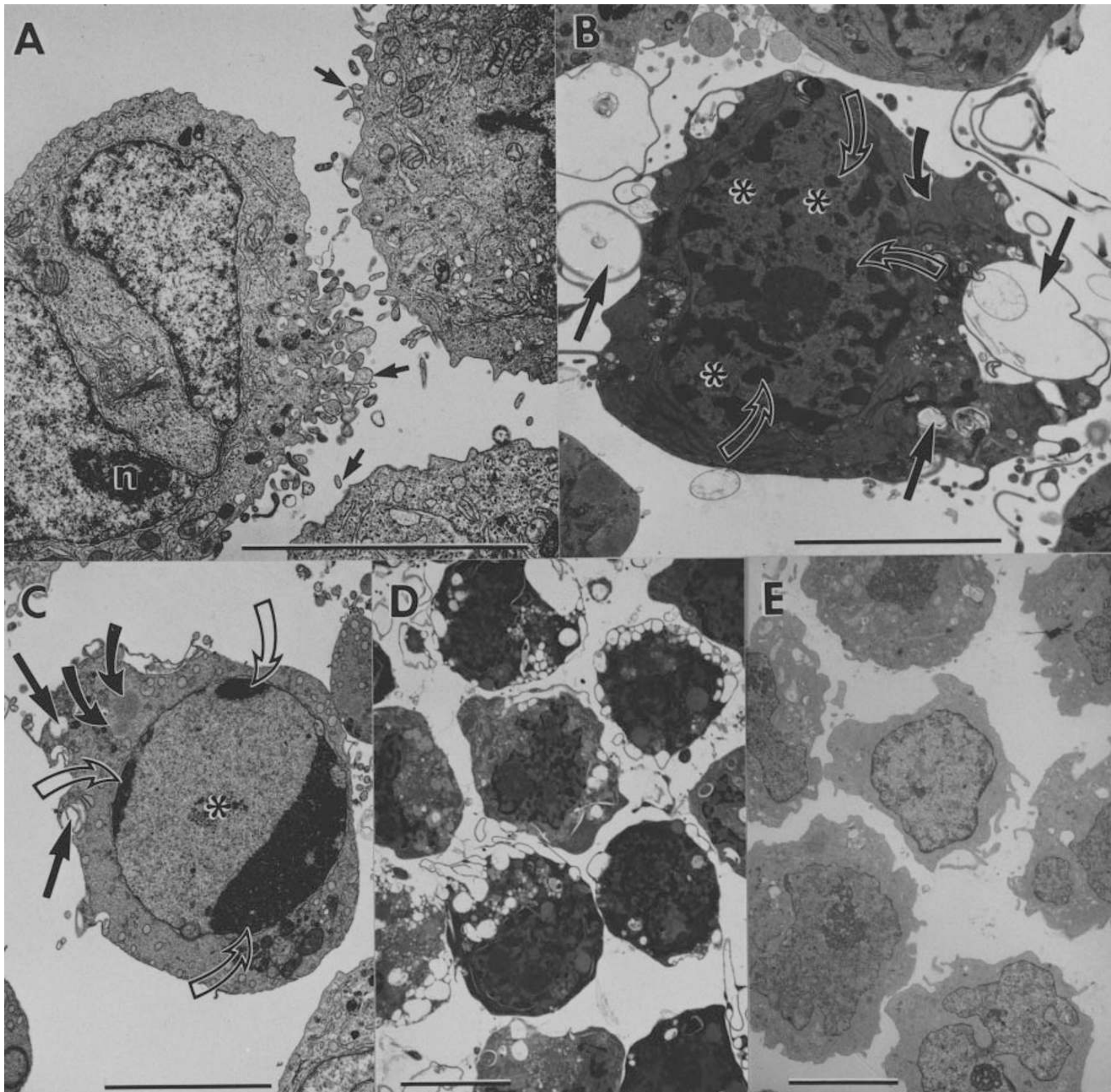

Figure 1 (**A**) Untreated THP.1 cells showing the presence of microvilli (arrow-heads) and a multi-lobed nucleus with distinct nucleolus (n). (**B**) Cell treated with TPCK (75 μM) showing dilation of the endoplasmic reticulum (solid arrows) and the accumulation of fine granular material (solid curved arrow). Numerous small clumps of partially condensed chromatin are distributed throughout the nucleoplasm (open curved arrows) and partial nucleolar disintegration has resulted in dispersal of the dense fibrillar compoment (*). (**C**) Cell exposed to cycloheximide (25 μM) in the presence of TLCK (100 μM) showing cytoplasmic condensation and cisternae of the endoplasmic reticulum which are dilated to produce electron-lucent vacuoles (solid arrows). The cell membrane is devoid of microvilli and the cytoplasm contains clumps fo fine granular material (solid curved arrows). The heterochromatin is condensed and marginated (open curved arrows) whereas the nucleolus is dispersed to reveal the dense fibrillar component (*). (**D**) Lower power showing several cells demonstrating the effects of