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The role of proteolysis in T cell apoptosis triggered by chelation of intracellular Zn^{2+}

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Abstract

Our previous work showed that chelation of intracellular Zn^{2+} with N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) induces apoptosis in rat thymocytes. The molecular mechanism involved in TPEN-triggered apoptosis remains unknown, except that it is a Ca^{2+}-independent process. In the present study, we show that TPEN is unable to induce DNA fragmentation when added to isolated thymocyte nuclei, indicating that activation of a cytoplasmic component is essential for TPEN-induced apoptosis. Since cytosolic proteases related to interleukin-1β-converting enzyme (ICE) are implicated as key activators of apoptosis in many different systems, we investigated the possible involvement of such proteases in TPEN-induced apoptosis. We found that treatment of thymocytes with TPEN caused an early degradation of nuclear poly(ADP-ribose) polymerase (PARP) and lamin prior to DNA cleavage. This could be inhibited by Z-Val-Ala-Asp-chloromethylketone (VADcmk), an inhibitor of ICE-like proteases, but not by an inhibitor of Ca^{2+}-regulated serine protease. Jurkat T cells also underwent extensive DNA fragmentation when incubated with TPEN. A cytosolic fraction, prepared from TPEN-treated Jurkat cells, produced extensive DNA fragmentation when applied to isolated thymocyte nuclei, whereas the cytoplasmic extract from untreated cells was ineffective either alone or together with TPEN. The apoptosis-inducing activity in cytosolic fraction from TPEN-treated Jurkat cells was blocked by incubating cells in the presence of VADcmk or another inhibitor of ICE-like proteases, Ac-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), which has been found to competitively inhibit CPP32/apopain. An increase in enzyme activity that cleaves Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC), a fluorogenic substrate of CPP32/apopain and Mch3α, was detected in TPEN-treated thymocytes and Jurkat cells. In addition, the proteolytic cleavage of CPP32 resulting in the formation of two active fragments (p17 and p12) was observed in cytosolic extracts from TPEN-treated Jurkat cells, but not in extracts which were prepared from cells treated with TPEN in the presence of VADcmk or DEVD-CHO. Our results suggest that activation of cytosolic ICE-like proteases is an essential step in TPEN-induced apoptosis, and that CPP32/apopain is critically involved in this process.

Keywords: thymocytes, Jurkat T cells, TPEN, Zinc, ICE-like proteases, CPP32/apopain

Abbreviations: TPEN, N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine; FBS, fetal bovine serum; VADcmk, Z-Val-Ala-Asp-chloromethylketone; AAPFcmk, Suc-Ala-Ala-Pro-Phe-chloromethylketone; ICE, interleukin-1β-converting enzyme; HMW, high molecular weight; DEVD-CHO, Ac-Asp-Glu-Val-Asp-aldehyde; DEVD-AMC, Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin; and PARP, poly(ADP-ribose) polymerase; FIGE, field inversion gel electrophoresis

Introduction

Cell death by apoptosis has become recognized as a common feature of many biological processes where cell deletion is a mechanism for altering tissue structure and function (Wyllie *et al*, 1980). Although extensive studies have been carried out to explore the mechanisms involved in this process, a common signaling pathway has not yet been found. It is becoming apparent that virtually every cell has a death program that can be triggered by external stimuli, and that the pathways by which apoptosis is triggered may vary from cell type to cell type and from inducer to inducer (McConkey and Orrenius, 1994). However, the appearance of volume shrinkage, plasma membrane blebbing, chromatin condensation, and DNA fragmentation in most cells undergoing apoptosis suggests that a final common program is involved in the execution of apoptosis (Wyllie *et al*, 1980; Arends *et al*, 1991).

Intracellular proteolysis has long been considered to be an important mechanism in the regulation of the levels of individual proteins, as well as overall growth and atrophy of tissues. Recent studies have shown that activation of intracellular proteases also plays a central role in triggering apoptosis (Martin and Green, 1995). Among the many proteases involved in apoptosis, much interest has been given to interleukin-1β-converting enzyme (ICE)-like proteases. ICE-like proteases are a family of cytoplasmic cysteine proteases which include ICE (Yuan *et al*, 1993; Miura *et al*, 1993), CED-3 (Yuan *et al*, 1993; Miura *et al*, 1993), NEDD-2/ICH-1 (Kumar *et al*, 1994; Wang *et al*, 1994), CPP32/YAMA/apopain (Fernandes-Alnemri *et al*, 1994; Tewari *et al*, 1995b; Nicholson *et al*, 1995), TX/ICH-2 (Faucheu *et al*, 1995; Kamens *et al*, 1995), Mch-2 (Fernandes-Alnemri *et al*, 1995a), and Mch-3 (Fernandes-Alnemri *et al*, 1995b). Overexpression of any one of these leads to apoptotic cell death, whereas

blocking their activity with the specific viral inhibitor, *CrmA*, or with non-hydrolyzable peptide substrates protects cells from apoptosis.

Thus, the involvement of ICE-like proteases in apoptosis induced by diverse stimuli is now well established (Kumar, 1995). Some of the substrates for ICE-like proteases in apoptosis have been identified, these include poly(ADP-ribose) polymerase (PARP) (Kaufmann *et al*, 1993; Lazebnik *et al*, 1994; Fernandes-Alnemri *et al*, 1995b; Gu *et al*, 1995), sterol regulatory element-binding proteins (Wang *et al*, 1995), the 70 kDa protein component of the U1 small nuclear ribonucleoprotein (Tewari *et al*, 1995a), and a component of microfilament system, Gas2 (Brancolini *et al*, 1995). Cleavage of nuclear lamins is another proteolytic event commonly observed in cells undergoing apoptosis (Oberhammer *et al*, 1994; Neamati *et al*, 1995; Lazebnik *et al*, 1995). The proteases responsible for the degradation of nuclear lamins during apoptosis are not fully identified. A Ca^{2+}-regulated serine protease has been found to be responsible for the lamin degradation in the nuclear scaffold extract (Clawson *et al*, 1992). Recently, Lazebnik *et al* (1995) also reported that lamin cleavage during apoptosis requires the action of a second ICE-like protease which exhibits kinetics of cleavage and a profile of sensitivity to specific inhibitors that is distinct from PARP-cleaving CPP32/YAMA.

Zn^{2+} is a universal inhibitor of apoptosis (Sunderman, 1995). However, the sites and the mode of inhibition of apoptosis by Zn^{2+} are still not clear. An early study by Cohen and Duke (1984) showed that Zn^{2+} inhibits the Ca^{2+}/Mg^{2+}-dependent endonuclease, which is intrinsically present in thymocyte nuclei and thought to be responsible for the internucleosomal DNA fragmentation induced by glucocorticoids. It was initially assumed that the inhibitory effects of Zn^{2+} on apoptosis were due to the inhibition of Ca^{2+}/Mg^{2+}-dependent endonuclease. However, it was later found that Zn^{2+} also inhibits Ca^{2+}-independent forms of apoptosis (Lazebnik *et al*, 1993). Thus, inhibition of Ca^{2+}/Mg^{2+}-dependent endonuclease activity alone is not sufficient to explain the effects of Zn^{2+} on apoptosis.

In a previous study, we demonstrated that chelation of intracellular Zn^{2+} with N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylenediamine (TPEN) is sufficient to induce apoptosis in thymocytes (McCabe *et al*, 1993). Although the pathway whereby TPEN triggers apoptosis in these cells is unknown, it does not require protein synthesis (McCabe *et al*, 1993) and is Ca^{2+}-independent (Jiang *et al*, 1995). In our present study, the mechanisms involved in TPEN-induced thymocyte apoptosis have been investigated further. We report that the induction of apoptosis by TPEN is not due to direct effects on the nucleus. Instead, the activation of cytosolic components, including ICE-like proteases, is essential for TPEN-triggered apoptosis.

