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Multiple Proteases Are Involved in Thymocyte Apoptosis

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To investigate the involvement of proteases in apoptosis, rat thymocytes were treated with the glucocorticoid hormone methylprednisolone or the topoisomerase II inhibitor etoposide in the presence of selective substrate inhibitors of either interleukin-1β-converting enzyme (ICE), (Z-Val-Ala-Asp-chloromethylketone, VADcmk) or Ca^{2+}-regulated serine protease (Suc-Ala-Ala-Pro-Phe-chloromethylketone, AAPFcmk). VADcmk protected from lamin proteolysis, chromatin fragmentation, cell shrinkage, and formation of apoptotic nuclei in both methylprednisolone- and etoposide-treated thymocytes when present during the initiation of the apoptotic process. AAPFcmk prevented lamin breakdown, chromatin fragmentation, and apoptotic morphological changes in thymocytes treated with methylprednisolone, but not with etoposide. Both MPS- and etoposide-treated thymocytes exhibited enhanced ICE-like protease activity which was maximal 1 h after treatment. This increase in proteolytic activity was blocked by VADcmk, but not AAPFcmk. Our findings suggest that ICE-like protease activity is critically involved in the early phase of both methylprednisolone- and etoposide-induced apoptosis in thymocytes, whereas the Ca^{2+}-regulated serine protease is an obligatory component of the proteolytic cascade in methylprednisolone-induced apoptosis. © 1995 Academic Press, Inc.

INTRODUCTION

Apoptosis is a special form of cell death with characteristic morphological features which were established in early studies by Kerr *et al.* [1] and shown to include cell shrinkage, cytoplasm blebbing, organelle relocation, chromatin condensation, and cell fragmentation into apoptotic bodies. The alterations of nuclear ultrastructure are associated with rearrangement and fragmentation of the chromatin, typically resulting in the generation of both high-molecular-weight (HMW; 50–300 kbp) and oligonucleosome-length DNA fragments [2–5]. Such a fragmentation pattern can be reproduced by addition of divalent cations (Ca^{2+} plus Mg^{2+}) to isolated nuclear preparations [6]. Normally, the apoptotic process ends with phagocytosis of the dead cell or its remnants [1].

There is increasing evidence that the biochemical machinery involved in the killing and degradation of the cell undergoing apoptosis is expressed constitutively and available for activation by various signals. In fact, recent studies with enucleated cells (cytoplasts) have suggested that the apoptotic process can be initiated and proceed also in the absence of the nucleus, and that there may be a "cytoplasmic regulator" of the process [7, 8]. However, the nature of this regulator is yet unknown.

Both nucleases and proteases have been implicated in the killing and degradation of the apoptotic cell. Whereas much of the early work aimed at the identification of endonucleases involved the degradation of chromatin [see ref. 9 for review], recent studies have focused more on the role of proteases in apoptosis. Although it has been known for some time that serine proteases (granzymes) participate in the apoptotic killing of target cells by cytotoxic T lymphocytes [10], the interest in a more general role of proteases in apoptosis was stimulated markedly by the observation that the product of the cell death gene, ced-3, in the nematode *Caenorhabditis elegans* shares sequence homology with the well-characterized human protein interleukin-1β-converting enzyme (ICE) and that both proteins contain a conserved pentapeptide domain in the active site [11, 12]. Overexpression of ICE resulted in the apoptotic death of approximately 95% of transfected cells. Conversely, introduction of the crmA gene (which encodes a serine protease inhibitor (serpin) that binds ICE and inhibits its proteolytic activity) into cells expressing ICE resulted in cell survival [13]. However, the specificity of this protective effect has not been clearly established. This is also true for most of the other inhibitors of various proteases (cysteine and serine proteases, calpains, etc.) which have been found to block apoptosis in thymocytes and other cell types [14–17].

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It is known that apoptosis shares certain features with mitosis, e.g., chromatin condensation and lamin degradation. Lamin breakdown has been observed in several models of apoptosis [18–20], although it is unclear how it is brought about and what role it plays in chromatin fragmentation. However, recently Clawson *et al.* [21] have isolated a Ca^{2+}-regulated serine protease (CRP) associated with both the nuclear scaffold and endoplasmic reticulum which catalyzes the cleavage of lamins and possibly other proteins. Inhibition of CRP was subsequently reported to block chemical transformation in fibroblasts [22].

The present study was designed to investigate the contribution of ICE-like protease(s) and of the Ca^{2+}-regulated serine protease in thymocyte apoptosis triggered by either methylprednisolone or the topoisomerase II inhibitor, etoposide. High-affinity substrate inhibitors of both proteases were employed. Our results demonstrate that both ICE-like protease(s) and CRP can be involved in thymocyte apoptosis, depending on how this process is triggered.