exposure to TPCK (75 μM). (**E**) Cells exposed in parallel with those shown in **D**, but in the presence of Z-VAD.FMK (20 μM) show inhibition of all the ultrastrucural changes of apoptosis. All bars = 5 μm.

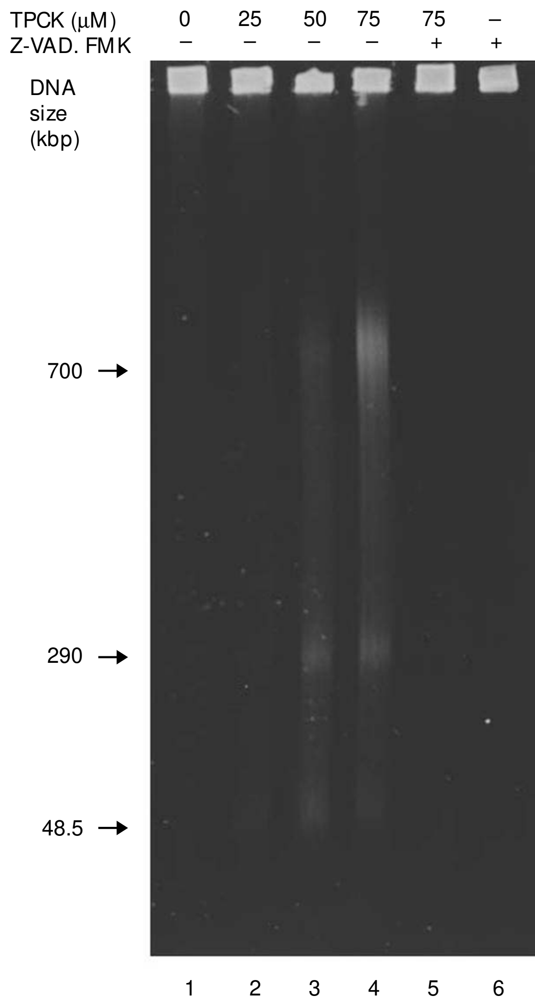

Figure 2 TPCK induces a concentration-dependent formation of large kilobasepair fragments of DNA. Cells were incubated for 4 h either alone (lane 1) or with the indicated concentrations of TPCK (lanes 2–4). Cells were also treated with Z-VAD.FMK (20 μM) in the presence (lane 5) or absence (lane 6) of TPCK (75 μM).

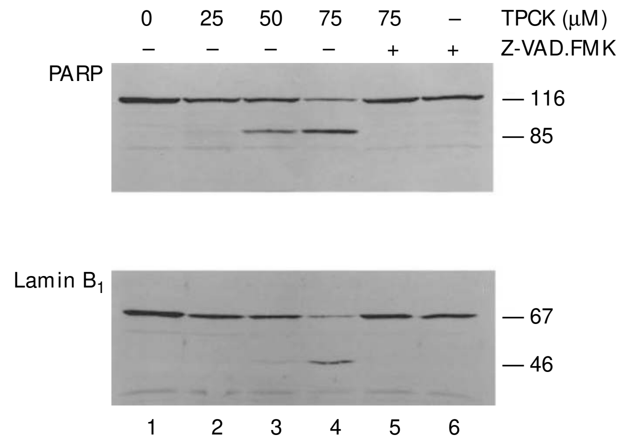

Figure 3 TPCK causes a concentration-dependent proteolysis of PARP and lamin B_1. Cells were incubated either alone (lane 1) or with the indicated concentrations of TPCK (lanes 2–5). PARP and lamin B_1 were detected by Western blotting. The lines in the upper figure indicate intact PARP (116 kDa) and its 85 kDa proteolytic product. The lines in the lower figure indicate lamin B_1 (67kDa) and its ~46 kDa proteolytic fragment. Cells were also treated with Z-VAD.FMK (20 μM) in the presence (lane 5) or absence of TPCK (lane 6).