Results

Lack of effects of TPEN on thymocyte nuclei

Since Zn^{2+} inhibits endonuclease activity (Cohen and Duke, 1984) and stabilizes chromatin structure (Crompton *et al*, 1992), it appears possible that chelation of Zn^{2+} by TPEN could induce DNA fragmentation by either de-repressing endonuclease activity or destabilizing chromatin structure. To examine this, the direct effects of TPEN on isolated thymocyte nuclei were investigated. Isolated rat thymocyte nuclei were incubated with 10 μM TPEN for 2 h prior to quantitation of DNA cleavage. Ca^{2+}, which has previously been shown to induce extensive DNA fragmentation in isolated thymocyte nuclei, was used as a positive control. As shown in Figure 1, while Ca^{2+} resulted in a dose-dependent increase in DNA fragmentation, TPEN failed to induce DNA fragmentation in isolated thymocyte nuclei. Moreover, TPEN had no additional effect on Ca^{2+}-induced DNA fragmentation in thymocyte nuclei. Similar results were obtained with prolonged incubation times (data not shown). Thus, although the addition of Zn^{2+} inhibits Ca^{2+}-induced DNA fragmentation (Cohen and Duke, 1984), the chelation of Zn^{2+} by TPEN did not enhance Ca^{2+}-induced DNA fragmentation in isolated nuclei. This result is in agreement with our previous finding that TPEN-induced thymocyte apoptosis is Ca^{2+}-independent (Jiang *et al*, 1995). Together, these findings suggest that the hypothesis is not true, that TPEN induces chromatin fragmentation by either removing endogenous Zn^{2+} inhibition from Ca^{2+}/Mg^{2+}-dependent endonuclease(s) or changing chromatin conformation and thereby increasing its susceptibility to cleavage by Ca^{2+}/Mg^{2+}-dependent endonuclease(s). An alternative hypothesis, namely that TPEN treatment activates a cytosolic apoptosis-promoting activity, was therefore investigated.

Involvement of ICE-like proteases in TPEN-induced thymocyte apoptosis

Among the possible cytosolic factors which could trigger apoptosis in TPEN-treated thymocytes are the ICE-like

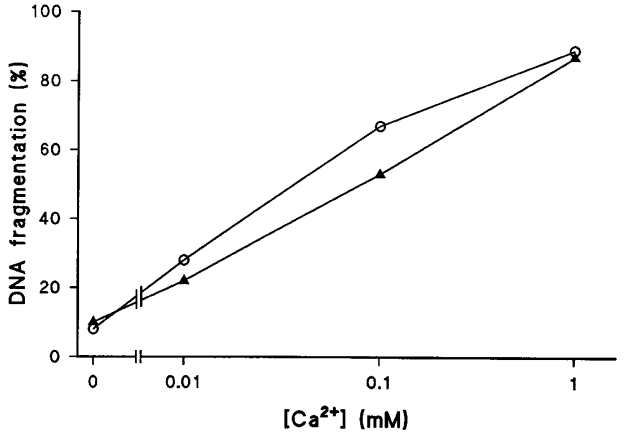

Figure 1 Lack of effect of TPEN on isolated thymocyte nuclei. Thymocyte nuclei were untreated (open circle) or treated with 10 μM TPEN (closed triangle) in the presence or absence of different concentrations of $CaCl_2$ for 2 h. DNA fragmentation was quantitated by diphenylamine assay as described in Materials and Methods. The results are the means of duplicate samples from one of three separate nuclei preparations.

proteases, since they are involved in many other examples of apoptosis (Martin and Green, 1995). Previous studies using cell-free systems suggest that ICE-like proteases are the active components in cytosolic extracts which cause nuclei to undergo apoptotic degradation (Martin *et al*, 1995; Schlegel *et al*, 1996). To investigate the possible involvement of ICE-like proteases in TPEN-triggered apoptosis, the effects of the protease inhibitor, Z-Val-Ala-Asp-chloromethylketone (VADcmk), was studied in this system. Thymocytes were treated with TPEN in the presence or absence of VADcmk. Thereafter, the formation of high molecular weight (HMW) and oligonucleosomal DNA fragments was examined. As shown in Figure 2a and b, in the absence of VADcmk, TPEN induced the cleavage of DNA into HMW fragments (700, 300, and 50 kbp in length), which were then further degraded into oligonucleosomal DNA fragments. In the presence of VADcmk, the TPEN-induced formation of HMW and oligonucleosomal DNA fragments was significantly reduced.

PARP and lamin degradation and lack of involvement of a Ca^{2+}-regulated serine protease in TPEN-induced apoptosis

One of the substrates for ICE-like proteases is PARP (Lazebnik *et al*, 1994; Fernandes-Alnemri *et al*, 1995b; Gu *et al*, 1995). Cleavage of intact PARP (116 kDa) to an 85 kDa fragment was observed in several apoptotic systems. In the present study, the proteolysis of PARP was studied in rat thymocytes treated with TPEN. As shown in Figure 3a, in control cells after incubation for 4 h, the majority of PARP was intact (116 kDa), although a small amount of 85 kDa fragments could be detected, possibly resulting from spontaneous apoptosis occurring in these cells. Treatment of thymocytes with TPEN induced a loss of intact PARP and was accompanied by an increase in the formation of 85 kDa fragments. VADcmk prevented the cleavage of PARP induced by TPEN, suggesting that this cleavage is also mediated by ICE-like proteases.