MATERIAL AND METHODS

Chemicals. Suc-Ala-Ala-Pro-Phe-chloromethylketone (AAPFcmk), Z-Val-Ala-Asp-chloromethylketone (VADcmk), and enzyme overlay membrane impregnated with YVADafc were from Enzyme System Products (CA). DMSO, *N*-lauroylsarcosine, methylprednisolone, spermine, and spermidine were obtained from Sigma. Proteinase K was from Boehringer-Mannheim. All agarose types were purchased from FMC BioProducts. EDTA, EGTA, and all inorganic salts were from Fluka. Etoposide was from Bristol–Meyers and anti-lamin antibodies were obtained from Novocastra. Goat anti-mouse IgG was from Pierce. ECL was from Amersham Corp. and nitrocellulose filters were from Sartorius.

Cell culture and confocal microscopy. Male, 3-week-old Sprague–Dawley rats (50–65 g) were used in all experiments. Rats were killed by cervical dislocation. Thymuses were removed and thymocyte suspensions (5×10^6 cells/ml) were prepared and maintained in RPMI 1640 medium plus 2% fetal calf serum. Thymocyte suspension was treated with either 0.1 μM MPS or 5 μM etoposide. All substances to be tested as possible inhibitors of apoptosis were added to the cell suspension before treatment with MPS or etoposide, unless otherwise indicated. All reagents were dissolved in DMSO or ethanol, and equivalent quantities of DMSO or ethanol, never exceeding 1%, were added to controls. Cell volume profiles were determinated by Coulter counting as previously described [23]. Cell suspensions were diluted with Isoton II solution and counted in a model Z1 Coulter counter.

At different time intervals, samples of thymocyte suspension were harvested by centrifugation, resuspended in PBS, and added to coverslips coated with poly-L-lysine. After fixation with methanol, cells were stained with propidium iodide. Images were then collected using a Bio-Rad MRC-600 (Bio-Rad Microscience Ltd, Hemel Hempstead, England) scanning assembly, incorporating a krypton–argon laser and coupled to a Diaphot Nikon microscope equipped with a 60 × 1.4 NA, Nikon plan Apo lens.

Field-inversion gel electrophoresis (FIGE). Following treatment, cells were resuspended in a solution containing 0.15 *M* NaCl, 2 m*M* KH_2PO_4, pH 6.8, 1 m*M* EGTA, and 5 m*M* $MgCl_2$. An equal volume of liquified 1% low-melting-point agarose solution was added to this suspension and the mixture was then aliquoted into gel plug casting forms. The resulting agarose blocks were introduced into the 1% agarose gel. FIGE was carried out using a horizontal gel chamber, a power supply, and Switchback pulse controller purchased from Hoefer Scientific Instruments. Electrophoresis was run at 180 V in 0.5 × TBE (45 m*M* Tris, 1.25 m*M* EDTA, 45 m*M* boric acid, pH 8.0), at 12°C, with the ramping rate changing from 0.8 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 *Hin*dIII fragments, λDNA, and λDNA concatemers (0.1–200 kbp) purchased from Sigma. DNA was stained with ethidium bromide, visualized using a 305-nm UV light source, and photographed using Polaroid 665 positive/negative film.

Conventional agarose gel electrophoresis. Aliquots from the cell suspensions were mixed with an equal volume of ice-cold lysis buffer (5 m*M* Tris–HCl, pH 8.0, 20 m*M* EDTA, 0.5% Triton X-100) and incubated at 4°C for 30 min. The resulting suspension was centrifuged at 15,000*g* for 15 min to separate cell debris containing intact chromatin (pellet) from DNA fragments (supernate). The DNA from the supernate was subsequently loaded on conventional 1.8% agarose gels. Electrophoresis was run with constant current set at 60 mA. The mixture of pBR 328 DNA–*Bgl*I and pBR 328 DNA–*Hin*fI was used as a marker. Gel staining and photography were performed as described above.

Identification of ICE-like protease activity. For identification of ICE-like activity proteins were extracted from cells with hypotonic buffer containing 25 m*M* Hepes, pH 7.5, 1 m*M* $MgCl_2$, 1 m*M* EDTA, 1 m*M* PMSF, 10 μg/ml pepstatin, and 10 μg/ml leupeptine. Postnuclear supernatant was adjusted with EDTA up to 6 m*M* and centrifuged at 20,000*g* for 20 min. Supernatant was adjusted with DTT up to 2 m*M* and proteins were separated on 7.5% nondenaturing PAGE. After electrophoresis, gels were incubated in buffer containing 10 m*M* Hepes, pH 7.5, 2 m*M* DTT, 0.1% Chaps, and 10% sucrose for 20 min at room temperature and then applied to the membrane impregnated with YVAD attached to afc. This sandwich was incubated at 37°C for various time periods. afc was liberated by protease activity, when membranes were irradiated with long-wavelength UV light. Results were registered by using Polaroid 665 positive/negative film.