induced by TPCK (Figure 1B), we examined the nuclear proteins PARP and lamin B_1. Proteolysis of PARP has been considered to be an early biochemical marker of apoptosis (Kaufmann *et al*, 1993). Intact PARP in control cells was present as a 116 kDa protein (Figure 3 lane 1), in agreement with previous findings (Kaufmann *et al*, 1993). Low concentrations of TPCK (25 μM), which did not induce apoptosis as assessed by flow cytometry (Table 1), did not increase proteolysis of PARP above that observed in control cells (Figure 3 lanes 1 and 2). Higher concentrations of TPCK induced a concentration-dependent proteolysis of PARP to yield a fragment of 85 kDa (Figure 3 lanes 3 and 4) concomitant with their induction of apoptosis (Table 1). Proteolysis of lamins has also been described as a common feature of apoptotic cells (Ucker *et al*, 1992; Lazebnik *et al*, 1995). Intact lamin B_1 was present as a ~67 kDa protein (Figure 3 lane 1). TPCK (50–75 μM) induced a concentration dependent formation of a ~46 kDa proteolytic fragment (Figure 3 lanes 3–4). Z-VAD.FMK completely inhibited TPCK-induced proteolysis of both PARP and lamin B_1 (Figure 3 lane 5) further supporting the suggestion that ICE-like proteases are required for such proteolysis to occur in TPCK induced apoptosis.

TPCK induces a DEVD.AFC cleaving activity together with processing of CPP32 and Mch 3

The induction of proteolysis of PARP and lamins by TPCK suggested that its induction of apoptosis was accompanied by an activation of ICE-like proteases. We therefore measured the CPP32/Mch 3-like protease activity, using Z-DEVD.AFC as substrate, in lysates from control cells or those exposed to different apoptotic stumuli. This model substrate was chosen because the DEVD tetrapeptide sequence is identical to the site of PARP cleaved by CPP32 (Lazebnik *et al*, 1994; Nicholson *et al*, 1995) and Mch 3 (Fernandes-Alnemri *et al*, 1995b). Lysates from control cells exhibited a small amount of Z-DEVD.AFC activity (Figure 4). In contrast, lysates from cells exposed to different apoptotic stimuli, TPCK, etoposide, cycloheximide either alone or in the presence of TLCK, demonstrated an increase in Z-DEVD.AFC cleaving activity (Figure 4) consistent with their ability to induce apoptosis (Figure 7 and Table 1). TLCK, at concentrations which did not induce apoptosis, did not cause an increase in this activity (Figure 4). TPCK caused a time- and concentration-dependent increase in Z-DEVD.AFC cleaving activity (data not shown). These data supported the involvement of a CPP32/Mch 3-like protease activity in the execution of apoptosis in THP.1 cells.

In order to confirm the activation of CPP32/Mch3 in TPCK-induced apoptosis, we investigated the ability of TPCK to induce processing of CPP32 and Mch 3. In untreated cells, immunoblots showed the presence of the 32 kDa precursor of CPP32 (Figure 5 lane 1) and the

~36 kDa precursor of Mch 3 (Figure 5 lane 1). Concentrations of TPCK (50–75 μM), which induced apoptosis, induced the formation of the p17 subunit of CPP32 and the p19 subunit of Mch 3 together with loss of their respective proforms (Figure 5 compare lanes 3 and 4 with lane 1). TPCK (75 μM) also induced the formation of an ~p19 subunit of CPP32 (Fernandes-Alnemri *et al*, 1996). TPCK (25 μM), which did not induce marked apoptosis, did not induce loss of the proforms of either CPP32 or Mch 3 or formation of their large subunits (Figure 5 lane 2). Z-VAD.FMK inhibited the processing of both CPP32 and Mch 3 preventing both the loss of the proforms and the appearance of the catalytically active large subunits (Figure 5 lane 5). Thus in intact cells, apoptogenic concentrations of TPCK induced the formation of the catalytically active subunits of CPP32 and Mch 3 and this was accompanied by an increase in DEVD.AFC cleaving activity in lysates. Taken together these results provide compelling evidence that TPCK induces apoptosis in THP.1 cells and that ICE-like protease(s) are key effectors in the execution of this apoptosis.