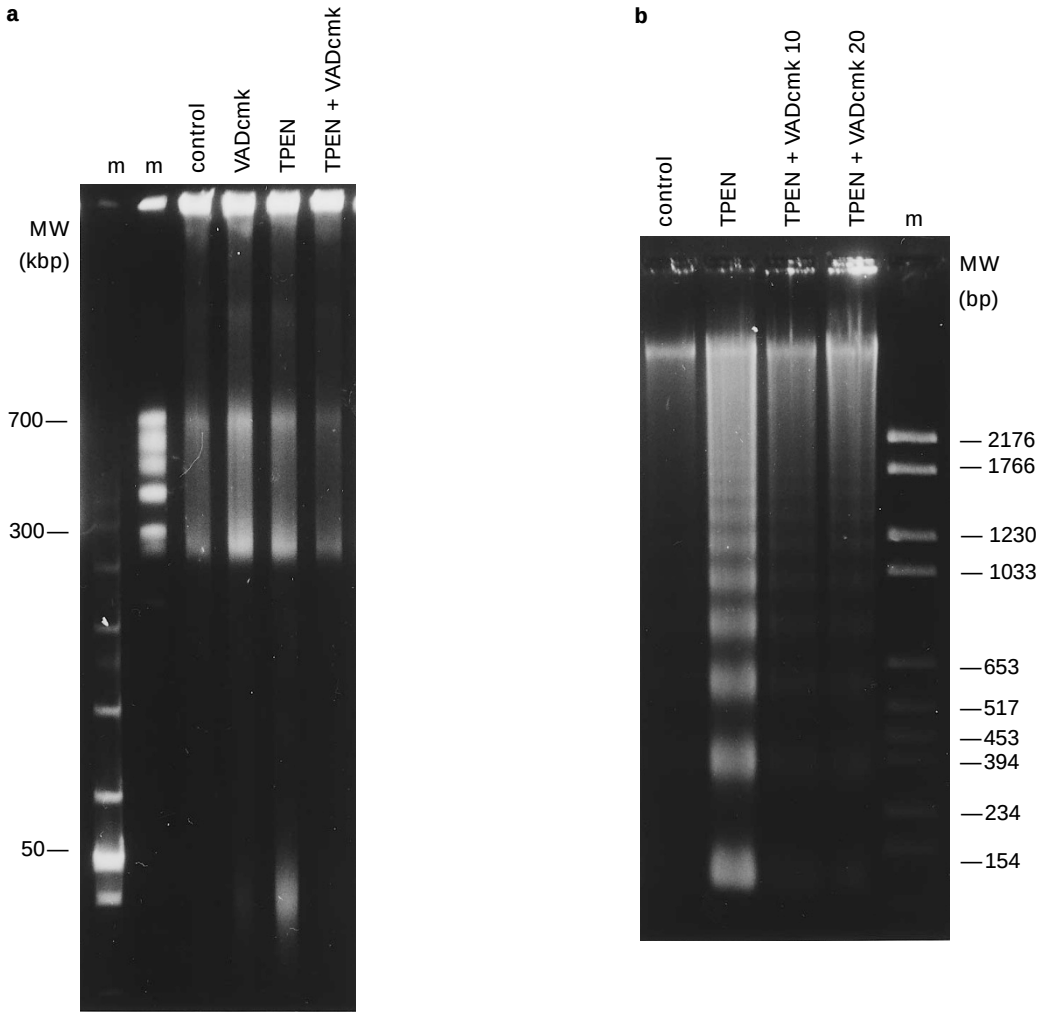

Figure 2 Involvement of ICE-like proteases in TPEN-induced apoptosis. Thymocytes were untreated or treated with 10 μM TPEN in the presence or absence of 10 or 20 μM VADcmk for 4 h. HMW (**a**) and oligonucleosomal (**b**) DNA fragments were examined by FIGE and conventional agarose gel electrophoresis, respectively. m=markers.

Lamin degradation occurs in cells undergoing mitosis (Gerace and Blobel, 1980). Recent studies have demonstrated that it is also an early event in apoptosis (Oberhammer *et al*, 1994; Neamati *et al*, 1995). As shown in Figure 3b, degradation of lamin B into 46 kDa, 34 kDa and 18 kDa fragments was observed in our system. Lamin degradation first appeared in thymocytes treated with TPEN for 1 h and was present during incubation for up to 4 h. VADcmk effectively abolished the TPEN-induced lamin breakdown, indicating that the activation of ICE-like proteases occurs prior to the lamin breakdown.

Clawson *et al* (1992) showed that a Ca^{2+}-regulated nuclear scaffold serine protease can cleave lamin. To investigate whether the TPEN-induced lamin degradation is also mediated by this protease, the effects of its specific inhibitor, Suc-Ala-Ala-Pro-Phe-chloromethylketone (AAPF-cmk) (Clawson *et al*, 1993) were then studied. As shown in Figure 3b, TPEN-induced lamin degradation was unaf-

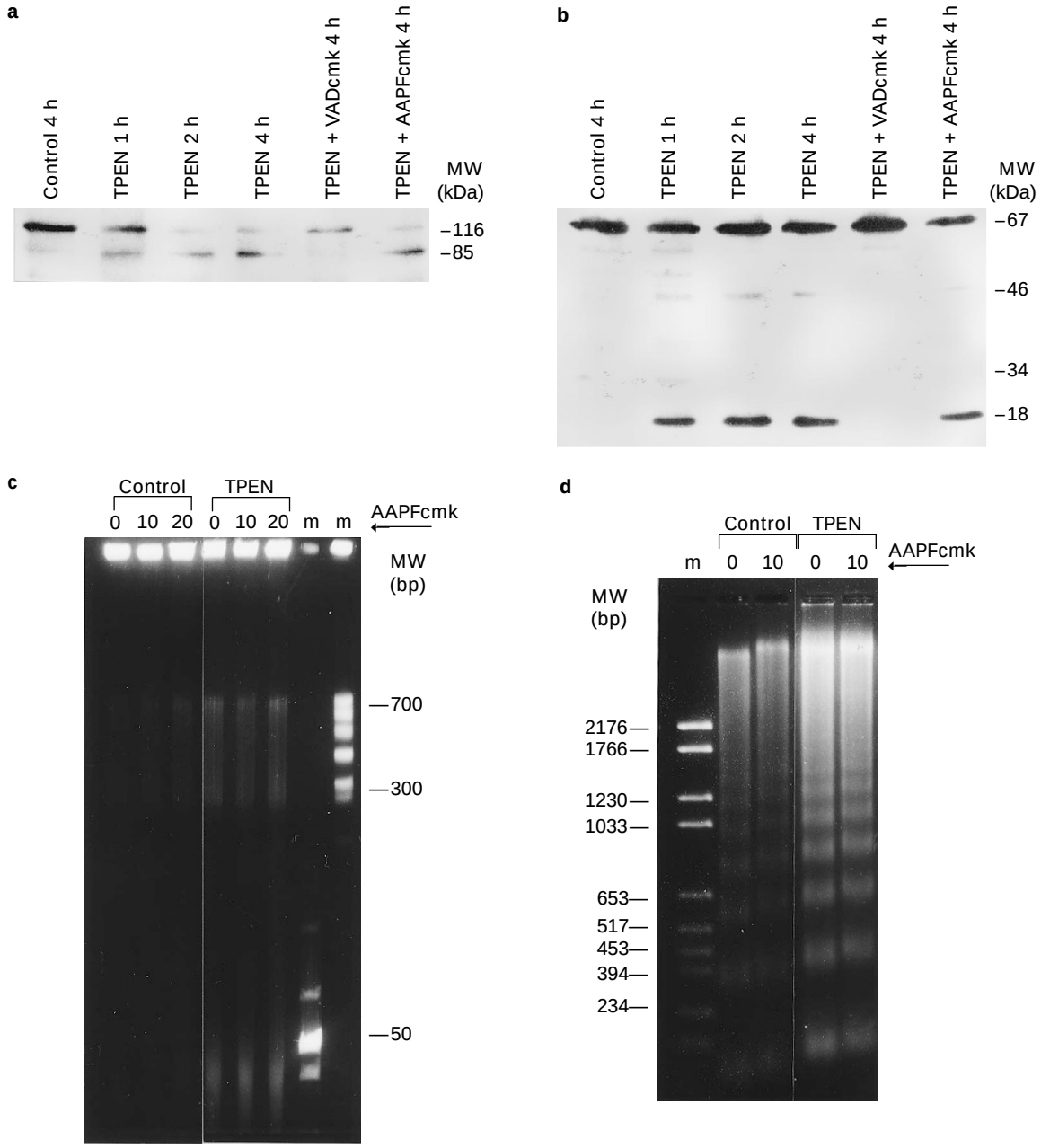

Figure 3 PARP and Lamin degradation and lack of effect of Ca^{2+}-regulated serine protease inhibitors. Rat thymocytes were untreated, treated with 10 μM TPEN alone for various times indicated, or treated with TPEN in the presence of 10 μM VADcmk or 10 μM AAPFcmk for 4 h. PARP (**a**) and Lamin B (**b**) content was determined by immunoblotting. Rat thymocytes were untreated or treated with 10 μM TPEN in the presence or absence of 10 or 20 μM AAPFcmk. After 4 h, the DNA was examined either by FIGE for the presence of HMW DNA fragments (**c**) or by agarose gel electrophoresis for the presence of DNA laddering (**d**) m=markers.

fected by AAPFcmk. Likewise, AAPFcmk was unable to prevent the formation of HMW and oligonucleosomal DNA fragments by TPEN (Figure 3c and d). In addition, we found that this protease was not involved in TPEN-induced PARP cleavage (Figure 3a). Lack of involvement of Ca^{2+}-regulated serine protease in TPEN-induced apoptosis is in agreement with our previous finding, which shows that Ca^{2+} is not a mediator of apoptosis in this system (Jiang *et al*, 1995).