Lamin breakdown. Lamin breakdown was determined by immunoblot analysis. Cells were homogenized in low salt buffer (10 m*M* Hepes, pH 8.0, 1 m*M* $MgCl_2$, 20 m*M* KCl, 1 m*M* DTT, 1 m*M* PMSF, 1 m*M* 1,10-phenanthroline, 20 μg/ml leupeptine, 5 μg/ml pepstatine, and 0.5% NP-40) and centrifuged at 2000*g* for 5 min at 4°C. The pellet was washed once with the same buffer without NP-40. Both supernatants were combined and centrifuged at 12,000*g* for 20 min at 4°C. Insoluble protein fraction from the debris was extracted with high salt buffer (0.4 *M* NaCl, 20 m*M* Hepes, pH 8.0, 1 m*M* $MgCl_2$, 0.5 m*M* EDTA, 0.5 m*M* DTT, 1 m*M* PMSF, 1 m*M* 1,10-phenanthroline, 20 μg/ml leupeptine, 5 μg/ml pepstatine, and 0.1% NP-40) by 40-min incubation on ice and centrifugation at 12,000*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% gels and electrophoretically transferred to nitrocellulose (0.2 μm) for 2 h at 100 V. Membranes were blocked overnight in a buffer (50 m*M* Tris, pH 7.5, 500 m*M* NaCl), containing 1% bovine serum albumin and 5% nonfat dried milk, and probed for 1.5 h with mouse anti-lamin B Ab or mouse anti-lamin A/C Ab (1:5000 in blocking solution without milk) and 1 h with goat anti-mouse IgG (1:10,000 in the same solution), and then visualized by ECL according to manufacturer's instructions.

RESULTS

The glucocorticoid hormone, methylprednisolone (MPS) (0.1 μM) and the topoisomerase II inhibitor, eto-

TABLE 1

Effects of Protease Inhibitors on Methylprednisolone- or Etoposide-Induced Cell Death in Rat Thymocytes

Treatment	DNA fragmentation[a]	Cell viability[b]
Control	15.4 ± 2	95 ± 2
MPS	30.7 ± 3[c]	79 ± 4[c]
+AAPFcmk 5 μM	27.5 ± 2	78 ± 4
+AAPFcmk 10 μM	18.3 ± 4[d]	88 ± 5[d]
+AAPFcmk 20 μM	14.2 ± 3[d]	89 ± 3[d]
+VADcmk 5 μM	25.5 ± 3	79 ± 3
+VADcmk 10 μM	21.1 ± 1[d]	86 ± 3[d]
+VADcmk 20 μM	15.3 ± 3[d]	90 ± 4[d]
Etoposide	33.8 ± 4[c]	76 ± 2[c]
+AAPFcmk 5 μM	28.6 ± 3	77 ± 4
+AAPFcmk 10 μM	24.1 ± 7	84 ± 5
+AAPFcmk 20 μM	24.0 ± 5	83 ± 3
+VADcmk 5 μM	29.5 ± 2	79 ± 3
+VADcmk 10 μM	21.1 ± 3[d]	90 ± 4[d]
+VADcmk 20 μM	15.8 ± 4[d]	91 ± 2[d]

Note. Thymocyte suspensions were prepared in RPMI 1640. Inhibitors were added to cells just before treatment with MPS or etoposide. Mean values ± SE from four separate experiments are presented.

[a] Percentage of total DNA resisting sedimentation at 20,000*g* after treatment.

[b] Percentage of cells excluding trypan blue after treatment.

[c] Significantly different from the control as determined by Student's paired *t* test, $P < 0.05$.

[d] Significantly different from the treatment with MPS or etoposide as determined by Student's paired test, $P < 0.05$.

poside (5 μM) were used to induce apoptosis in rat thymocytes. Apoptosis was monitored by the formation of HMW and oligonucleosome-length (DNA "laddering") DNA fragments as well as by changes in cell volume and appearance of cells with apoptotic morphology as determined by confocal microscopy. Inhibitors of proteases were added before or simultaneously with the inducers, and incubation was for 4 h. As shown in Table 1, both the inhibitor of ICE-like proteases, VADcmk, and the CRP inhibitor, AAPFcmk, inhibited MPS-induced DNA fragmentation in a dose-dependent manner. There were similar protective effects on cell viability by both inhibitors. For etoposide-treated thymocytes, VADcmk, but not AAPFcmk, protected from DNA fragmentation and loss of viability (Table 1). These findings were confirmed by analysis of DNA fragmentation using conventional gel electrophoresis (Fig. 1B). After 4-h incubation of thymocytes treated with either MPS or etoposide in the presence of VADcmk, there was no apparent DNA "laddering," whereas AAPFcmk efficiently prevented the formation of oligonucleosome-length DNA fragments only in MPS-treated cells. Similar to the effects on internucleosomal DNA cleavage, VADcmk dose-dependently inhibited the formation of 50-kbp fragments in both etoposide- and MPS-treated cells, whereas AAPFcmk failed to block the formation of 50-kbp fragments in etoposide-treated thymocytes (Fig. 1A).