TPCK inhibits internucleosomal cleavage of DNA induced by apoptotic stimuli

Our biochemical and ultrastructural data demonstrating that TPCK induced apoptosis is in marked contrast to many reports in the literature suggesting that it inhibits apoptosis (Ghibelli *et al*, 1995; Suffys *et al*, 1988; Ruggiero *et al*, 1987; Neamati *et al*, 1995 ; Weaver *et al*, 1993). Many of these studies have utilised an increase in internucleosomal cleavage as a marker of apoptosis. In order to resolve these differences, we investigated the effects of TPCK on internucleosomal cleavage induced by different apoptotic stimuli. Both etoposide alone (Figure 6 lane 3) and cycloheximide in the presence of TLCK (Figure 6 lane 5) induced internucleosomal cleavage of DNA compared with control cells (Figure 6 lane 1). These increases in internucleosomal cleavage were completely inhibited by a low concentration of TPCK (10 μM) (Figure 6 lanes 4 and 6). Thus, in agreement with other reports, TPCK inhibited internucleosomal cleavage of DNA.

TPCK potentiates cycloheximide-induced apoptosis but inhibits etoposide-induced apoptosis

Co-incubation of THP.1 cells with TLCK resulted in a marked potentiation of the induction of apoptosis by a number of stimuli including cycloheximide but resulted in the inhibition of apoptosis induced by etoposide (Zhu *et al*, 1995). These results suggested that a serine protease may act as a regulator of both cell survival and apoptosis in THP.1 cells. In order to explore further the nature of this putative protease, we examined whether low concentrations of TPCK, which did not induce apoptosis alone, exerted similar effects. THP.1 cells were incubated with cycloheximide (25 μM) or etoposide (25 μM) either alone or in the presence of TPCK (5 μM) or TLCK (100 μM). Both TPCK and TLCK caused a potentiation of cycloheximide-induced apoptosis but both inhibited etoposide-induced apoptosis, as assessed by flow cytometry (Figure 7). Similar effects were observed with a

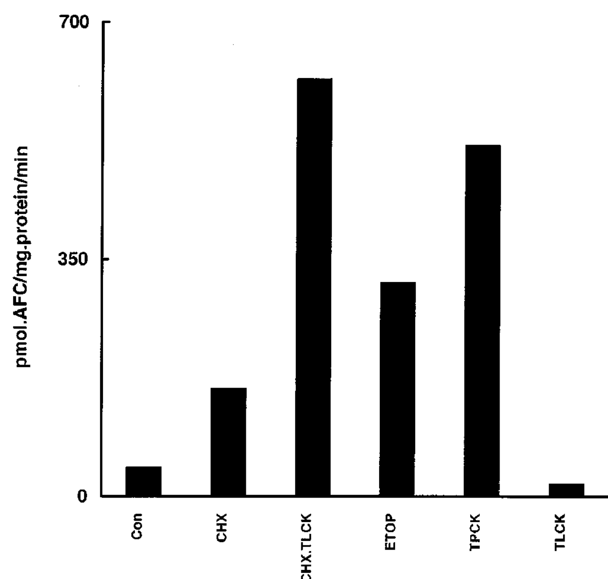

Figure 4 The induction of apoptosis was accompanied by an increase in Z-DEVD-AFC cleavage activity. THP.1 cells were incubated alone (Con) or in the presence of the indicated agents for 4 h. Lysates from these cells were assayed for Z-DEVD-AFC cleavage activity. Cychoheximide (25 μM) (CHX) induced an increase in the Z-DEVD-AFC cleavage activity, which was markedly enhanced by TLCK (100 μM). TPCK (75 μM) alone and etoposide (25 μM) (ETOP) caused a marked increase in Z-DEVD-AFC cleavage activity, while TLCK (100 μM) alone showed no effect on this activity.