Induction of DNA fragmentation in isolated thymocyte nuclei by a cytosolic extract from TPEN-treated Jurkat cells

Dissection of the molecular events which occur during apoptosis is facilitated by the use of cell-free systems (Earnshaw *et al*, 1995). In our present study, this approach was applied to examine whether the cytosolic extracts from TPEN-treated cells were capable of inducing DNA fragmentation in isolated nuclei from untreated cells. Jurkat cells, which have more cytoplasm than thymocytes, were used as a source of cytosolic extract. We first examined whether TPEN was able to trigger apoptosis in these cells. Jurkat cells were incubated in the presence of 2.5 or 10 μM TPEN for various time periods and the DNA was analyzed. As shown in Figure 4, DNA cleavage into oligonucleosomal fragments was observed in Jurkat cells after treatment with 10 μM TPEN for 4 h. The cytosolic fractions were extracted from Jurkat cells, untreated or treated with TPEN for 4 h, and their ability to induce DNA fragmentation in isolated untreated thymocyte nuclei was examined. As shown in Figure 5a, the cytosolic extract from TPEN-treated Jurkat cells was found to produce DNA fragmentation in isolated thymocyte nuclei, while an identically prepared cytosolic extract from untreated Jurkat cells was incapable of inducing DNA fragmentation. Since TPEN was unable to cause DNA fragmentation in isolated thymocyte nuclei (Figure 1), the possibility that TPEN contamination of the Jurkat cytosols caused DNA fragmentation can be effectively excluded. The addition of 10 μM TPEN to the cytosol fraction from untreated Jurkat cells also failed to induce DNA cleavage in thymocyte nuclei (Figure 5b).

Effects of inhibitors of ICE-like proteases on the apoptosis-inducing activity in TPEN-treated Jurkat cells

Similar to the rat thymocyte experiments, VADcmk was effective in inhibiting the TPEN-induced DNA fragmentation in Jurkat cells (Figure 6). DNA fragmentation induced by TPEN was also blocked by another inhibitor of ICE-like proteases, Ac-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO), which has been found to competitively inhibit CPP32/apopain (Nicholson *et al*, 1995). To investigate whether the inhibitors of ICE-like proteases could also affect the apoptosis-inducing activity present in cytosolic extract from TPEN-treated Jurkat cells, these inhibitors were added to cell cultures before or simultaneously with TPEN. After 4 h, the cytosolic extracts were prepared from each sample. As shown in Figure 7, no apoptosis-inducing activity was detected in the cytosolic extract from cells treated with TPEN in the presence of VADcmk or DEVD-CHO. The involvement of ICE-like proteases in TPEN-treated cytosolic extract was further examined by incubating nuclei with this extract in the presence of VADcmk or DEVD-CHO. A partial decrease in DNA fragmentation was observed in the samples which included VADcmk or DEVD-CHO (data not shown), suggesting that ICE-like proteases are components of the active cytosolic extract. Similar to that observed with rat thymocytes, AAPFcmk was unable to prevent TPEN-induced DNA fragmentation in Jurkat cells, indicating the lack of involvement of Ca^{2+}-regulated serine protease in this process (Figure 6).

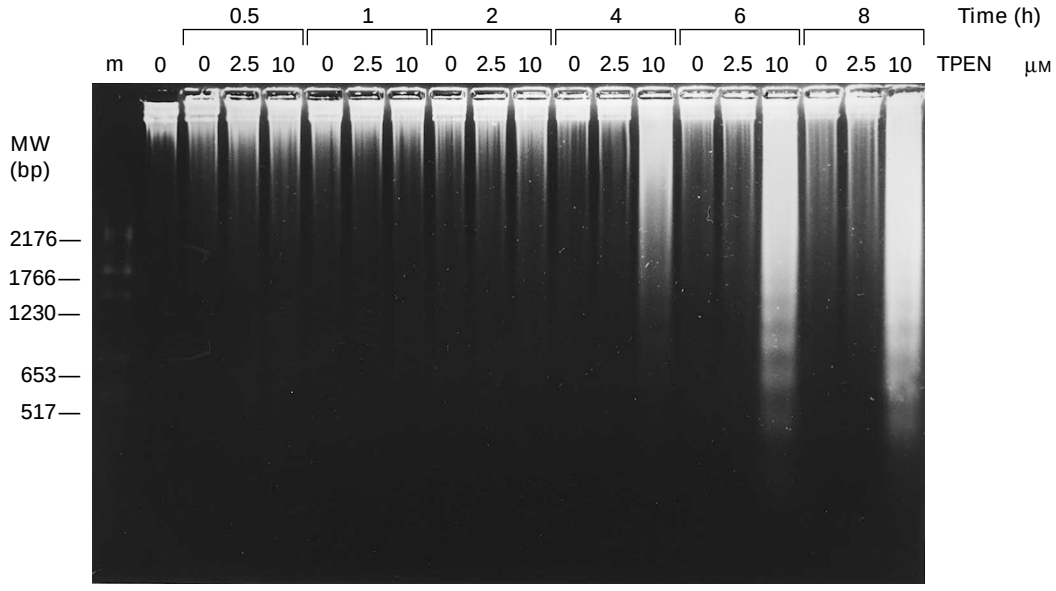

Figure 4 Time course of TPEN-induced DNA fragmentation in Jurkat cells. Jurkat cells were untreated or treated with 2.5 or 10 μM TPEN for various time periods. The formation of oligonucleosomal DNA fragments was examined by agarose gel electrophoresis. m=marker.