Cell shrinkage is a characteristic feature of apoptosis which was monitored after 4-h incubation using a Coulter counter. As shown in Fig. 2, both protease inhibitors protected from cell shrinkage induced by MPS. Similar results were obtained with VADcmk in etoposide-treated thymocytes (Fig. 2B), whereas AAPFcmk, even at 50 μM concentration, failed to prevent cell

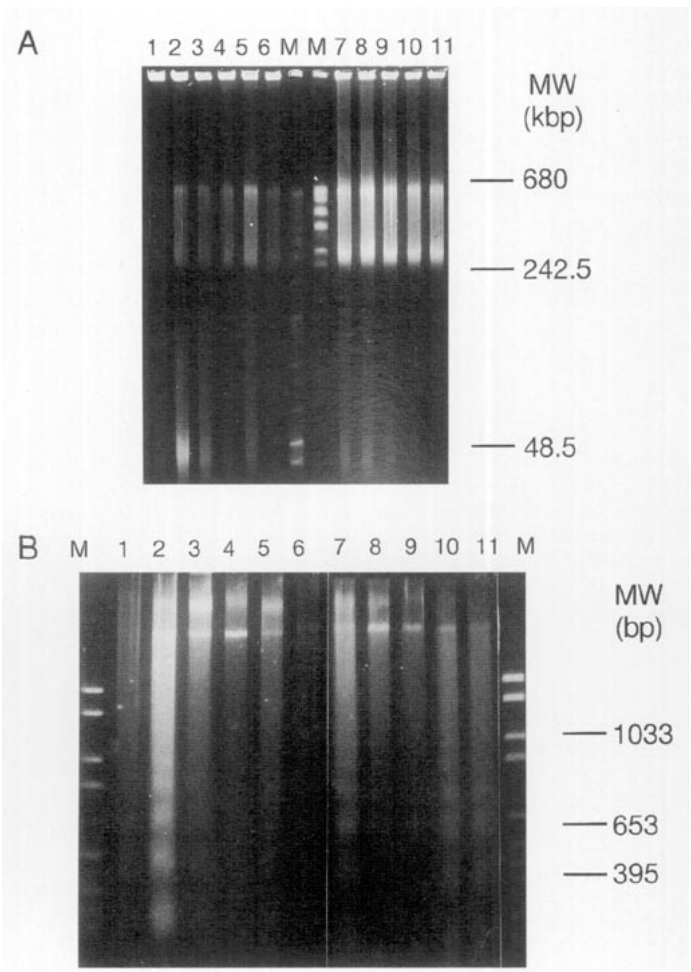


FIG. 1. Effect of protease inhibitors on formation of high- and low-molecular-weight DNA fragments in rat thymocytes treated with methylprednisolone or etoposide. (A) Untreated (lane 1) and MPS-treated (lanes 2–6) or etoposide-treated (lanes 7–11) rat thymocytes were incubated in presence of 10 μM (lanes 3 and 8), or 20 μM (lanes 4 and 9) AAPFcmk; or 10 μM (lanes 5 and 10), or 20 μM (lanes 6 and 11) VADcmk for 2 h at 37°C and examined for HMW DNA fragments by FIGE. M, markers (see Materials and Methods). (B) Untreated (lane 1) and MPS-treated (lanes 2–6) or etoposide-treated (lanes 7–11) rat thymocytes were incubated in presence of 10 μM (lanes 3 and 10) or 20 μM (lanes 4 and 11) AAPFcmk; or 10 μM (lanes 5 and 8), or 20 μM (lanes 6 and 9) VADcmk for 4 h at 37°C and examined for DNA laddering by conventional gel electrophoresis. M, marker (see Materials and Methods).