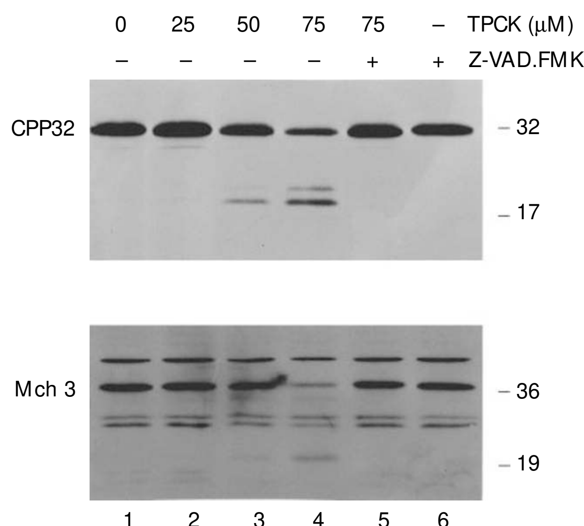

Figure 5 TPCK induces concentration-dependent processing of CPP32 and Mch 3. Cells were incubated either alone (lane 1) or with the indicated concentrations of TPCK (lanes 2–5). CPP32 and Mch 3 were detected by Western blotting. The lines in the upper figure indicate proCPP32 and its p17 subunit whilst those in the lower figure indicate proMch 3 and its p19 subunit. Cells were also treated with Z-VAD.FMK (20 μM) in the presence (lane 5) or absence of TPCK (lane 6).

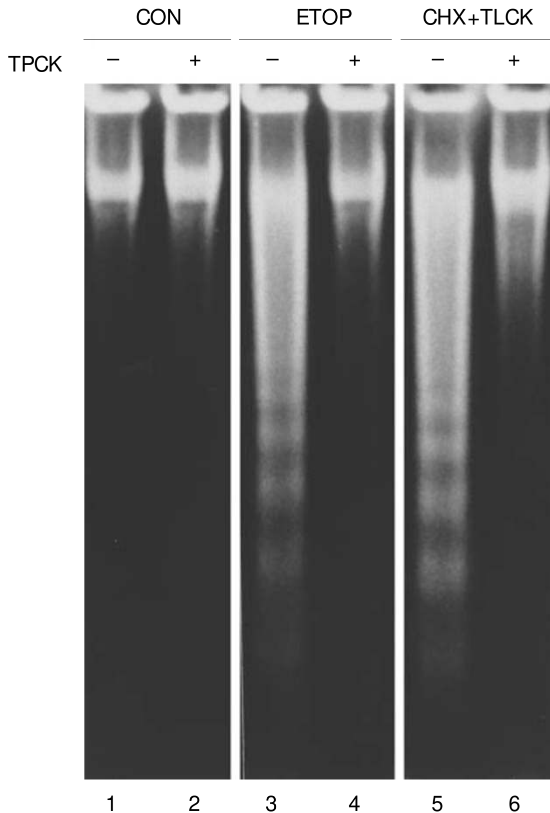

Figure 6 TPCK inhibits internucleosomal cleavage of DNA induced by etoposide and cycloheximide. Cells were cultured for 4 h, with control medium (lane 1), with etoposide (25 μM) (lanes 3 and 4) or with cycloheximide (25 μM) and TLCK (100 μM) (lanes 5 and 6). TPCK (10 μM) was added to replicates of these incubations (lanes 2, 4 and 6).

range of TPCK concentrations (5–20 μM) (data not shown). Neither TPCK (5 μM) nor TLCK (100 μM) alone induced apoptosis (Figure 7). These results suggested that a common TPCK and TLCK target may be involved in the regulation of apoptosis.

As TLCK and TPCK are site-directed inhibitors of trypsin- and chymotrypsin-like proteases respectively, we investigated whether their effects on apoptosis were related to their inhibition of these proteases. Lysates from THP.1 cells were assayed, using Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC as substrates, for trypsin- and chymotrypsin-like serine proteases activity respectively (Kunugi *et al*, 1985; Sawada *et al*, 1984). TPCK (10 μM), a concentration which modulated cycloheximide- and etoposide- induced apoptosis in a similar manner to TLCK (100 μM) did not affect these activities (Table 2). Thus the target for TPCK and TLCK, which modulates apoptosis, is not related to these activites. In contrast, concentrations of TPCK (50–75 μM), which induced apoptosis (Table 1), caused a decrease in Boc-Val-Leu-Lys-AMC cleavage activity, but not in Suc-Ala-Ala-Pro-Phe-AMC cleaving activity (Table 2), suggesting that TPCK inhibited a cellular target(s) possibly a protease(s), which is important in cell survival.