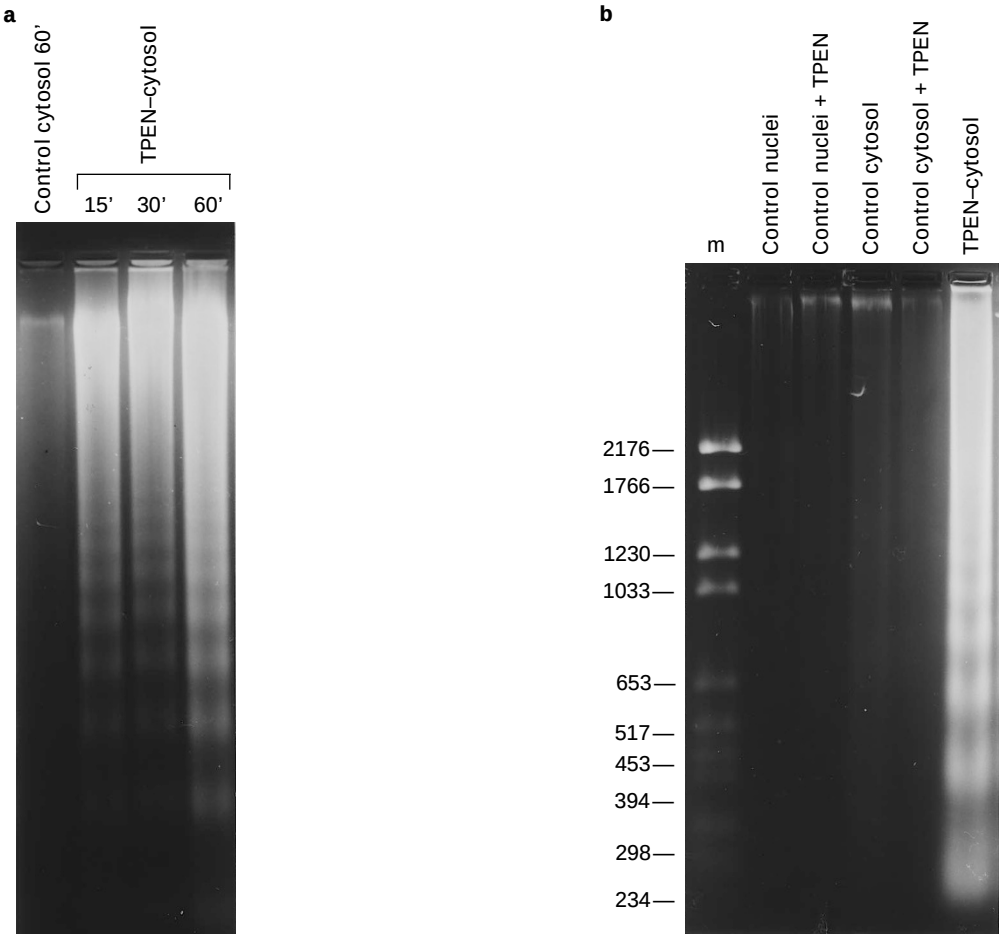

Figure 5 Induction of DNA fragmentation in isolated rat thymocyte nuclei by cytoplasmic extract from TPEN-treated Jurkat cells. Cytosolic extracts were prepared from Jurkat cells untreated (control cytosol) or treated with TPEN (TPEN cytosol) for 4 h. Isolated thymocyte nuclei were incubated with these cytosolic extracts for various times indicated (**a**), or incubated with nothing, 10 μM TPEN, or these cytosolic extracts in the absence or presence of TPEN for 1 h (**b**). The formation of oligonucleosomal DNA fragments was then assayed by conventional agarose gel electrophoresis as described in Materials and Methods. m=marker.

Involvement of CPP32/apopain in TPEN-triggered apoptosis

In order to identify the ICE-like proteases involved in TPEN-induced apoptosis, the enzyme activity that cleaves Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC), a fluorogenic substrate of CPP32/apopain and Mch3α, was measured in both TPEN-treated thymocytes and Jurkat cells using a continuous fluorometric assay. As shown in Table 1, an increase in AMC release, which corresponded to the increase in enzyme activity, was observed in both types of cells treated with TPEN. Exposure of rat thymocytes to TPEN for 40 min induced AMC release, whereas in control cells no enzyme activity was detected at this time point. Prolonged incubation induced an increase in AMC release in control rat thymocytes, which correlated with spontaneous apoptosis in these cells. However, a greater increase in enzyme activity was observed in the TPEN-treated thymocytes at these time points. The increase in AMC release was inhibited by including 50 nM DEVD-CHO in the reaction mixture (data not shown). In untreated Jurkat cells no enzyme activity was detected throughout the time of investigation. However, incubation of Jurkat cells for 40 min in the presence of TPEN caused a release of AMC from substrate, which increased several fold in 2 h (Table 1). Compared to thymocytes, the increase in the enzyme activity by TPEN in Jurkat cells was much less. In cytosolic extracts from TPEN-treated Jurkat cells, the enzyme activity that cleaves DEVD-AMC was also detected (Table 2). The addition of DEVD-CHO to this extract partially inhibited the enzyme activity. The control cytosol from untreated Jurkat cells contained little enzyme activity. The addition of TPEN to control cytosol failed to increase the AMC release.

The proenzyme, CPP32, has been identified as a native 32 kDa protein. The activation of this enzyme includes the cleavage of 32 kDa precursor resulting in two subunits of relative molecular mass 17 kDa and 12 kDa, respectively, followed by the formation of tetramers (Nicholson *et al*, 1995). Immunoblot analysis using antibody against the 32 kDa protein shows the presence of native polypeptide in

the extracts from untreated Jurkat cells and the disappearance of this protein in cells treated with TPEN (Figure 8a). However, in the extracts prepared from cells that were treated with TPEN in the presence of VADcmk or DEVD-CHO, the native CPP32 was detected. Since this antibody was unable to recognize the cleavage products of CPP32, in the next experiment membranes were probed with the monoclonal antibodies to p17 and p12 (Figure 8b and c). The native CPP32 protein was again found in the extracts from untreated cells. In the cytosolic extracts from TPEN-treated Jurkat cells, the proteins with molecular mass corresponding to both p17 and p12 were present. It is known that CPP32 is initially processed with the formation of p20 and p12, followed by conversion of p20 to p17 (Nicholson *et al*, 1995). Immunoblots stained with antibody against p17 and p12 show the presence of p20 in the cytosolic extracts from TPEN-treated Jurkat cells (Figure 8b and c). The formation of p17 and p12 was effectively blocked and these proteins were not detected in the cytosolic extract from cells that were treated with TPEN in the presence of VADcmk or DEVD-CHO (Figure 8b and c).

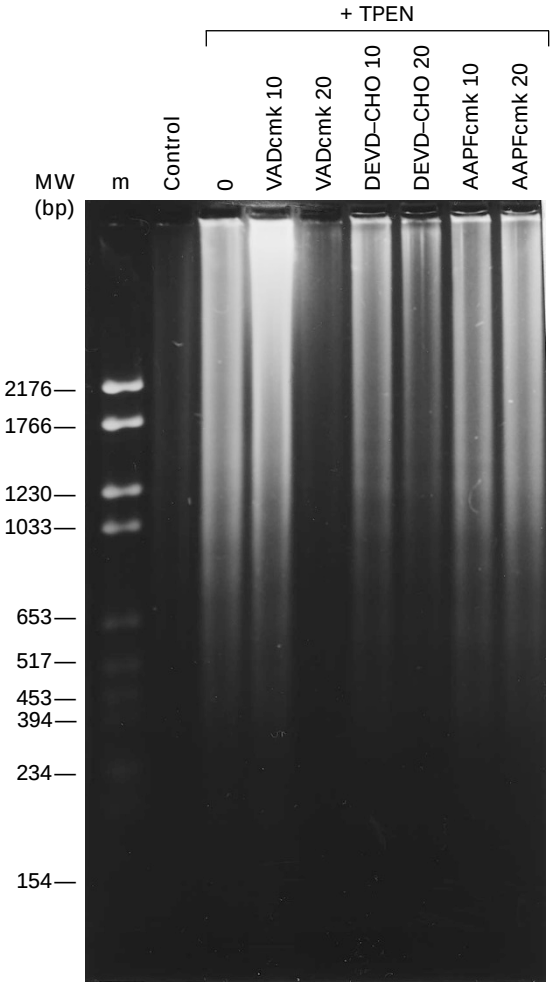

Figure 6 Effects of inhibitors of ICE-like proteases on TPEN-induced DNA fragmentation in Jurkat cells. Jurkat cells were untreated or treated with 10 μM TPEN in the presence or absence (μM) of VADcmk, DEVD-CHO, or AAPFcmk for 6 h. Oligonucleosomal DNA fragmentation was examined by conventional agarose gel electrophoresis. m=marker.