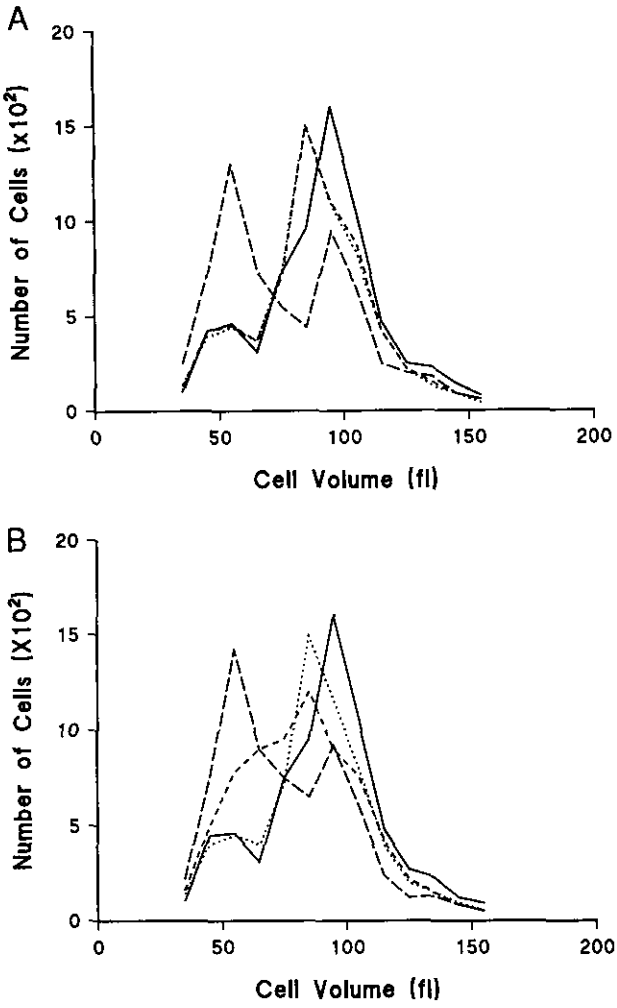


FIG. 2. Effect of protease inhibitors on volume changes in rat thymocytes treated with methylprednisolone (A) or etoposide (B). Cell volume was evaluated in a Coulter counter at 4-h incubation of untreated rat thymocytes (solid line), or thymocytes treated with MPS or etoposide (long-dashed line) in presence of 10 μM AAPFcmk (short-dashed line) or 10 μM VADcmk (dotted line). Data from one of three independent experiments are presented.

shrinkage induced by etoposide. Higher concentrations of VADcmk and AAPFcmk were toxic to the thymocytes and could therefore not be used (data not shown).

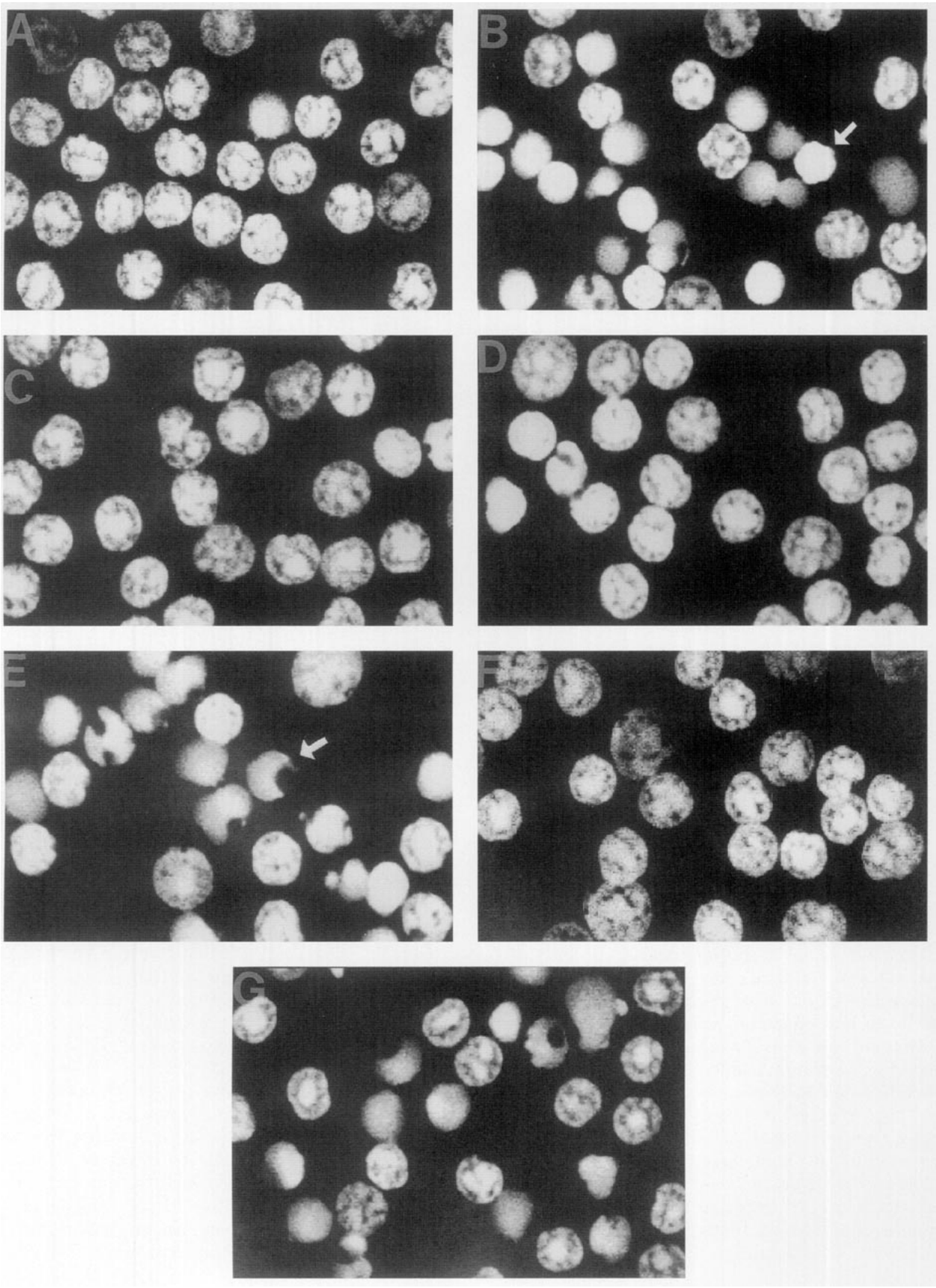
The protective effects of both protease inhibitors in MPS-treated cells were confirmed by morphological studies (Fig. 3). Confocal microscopy images of propidium iodide-stained thymocytes revealed that nuclei appeared to be reduced in size in more than 50% of cells after 4-h treatment with MPS or etoposide. The chromatin was typically condensed and aggregated against one pole of the nucleus. These cells were highly fluorescent without any residual appearance of normal chromatin organization. (Figs. 3B, 3E). In contrast, in the presence of VADcmk most cells treated with MPS or etoposide kept their morphology which was not significantly different from control cells, but a few cells were highly fluorescent (Figs. 3C, 3F). AAPFcmk was somewhat less effective than VADcmk in preventing the formation of apoptotic nuclei after MPS treatment (Fig. 3D). Etoposide-treated cells incubated in the presence of AAPFcmk still showed apoptotic morphology (Fig. 3G).