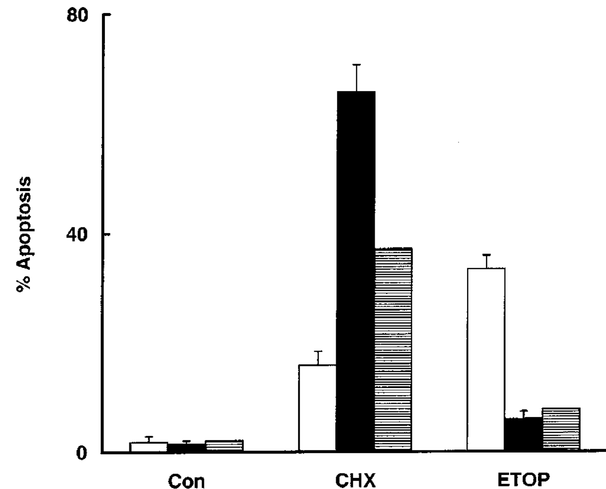

Figure 7 TLCK and TPCK potentiate cycloheximide-induced apoptosis and inhibit etoposide-induced apoptosis. THP.1 cells were incubated alone (Con) or with cycloheximide (CHX) (25 μM) or etoposide (ETOP) (25 μM) for 4 h in the absence (open boxes) or presence of TLCK (100 μM) (black boxes) or TPCK (5 μM) (hatched boxes). Apoptosis was assessed by flow cytometry. Results are expressed as either the mean $\pm$ s.e.m. of at least three experiments or the mean value of two experiments.

Table 2 Effect of protease inhibitors on trypsin-like and chymotrypsin-like protease activities

	Boc-Val-Leu-Lys-AMC	Suc-(Ala)$_2$-Pro-Phe-AMC
Control	145$\pm$46	993$\pm$82
TPCK 10 μM	143$\pm$17	989$\pm$45
TPCK 25 μM	87$\pm$34	844$\pm$64
TPCK 50 μM	39$\pm$16	690$\pm$96
TPCK 75 μM	38$\pm$16	978$\pm$23
TLCK 100 μM	108$\pm$25	1396$\pm$441

THP.1 cells were treated with TLCK or TPCK for 1 h. Lysates from control cells and those treated with these inhibitors were measured for Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC cleavage activities. The results are presented as mean pmol AMC/mg. protein/min $\pm$ s.e.m. from at least three experiments.

Discussion

Concentration dependent modulation of apoptosis by TPCK

TPCK was initially synthesised as a specific inhibitor of chymotrypsin-like proteases (Schoellmann and Shaw, 1963). Subsequent studies have shown that it can also affect some cysteine proteases as well as thiols in general (Shaw, 1990). In this study, we demonstrate marked concentration dependent effects of TPCK on apoptosis in THP.1 cells.

High concentrations of TPCK induce apoptosis

TPCK (50–70 μM) induced apoptosis assessed by several criteria including flow cytometry (Table 1), formation of large kilobase pair fragments of DNA (Figure 2), proteolysis of

PARP and lamin B_1 (Figure 3) and processing of CPP32 and Mch 3 (Figure 5). These biochemical changes were accompanied by the induction of a distinct ultrastructure, with several features characteristic of early apoptotic changes (Kerr *et al*, 1987; Walker *et al*, 1988), including dilation of the endoplasmic reticulum, partial nucleolar disintegration and condensation of chromatin (Figure 1B). Thus, TPCK alone has induced almost all the morphological and biochemical changes associated with fully apoptotic cells except for internucleosomal cleavage and the full condensation of the chromatin. These data are in good agreement with our previous studies, with thymocytes treated either with TPCK or dexamethasone in the presence of zinc, when an early apoptotic morphology was observed in the absence of internucleosomal cleavage of DNA but associated with similar sized large kilobase pair fragments of DNA (Brown *et al*, 1993; Cohen *et al*, 1992b; Fearnhead *et al*, 1995a).