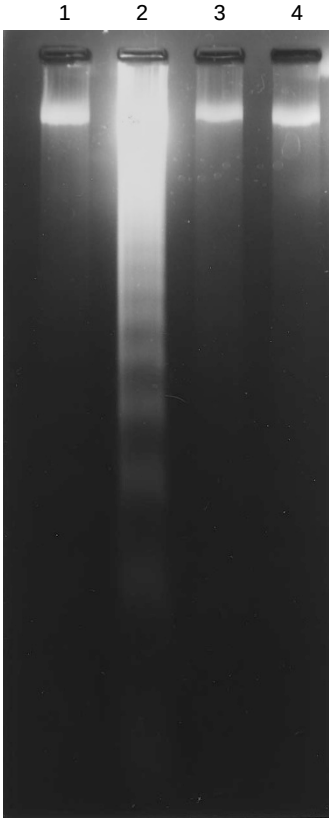

Figure 7 Effects of inhibitors of ICE-like proteases on apoptosis-inducing activity in TPEN-treated Jurkat cells. Jurkat cells were untreated (lane 1) or treated with 10 μM TPEN (lanes 2–4) in the absence (lane 2) or presence of 10 μM VADcmk (lane 3) or 10 μM DEVD-CHO (lane 4) for 4 h. Cytosolic extracts were prepared from each sample and incubated with isolated thymocyte nuclei for 1 h. Their ability to induce DNA fragmentation in isolated thymocyte nuclei was examined by conventional agarose gel electrophoresis.

Table 1 DEVD-AMC cleavage in TPEN-treated rat thymocytes and Jurkat cells

	AMC release (pmol/min)	
Time (min)	**Control**	**TPEN**
(A) Thymocytes		
40	0	8.6
100	18.9	36.8
140	31.0	62.2
(B) Jurkat cells		
40	0	2.5
90	0	4.3
120	0	6.6
150	0	7.6

Thymocytes and Jurkat cells (5×10^6 cells/ml) were untreated or treated with 10 μM TPEN for the various times indicated. AMC release was measured as described in Materials and Methods. The results are from one representative experiment out of two.

Discussion

Our present study shows that in TPEN-triggered thymocyte apoptosis DNA fragmentation is not induced by the direct effects of TPEN on nuclei, but rather by the activation of cytosolic effectors. Activation of these cytosolic effectors by TPEN occurs only in intact cells, since the addition of TPEN to cytosolic extract from untreated cells was unable to induce DNA fragmentation in isolated nuclei. It suggests that TPEN cannot directly activate the cytosolic component(s) required to induce DNA fragmentation in the isolated nuclear fraction. This is unlike the systems described by Bertrand *et al* (1994) and Martin *et al* (1995). In their respective systems, they found that apoptotic activity could be induced by cytoplasmic extract from untreated cells following either the addition of staurosporine, a broad-spectrum protein kinase inhibitor, or the addition of ceramide, a lipid second messenger. However, although ceramide could induce apoptotic activity in normal cell extracts, the morphological changes in the nuclei were not as dramatic as those observed when extracts from UV- or anti-Fas antibody-primed cells were employed (Martin *et al*, 1995).

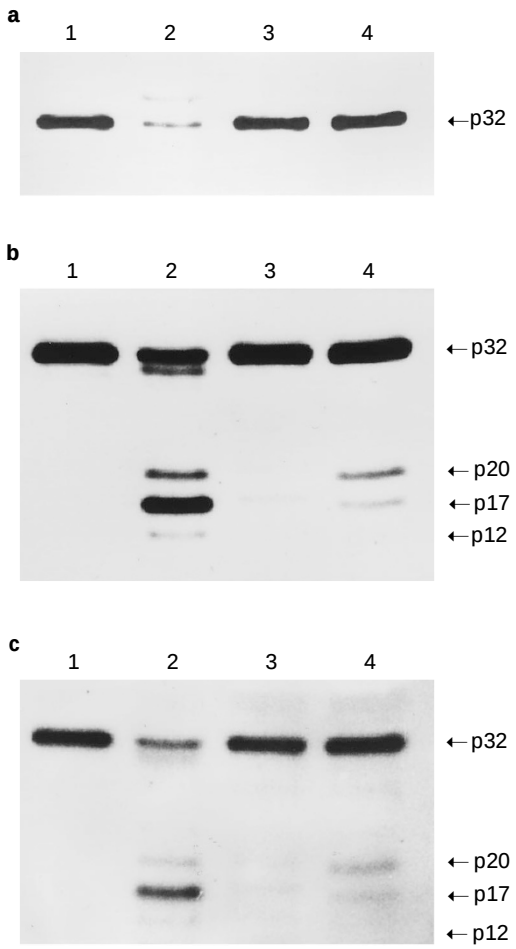

Figure 8 CPP32 cleavage in cytosolic extract from TPEN-treated Jurkat cells and the effects of inhibitors of ICE-like proteases. Jurkat cells were untreated (lane 1) or treated with 10 μM TPEN (lanes 2–4) in the absence (lane 2) or presence of 10 μM VADcmk (lane 3) or 10 μM DEVD-CHO (lane 4) for 4 h. Cytosolic extracts were prepared from these cells. The presence of CPP32 and the fragments of its proteolytical cleavage was determined by immunoblotting using anti-CPP32 (**a**), anti-p17 (**b**), or anti-p12 (**c**) antibodies.

Table 2 DEVD-AMC cleavage in cytosolic extracts from TPEN-treated Jurkat cells

Time (min)	AMC release (pmol/min)
Control cytosol	0.64
Control cytosol+TPEN	0.56
TPEN cytosol	38.3
TPEN cytosol+DEVD-CHO	15.4

Cytosolic extracts were prepared from Jurkat cells untreated (control cytosol) or treated with 10 μM TPEN (TPEN cytosol) for 4 h. Cytosolic extracts were then incubated with or without 10 μM TPEN for 1 h. AMC release was measured in the absence or presence of 50 nM DEVD-CHO as described in Materials and Methods. The results are from one representative experiment out of two.

From our results, it is clear that the cytosolic extract from TPEN-treated cells contains ICE-like proteolytic activity. Whether ICE-like proteases are also required for the formation of the apoptosis-inducing activity in this cytosolic extract is not known. The apoptosis-inducing activity in cytosolic fraction from TPEN-treated Jurkat cells was blocked by incubating cells in the presence of inhibitors of ICE-like proteases. The abolishment of apoptosis-inducing activity by protease inhibitors may be due to the inhibition of production of active effectors of apoptosis in TPEN-treated Jurkat cells. However, we cannot exclude the possibility that active effectors were generated but that their effect on the isolated nuclei was blocked by the inhibitors present in the cytosol.

Currently, we do not know how ICE-like proteases are activated in TPEN-treated cells. An *in vitro* study by Black *et al* (1989) showed that the metal chelators EDTA and 1,10-phenanthroline, which both chelate Zn^{2+}, completely inhibit ICE activity. Moreover, we found that TPEN was unable to directly activate cytosolic components, i.e. proteases, from untreated Jurkat T cells to induce DNA fragmentation in isolated nuclei. Thus, the increase of ICE-like protease activity in the TPEN system was probably not due to a direct effect of TPEN. The inhibition of DNA fragmentation by DEVD-CHO both *in vivo* and *in vitro* indicates the involvement of CPP32 in TPEN-induced apoptosis. DEVD-CHO has low membrane permeability (Nicholson *et al*, 1995), and its effect on intact Jurkat cells may be due to the high concentration which we used (10 μM). However, the involvement of CPP32 in TPEN-induced apoptosis was strongly supported by an increase in enzyme activity which cleaves DEVD-AMC, a fluorogenic substrate of CPP32/apopain, and the cleavage of CPP32 into p17 and p12 fragments. The activation of a protease which cleaves DEVD-AMC was observed in both thymocytes and Jurkat cells treated with TPEN. This activity was inhibited by DEVD-CHO. It is known that CPP32 activation involves proteolysis and the formation of two polypeptides, p17 and p12. In fact, the active cytosolic extract from Jurkat cells treated with TPEN contained a small amount of the native CPP32 and greater amounts of p17 and p12. The activation of CPP32 registered as a formation of p17 and p12, was blocked by two inhibitors, VADcmk and DEVD-

CHO. Therefore, TPEN-triggered apoptosis is also mediated by the activation of a CPP32-like protease.