To investigate the selectivity of the substrate inhibitors used, experiments were performed in which enzyme activity was measured using membranes impregnated with specific substrate (YVADafc) for ICE-like protease(s). In thymocytes undergoing apoptosis this activity was elevated and peaked at 1 h after treatment with MPS or etoposide (Fig. 4); it then returned to control level at 4 h (data not shown). Incubation of cells with VADcmk prevented this increase in ICE-like protease activity, whereas AAPFcmk failed to do so (Fig. 4).

Lamin breakdown has been observed recently in several models of apoptosis [18–20, 24]. One candidate enzyme which could be responsible for lamin degradation during apoptosis is the Ca^{2+}-regulated serine protease associated with the nuclear scaffold [21]. All known classes of lamins (A, B, C) can be degraded by CRP, although the rate of degradation of lamins A/C is faster than that of lamin B ($t_{1/2}$ = 21–24 min versus 38 min for lamin B) [25]. In agreement with the previous finding that lymphoid cells lack lamins A/C [26], we found that lamins A/C are not expressed in rat thymocytes (data not shown). However, treatment with either MPS or etoposide resulted in time-dependent degradation of lamin B, which was apparent 1 h post-treatment and continued during incubation of the cells for up to 4 h (Fig. 5A). Lamin breakdown was more pronounced in etoposide- than MPS-treated cells and was also observed in thymocytes spontaneously undergoing apoptosis (data not shown). Lamin degradation preceded the formation of HMW DNA fragments. Pre-incubation of the cells with either VADcmk or AAPFcmk prevented lamin degradation in cells treated with MPS, whereas VADcmk, but not AAPFcmk, was also effective in preventing lamin breakdown in etoposide-treated thymocytes (Fig. 5B).