Z-VAD.FMK inhibits apoptosis by blocking DNA fragmentation, proteolysis of PARP and processing of CPP32 in different systems including Jurkat cells, thymocytes, hepatocytes and THP.1 cells (Cain *et al*, 1996; Chow *et al*, 1995; Fearnhead *et al*, 1995b; Slee *et al*, 1996; Zhu *et al*, 1995). In this study, Z-VAD.FMK inhibited all the biochemical and morphological features of apoptosis induced by TPCK including accumulation of large kilobase pair fragments, proteolysis of PARP and lamin B_1 and processing of CPP32 and Mch 3 (Figures 1–5). These results suggest that ICE-like proteases, including CPP32 and Mch 3, may be key effectors in the execution phase of TPCK-induced apoptosis. Other studes have implicated a number of different ICE-like proteases in the execution phase of apoptosis (reviewed in Kumar 1995; Martin and Green, 1995).

The activation of a CPP32/Mch 3-like protease together with PARP degradation was observed in TPCK-induced apoptosis in intact THP.1 cells (Figures 3 and 5) even though TPCK inhibits the ability of bacterially expressed recombinant Mch 3 and CPP32 to cleave PARP (Fernandes-Alnemri *et al*, 1995b). This difference may be due either to the concentration of TPCK (100 μM) required to inhibit recombinant CPP32 and Mch 3 (Fernandes-Alnemri *et al*, 1995b) or to an inability to achieve adequate concentrations of TPCK within intact cells. It is therefore unlikely that the intracellular target(s) of TPCK is CPP32 or Mch 3 but it may be a protease upstream of CPP32/Mch 3, the inhibition of which results in activation of the cell death programme. As TPCK inhibited Boc-Val-Leu-Lys-AMC cleavage activity (Table 2), this may represent one of the TPCK targets whose inhibition results in apoptosis. The protease responsible for this cleavage activity and the mechanism whereby TPCK induces apoptosis are not known. TPCK also induces apoptosis in murine B cells and HL60 cells possibly by a decrease in c-*myc* expression or by inhibition of proteasome function as a result of inhibiting its chymotrypsin-like protease activity (Wu *et al*, 1996; Drexler, 1997). Due to multiple functions of chloromethyl ketone groups, TPCK may affect apoptosis by different mechanisms in different cell types.

Low concentrations of TPCK inhibit internucleosomal cleavage and differentially modulate apoptosis dependent on the stimulus

Low concentrations of TPCK (5–10 μM) inhibited the internucleosomal cleavage of DNA induced by different apoptotic stimuli (Figure 6) in agreement with its effects in several different systems (Bruno *et al*, 1992; Chow *et al*, 1995; Fearnhead *et al*, 1995a; Hara *et al*, 1996; Neamati *et al*, 1995). This may be related to the ability of TPCK to inhibit a 24 kDa protease, which can initiate internucleosomal cleavage of DNA (Wright *et al*, 1994). Previously we have shown that TLCK potentiated apoptosis induced by some apoptotic stimuli, including cycloheximide, but inhibited apoptosis induced by etoposide (Zhu *et al*, 1995). In this study, a similar effect was observed with TPCK (5–10 μM) (Figure 7). Thus both TPCK and TLCK probably modulate apoptosis by affecting a common target. We were unable to characterise further this target using trypsin- and chymotrypsin-like substrates (Table 2).

In summary we demonstrate, that in a single cell type, TPCK has several distinct effects on apoptosis dependent both on the apoptotic stimulus used and on the concentration of TPCK. TPCK induces apoptosis in THP.1 cells and Z-VAD.FMK inhibits this process, indicating a critical role for ICE-like proteases in the execution phase of apoptosis.