One of the proteins that is known to be cleaved by CPP32/apopain is PARP. PARP is an enzyme involved in DNA repair (D'Murcia and D'Murcia, 1994). Using NAD^+ as a substrate, this enzyme links poly(ADP-ribose) moieties to cleave sites in the DNA backbone, presumably allowing for the short term maintenance of structural integrity until DNA polymerases can make permanent repairs. Importantly, PARP is a zinc binding enzyme and has two zinc fingers near its N-terminus, defining the domain that binds to DNA breaks with nanomolar affinity (D'Murcia and D'Murcia, 1994). Activation of CPP32/apopain induces the cleavage of PARP from the 116 kDa active form to an 85 kDa carboxy-terminal fragment and a small fragment containing the zinc fingers (Lazebnik *et al*, 1994). It is suggested that the generation of these small fragments might enhance cell death in conjunction with DNA fragmentation (Lindahl *et al*, 1995). In TPEN-treated rat thymocytes, PARP underwent a similar pattern of cleavage, which could be blocked by the inhibitor of ICE-like proteases. These data suggest that the TPEN-induced PARP cleavage is mediated by ICE-like proteases, but not by a direct effect of TPEN on PARP.

It is not clear whether the activation of ICE-like proteases also contributes to the activation of proteases involved in lamin degradation. Our data show that TPEN-induced lamin degradation was inhibited by VADcmk, suggesting that the activation of ICE-like proteases occurs prior to lamin degradation. The lamin degradation in TPEN-induced apoptosis was not due to an activation of the Ca^{2+}-regulated serine protease, as demonstrated by the lack of effect of AAPFcmk. It is possible that another Ca^{2+}-independent protease may be used to cleave lamin in the TPEN system. A recent study by Lazebnik *et al* (1995) demonstrated that a Ca^{2+}-regulated serine protease is not the only enzyme capable of cleaving lamin during apoptosis. In their study, no inhibition of lamin cleavage by AAPFcmk was observed, which is similar to what we observed in the TPEN system. Nevertheless, we found the involvement of Ca^{2+}-regulated serine protease in systems where apoptosis is associated with an increase of cytosolic free Ca^{2+} concentration, such as thymocyte apoptosis induced by glucocorticoid and thapsigargin (Zhivotovsky *et al*, 1995; S. Jiang, B. Zhivotovsky, A. Gahm, and S. Orrenius unpublished data).

TPEN induces neither oligonucleosomal DNA fragmentation in isolated rat thymocyte nuclei (Figures 1 and 5) nor single or double strand DNA breaks in isolated liver nuclei (Burkitt *et al*, 1996). Nevertheless, it is possible that in whole cells TPEN can result in early damage to DNA not detectable as HMW or oligonucleosomal DNA fragments. Thus, we can not exclude the possibility that early DNA damage serves as a signal thereby initiating the activation of cytosolic ICE-like proteases, as suggested in apoptosis induced by the topoisomerase inhibitor, etoposide (Zhivotovsky *et al*, 1995). We found that TPEN- and etoposide-induced apoptosis share many features. Both involve an early activation of ICE-like proteases and degradation of nuclear lamins prior to DNA cleavage, which are not prevented by a Ca^{2+}-regulated serine protease inhibitor (Zhivotovsky *et al*, 1995). The signal initiating the activation of cytosolic ICE-like proteases could be also from the cell surface. Zinc has been found to significantly affect the cell membrane by interfering with macromolecular components of the membrane, thus changing their conformation or enzyme-substrate specificity (Bettger and O'Dell, 1981). Therefore, it is possible that chelation of Zn^{2+} by TPEN may activate ICE-like proteases by a pathway similar to that triggered by receptor-mediated apoptotic stimuli, for example, anti-Fas antibody.

In conclusion, our study demonstrates that the mechanism whereby TPEN triggers apoptosis does not involve its direct effects on nuclei. An activation of cytosolic effectors, which include CPP32/apopain, is essential for initiating apoptosis in the TPEN system. Our results provide strong evidence that, as in several other systems, ICE-like proteases play a central role in TPEN-induced apoptosis.

Materials and Methods

Materials

RPMI 1640 medium and fetal bovine serum (FBS) were obtained from Gibco BRL (Gaithersburg, MD, USA). TPEN and Pulse markers - chromosomes from *Saccharomyces cerevisiae* (225–2200 kbp) and a mixture of λDNA HindIII fragments, λDNA, and λDNA concatemers (0.1–200 kbp) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Molecular weight markers VI (pBR 328 DNA/*Bgl* I - *Hinf* I) and proteinase K were obtained from Boehringer-Mannheim (Mannheim, Germany). RNAse and pronase were purchased from Calbiochem (La Jolla, CA, USA). VADcmk and AAPFcmk were obtained from Enzyme System Products (Dublin, CA, USA). ECL was from Amersham Corp. (Buckinghamshire, UK). Antibody against lamin B1 was generously provided by Dr. Scott H. Kaufmann (The Mayo Clinic, Rochester, MN, USA). Anti-PARP antibody was kindly provided by Dr. Guy G. Poirier (Laval University, Quebec, Canada). Anti-p17 and p12 antibody were kindly donated by Dr. Donald W. Nicholson (Merck Frosst Center for Therapeutic Research, Quebec, Canada). Goat anti-mouse IgG-HRP was from Pierce (Rockford, IL, USA). Human anti-CPP32 antibody was from Transduction Laboratories (Lexington, KY, USA). Goat anti-chicken IgG-HRP was from Southern Biotechnology Associates, Inc. (Birmingham, AL, USA). Rabbit anti-human IgG-HRP was from Dakopatts A/S (Denmark). DEVD-CHO was from Merck Research Laboratory (Rahway, NJ, USA). DEVD-AMC was from Bachem (Bubendorf, Switzerland). All other reagents were from local sources and were of the highest purity available.

Cells and culture conditions

Cells were cultured in RPMI 1640 medium supplemented with FBS, 2 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin in a humidified incubator (37°C) at an atmosphere of 5% CO_2 in air. Thymocytes were prepared from thymic glands from male Sprague-Dawley rats (75–85 g) as described previously (McCabe *et al*, 1993) and incubated with the aforementioned agents at a density of 5×10^6 cells/ml in RPMI 1640 medium supplemented with 2% FBS. Human leukemia T cell line, Jurkat, was obtained from ATCC (Rockville, MD, USA) and maintained in suspension culture using RPMI 1640 medium supplemented with 10% FBS.

Isolation of thymocyte nuclei

Rat thymocyte nuclei were prepared as described previously by Alnemri and Litwack (1990) with the following modifications: 1) 0.02% NP-40 was added to thymocytes before homogenization and in the buffer used to prepare nuclei and 2) PMSF was omitted. The isolated nuclei were then resuspended in buffer B (5 mM $MgCl_2$, 2.1 M sucrose, 50 mM Tris, pH 7.5) at a final density of $3-4\times10^8$ nuclei/ml before use.