DISCUSSION

The requirement of proteolysis for both induction and perpetuation of apoptosis now is well documented [11, 14–16, 27]. Recently, to mimic the injection of granzymes into target cells by cytotoxic T cells, Williams and Henkart [28] showed that internalization of several proteases into different cell types caused cell lysis associated with typical apoptotic features. However, the identity of the protease(s) involved in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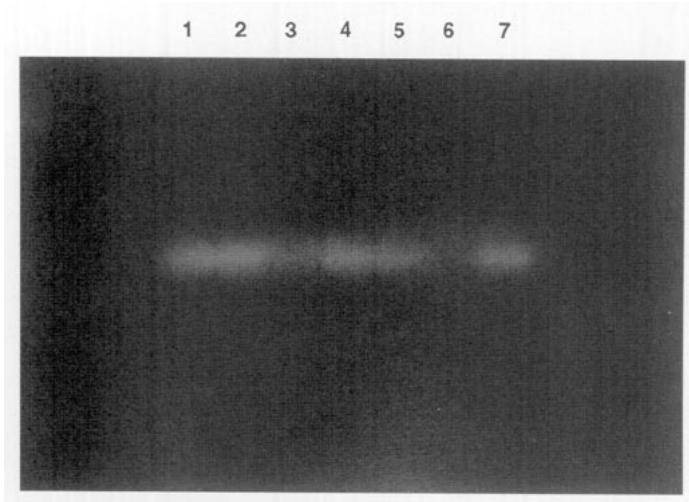

FIG. 4. Effect of protease inhibitors on ICE-like activity in rat thymocytes treated with methylprednisolone or etoposide. Untreated (lane 1) and MPS-treated (lanes 2–4) or etoposide-treated (lanes 5–7) rat thymocytes were incubated in presence of 10 μM VADcmk (lanes 3 and 6) or 10 μM AAPFcmk (lanes 4 and 7) for 1 h at 37°C and examined for ICE-like activity by incubation of nondenaturing gels with membranes impregnated with YVADafc. afc was liberated by protease activity, when membranes were irradiated with long-wavelength UV light.

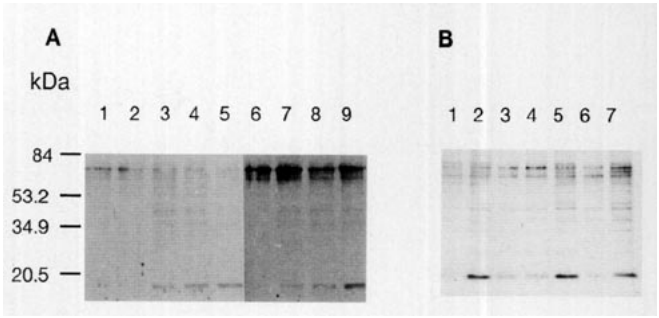

FIG. 5. Effect of protease inhibitors on lamin breakdown in rat thymocytes treated with methylprednisolone or etoposide. (A) Untreated (lane 1) and MPS-treated (lanes 2–5) or etoposide-treated (lanes 6–9) rat thymocytes were incubated for 1 (lanes 2 and 6), 2 (lanes 3 and 7), 3 (lanes 4 and 8), and 4 h (lanes 5 and 9), and lamin B content was determined by immunoblotting. (B) Untreated (lane 1) and MPS-treated (lanes 2–4) or etoposide-treated (lanes 5–7) rat thymocytes were incubated in presence of 10 μM VADcmk (lanes 3 and 6) or 10 μM AAPFcmk (lanes 4 and 7) for 4 h at 37°C and examined for lamin breakdown by immunoblotting.

apoptosis, the mechanism(s) of activation, and their subcellular targets, are still poorly understood.

In the present work we used highly selective substrate inhibitors in an attempt to estimate the involvement of ICE-like protease and Ca^{2+}-regulated serine protease activities in apoptosis. Our data show that the inhibitor of ICE-like protease activity, VADcmk, can prevent the morphological and biochemical changes characteristic of apoptosis in rat thymocytes treated with MPS or etoposide. The inhibitor was only effective when added before treatment with the apoptosis-inducing agents. Similar results were obtained with another inhibitor of ICE-like protease activity (Boc-aspartyl-(benzyl)chloromethylketone, BACMK), which was effective at 2.5 μM concentration. This observation is consistent with data published recently [29].

The involvement of ICE-like protease activity in the early phase of thymocyte apoptosis is further supported by our experiments with membranes impregnated with YVADafc, demonstrating that this activity was increased in thymocytes treated with MPS or etoposide. Incubation of cells with VADcmk prevented enzyme activation, whereas the presence in the incubation medium of AAPFcmk did not affect ICE-like protease activity in cells treated with MPS or etoposide. Thus, it appears that VADcmk can protect cells from apoptosis by inhibiting the activation of ICE-like protease(s) which is critical for the propagation of the apoptotic process. The lack of inhibition of ICE-like protease activity in cells treated with AAPFcmk further suggests that CRP was not required for activation of ICE-like protease(s) in treated cells.