Materials and Methods

Materials

Media and sera were from Gibco (Paisley, UK). The antibody (318) to PARP was a kind gift from Dr. G. Poirier, Quebec, Canada. TPCK and TLCK were from Boehringer Mannheim (Mannheim, Germany). Z-VAD.FMK and benzyloxycarbonyl-Asp-Glu-Val-Asp-7-amino-4-triflur-omethyl coumarin (Z-DEVD.AFC) were from Enzyme Systems Inc. (Dublin, CA, USA). Boc-Val-Leu-Lys-7-amino-4-methylcoumarin (Boc-Val-Leu-Lys-AMC), Suc-Ala-Ala-Pro-Phe-7-amino-4-methylcoumarin (Suc-Ala-Ala-Pro-Phe-AMC) were from Bachem Bioscience, Essex, U.K. Other chemicals were obtained from Sigma Chemical Company (Poole, UK).

Cell culture and electron microscopy

THP.1 cells were maintained as a suspension culture as described previously (Zhu *et al*, 1995). Logarithmically growing cultures of THP.1 cells were used for all experiments and they were seeded at a density of $0.5-0.8\times10^6$/ml one day prior to the experiment. Cell pellets were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at 4°C overnight and postfixed with 1% osmium tetroxide/1.7% potassium ferrocyanide for 90 min. After fixation, cells were stained en bloc for 1 h with 2% aqueous uranyl acetate, dehydrated, and embedded in Araldite. Sections were stained with lead citrate and examined using a Zeiss 902A electron microscope (Cohen *et al*, 1992b).

Quantification of apoptotic cells by flow cytometry

Normal and apoptotic cells were separated and quantified by a flow cytometric method as originally described for thymocytes (Sun *et al*,

1992) and subsequently used for THP.1 cells (Zhu *et al*, 1995) cells were stained with Hoechst 33342 and propidium iodide and analysed using a Becton Dickinson flow cytometer with Lysis II software.

DNA analysis

Internucleosomal cleavage of DNA was detected by the method of Sorenson *et al* (1990). Formation of large kilobase pair fragments of DNA was detected by field inversion gel electrophoresis (Brown *et al*, 1993).

Preparation of cell lysates

Lysates were prepared from control or treated THP.1 cells. Cells were lysed by using 0.25% Nonidet P-40 (50×10^6 cells/100 μl) for 5 min on ice. The nuclear fraction was sedimented at 2000 g for 10 min and the supernatant was collected and stored at -80°C until assayed for protease activity. The protein concentration in the supernatant fraction (the lysate) was determined by the Bradford assay (Bio-Rad).

Measurement of protease activity

Z-DEVD.AFC, Boc-Val-Leu-Lys-AMC and Suc-Ala-Ala-Pro-Phe-AMC were used as substrates for measuring ICE-like, trypsin-like and chymotrypsin-like serine protease activities respectively (Kunugi *et al*, 1985; Sawada *et al*, 1984; Nicholson *et al*, 1995). Substrate (20 μM) was added to 1.25 ml of 50 mM Tris buffer, pH 7.4, at 37°C. Lysate (10 μl) was added to initiate the reaction. Fluorescence was monitored for 240 s using a Perkin Elmer fluorimeter at excitation and emission wavelengths of 380 and 460 nm for AMC, 400 and 505 nm for AFC, respectively. Standard curves were generated with AMC (0 – 1 μM) and AFC (0 – 1.6 μM). The protease activity was expressed as pmol AMC (or AFC)/mg. protein/min.

Western blot analysis

Cells (0.5×10^6) were prepared for SDS – PAGE as described (Harlow and Lane, 1988). Proteins were resolved on a 7% SDS (PARP), 10% (lamin B_1) or 15% (CPP32 and Mch 3) polyacrylamide gels and transferred onto nitrocellulose. Pro-CPP32 and its catalytically active P17 subunit were detected using a rabbit polyclonal antibody directed to the p17 subunit (Nicholson *et al*, 1995). Pro-Mch 3 and its catalytically active p19 subunit was detected using rabbit antiserum (MacFarlane *et al*, 1997). PARP was detected using rabbit antiserum (318), provided by Dr. G. Poirier (Quebec, Canada). Lamin B_1 and a proteolytic fragment were detected using a monoclonal antibody (Serotec Ltd., Oxford, UK). Detection was achieved with a secondary antibody (goat anti-rabbit IgGs) conjugated to horseradish peroxidase (diluted 1 : 2000 in the same buffer) and an ECL detection kit (Amersham Life Science, Bucks, UK).

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