Quantitation of DNA fragmentation

DNA fragmentation in thymocyte nuclei was measured by diphenylamine assay. Briefly described, thymocyte nuclei (5×10^6/ml) were incubated at 37°C in the incubating buffer (25 mM Hepes, pH 7.2, 2 mM potasium phosphate, 125 mM KCl, 4 mM $MgCl_2$) with 10 μM TPEN and/or various concentrations of $CaCl_2$ for 2 h. Aliquots of 0.8 ml nuclei suspension were added to 0.7 ml ice-cold lysis buffer containing 20 mM EDTA, 0.05% (v/v) Triton X-100 and 10 mM Tris-Cl at pH 8.0. The nuclei were allowed to lyse for 15 min at 4°C prior to centrifugation for 15 min at $13{,}000\times g$ to separate the intact chromatin (pellet) from DNA fragments in the supernatants. The DNA in the pellets and supernatants was digested with 5% trichloroacetic acid before being assayed using the diphenylamine reagent (Burton, 1956).

Analysis of oligonucleosomal DNA fragments

The formation of oligonucleosomal DNA fragments was examined by agarose gel electrophoresis. Briefly, after incubation with agents where indicated, 2×10^6 cells or nuclei were resuspended in 250 μl of TE buffer (10 mM Tris and 1 mM EDTA at pH 8.0). The suspensions were then mixed with an equal volume of lysis buffer (see above). After 30 min on ice, the lysates were centrifuged for 15 min at 4°C at $13{,}000\times g$ to separate cell debris containing intact chromatin (pellet) from DNA fragments (supernatant). The fragmented DNA in supernatant was precipitated overnight at -20°C with ethanol. Next, the DNA was resuspended in 20 μl TE buffer (see above), incubated subsequently with 1 μl 50 mg/ml RNAse A for 1 h at 37°C, and thereafter 1 μl 25 mg/ml proteinase K was added and incubated for 1 h at 37°C. The samples were electrophorezed for 4 h at 60 mA on 1.8% agarose gels. The separated DNA was stained with 1 μg/ml ethidium bromide and visualized by UV light.

Field inversion gel electrophoresis (FIGE)

Thymocytes were incubated with agents as previously indicated for 4 h, washed and then resuspended in 250 μl prewarmed phosphate buffered solution. An equal volume of liquified 1% low-melting point agarose solution was added to this suspension and gently mixed. One hundred microliters of mixture were dispensed to each well of the insert moulds, and allowed to cool on ice for 30 min. The resulting agarose plugs were dislodged from the insert moulds, transferred into a solution containing 1% lauryl sarcosine, 0.5 M EDTA, 10 mM Tris, and 1 mg/ml pronase, and incubated at 50°C with continuous agitation. After 24 h, the old solution was replaced with a fresh one and incubation was continued for another 24 h. In a similar solution as above but without pronase, the plugs were rinsed twice for 2 h per rinse and stored at 4°C until use. Before electrophoresis, the plugs were washed three times in a buffer containing 10 mM Tris and 1 mM EDTA. FIGE was carried out using a horizontal (HE 100B) gel chamber, a power supply (PS 500XT) and switchback pulse controller (PC 500, Hoefer Scientific Instruments, U.S.A.). The temperature was controlled by an LKB 2209 Multitemp constant temperature cooling system. Electrophoresis was run at 200 V in 1% agarose gel in $0.5\times$ TBE (4.45 mM Tris, 4.45 mM boric acid, and 1.25 mM EDTA, pH 8.5) at 14°C with the ramping rate changing from 0.8 s to 30 s over a 24 h period, applying a forward to reverse ratio of 3 : 1. DNA size calibration was performed using two sets of pulse markers: chromosomes from *Saccharomyces cerevisiae* (225–2200 kbp), and a mixture of λDNA HindIII fragments, λDNA and λDNA concatemers (0.1–200 kbp). DNA was stained with 1 μg/ml ethidium bromide and visualized using UV light.

Immunoblot analysis

For protein detection with Western Blot, cells were homogenized in low salt buffer (10 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 20 mM KCl, 1 mM DTT, 1 mM PMSF, 1 mM 1,10-phenanthroline, 20 μg/ml leupeptin, 5 μg/ml pepstatin, and 0.5% NP-40). Then, the cells were centrifuged at $2{,}000\times g$ for 5 min at 4°C. The pellets were washed once with a similar buffer as above but without NP-40. Both supernatants were combined and centrifuged at $12{,}000\times g$ for 20 min at 4°C. Insoluble protein fractions from the debris were extracted with high salt buffer (0.4 M NaCl, 20 mM HEPES, pH 8.0, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 1 mM 1,10-phenanthroline, 20 μg/ml leupeptin, 5 μg/ml pepstatin, and 0.1% NP-40) by incubating on ice for 40 min and thereafter centrifuging at $12{,}000\times g$ for 20 min at 4°C. Extracted proteins were solubilized for 5 min at 100°C in Laemmli's SDS-PAGE sample buffer. Polypeptides were resolved at 130 V on 12% or 15% SDS-PAGE gels and electrophoretically transferred to nitrocellulose (0.2 μm) for 2 h at 100 V. Membranes were blocked overnight in a buffer (50 mM Tris, 500 mM NaCl, pH 7.5) containing 1% bovine serum albumin and 5% non-fat dried milk. Then, the membranes were probed for 1.5 h with anti-lamin B1 Ab (1 : 500), anti-PARP Ab (1 : 5000), anti-CPP32 Ab (1 : 100), anti-p17 Ab (1 : 5000), or p12 (1 : 1000) Ab and 1 h with HRP-conjugated secondary antibodies (1 : 10,000), and then visualized by ECL according to manufacturer's instructions.

Preparation of cytosolic fraction

Jurkat cells (6×10^6 cells/ml) were treated with TPEN for 4 h. The cells were then washed with ice-cold RPMI 1640 medium (without FBS) and resuspended (6×10^6 cells per 10 μl) in buffer A (40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 10 mM HEPES, pH 7.0). After four cycles of freezing and thawing in ethanol: dry ice bath, the cell lysates were centrifuged for 30 min at $20{,}000\times g$ to pellet the membrane fraction. The supernatants were centrifuged for an additional 30 min at $100{,}000\times g$ to obtain supernatant fraction consisting mainly of cytosolic proteins. The protein concentrations in the supernatants were measured using the BCA protein assay kit (Pierce).

Reconstitution of the cell-free system

Using a cell-free system as described by Lazebnik *et al* (1993), isolated nuclei were incubated with cytosolic fractions prepared from untreated or TPEN-treated Jurkat cells. The reaction mixture, containing 145 μg of cytosolic proteins and 2×10^6 nuclei, was diluted to a final volume of 30 μl with buffer A (see above) plus 2 mM DTT, supplemented with an ATP-regeneration system. Samples were incubated at 37°C for the time periods indicated before analysis of the formation of oligonucleosomal DNA fragments.

Fluorometric assay of DEVD-AMC cleavage

The enzyme activity that cleaves DEVD-AMC, a specific fluorogenic substrate for CPP32/apopain, was measured by a continuous fluorometric assay, which was modified from a method described by Nicholson *et al* (1995). The reaction mixture contained 40 μM substrate, cell extract, and buffer (100 mM HEPES, pH 7.25, 10% sucrose, 0.1% CHAPS, 10 mM DTT). Liberation of AMC from the substrate was monitored continuously using an excitation wavelength of 355 nm and an emission wavelength of 460 nm. The enzyme activity was shown as pmol of AMC release per min.

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