Interestingly, the primary structure of ICE bears no sequence homology with any known member of the cysteine (or serine) protease superfamily. The highly restricted cleavage specificity for an aspartic acid residue, with no cleavage at glutamate or asparagine residues, suggests a role in processing rather than in general protein degradation [30]. The substrate specificity of ICE is unique among eukaryotic proteases and is shared with only one other known eukaryotic serine protease, granzyme B [31], although studies have shown that IL-1β precursor is not a substrate for granzyme B [32, 33]. Thus, rather than ICE itself, a group of enzymes resembling ICE may be the effector proteases in apoptosis. For example, Lazebnik *et al.* (34) have reported that cleavage of the nuclear enzyme, poly(ADP-ribose) polymerase, involves a protease resembling ICE. This enzyme was inhibited by

FIG. 3. Confocal microscopy images of propidium iodide-stained rat thymocytes treated with MPS (B–D) or etoposide (E–G) and incubated in presence of 10 μM VADcmk (C, F) or 20 μM AAPFcmk (D, G) at 37°C for 4 h. (A) Untreated control. Confocal microscopy was performed with excitation at 488 nm (argon laser). Scanning speed was set on slow (0.5 image/s) and three images of the same field were averaged. Seven separate images (A–G) are shown depicting chromatin changes in different cells. The arrows are pointing to typical apoptotic cells.

YVADcmk, but it could be distinguished from ICE by its substrate specificity.

Several genes encoding ICE-like cysteine proteases have now been implicated in apoptosis in various organisms and cell types. Thus, overexpression of ced-3, ICE, CPP32, and Nedd-2 proteins can cause apoptosis [11, 12, 35, 36]. Although transfection of the mammalian ICE and nematode ced-3 genes also causes apoptosis in rodent fibroblasts, other members of the ICE family are probably more important than ICE itself for induction of apoptosis by proteolysis of yet unidentified substrates. This assumption is strongly supported by the finding that mice deficient in ICE are defective in production of mature IL-1β; yet ICE-deficient mice proceed normally through development and thymocytes and macrophages from these animals undergo apoptosis normally [37, 38].

Recognized similarities between apoptosis and mitosis, such as chromatin condensation and disassembly of the nuclear lamins, have led to the suggestion that apoptosis is a defective form of mitosis (aberrant mitosis) [1, 39]. Moreover, in some cells activation of *cdc*-kinases, which orchestrate mitosis, is also seen during the early phase of apoptosis [40]. However, in rat thymocytes and in transfected embryonic fibroblasts activation of *cdc2* kinase is not required for apoptosis, indicating that chromatin condensation and degradation of lamins can occur by processes different from those that operate in mitosis [20, 41]. Indeed, it was recently reported that induction of apoptosis by glucocorticoids and by Fas ligation in human T cell lines is associated with activation of PITSLRE protein kinase [42], enzymes which are distantly related to members of the *cdc2* gene family, and which become targets of proteolysis during Fas-mediated apoptosis [42].

The nuclear scaffold may serve as a template for chromatin attachment and as a binding site for enzymes such as topoisomerase II. Recent work has demonstrated that proteolytic cleavage of lamins A/C accomplished by a nuclear scaffold protease activity was stringently regulated by Ca^{2+} [21]. This enzyme was resistant to calpain action and was characterized as a serine protease with a predominantly chymotrypticlike substrate preference. It was shown that AAPF peptide is an effective irreversible inhibitor of CRP when coupled to a chloromethyl ketone moiety [22]. In the present study, AAPFcmk, depending on concentration, prevented lamin degradation as well as other signs of apoptosis in MPS-treated thymocytes. Further, our data suggest that lamin degradation may precede chromatin cleavage in apoptosis; lamin breakdown was apparent 1 h after treatment of cells with either MPS or etoposide (cf. Fig. 5), whereas formation of HMW DNA fragments was seen at 90 min (data not shown). As mentioned above, cells of the cellular immune and hemopoietic systems do not express lamins A/C in contrast to other somatic cells [26]. In these cells, B lamins are sufficient to build the nuclear lamina structure. Kaufmann [43] has described the presence of two additional lamins in the rat, designated as lamins D and E, which run close to lamin B on the gels and share at least one epitope with lamin B. The pattern of lamin fragments observed in our study was different from that described previously in fibroblasts by Oberhammer *et al.* [20]. In our experiments lamin underwent step-wise degradation with formation of 46-, 34-, and 18-kDa fragments. This discrepancy may be explained by the observation that cleavage products of lamins differ between cell types, as evidenced by use of selective antibodies [24].

Preincubation of thymocytes with either VADcmk or AAPFcmk prevented lamin degradation in cells treated with MPS (cf. Fig. 5). VADcmk was also effective in blocking lamin breakdown in thymocytes treated with etoposide. The fact that AAPFcmk failed to prevent this and other steps of the apoptotic process in etoposide-treated cells suggests that CRP activity was not required for perpetuation of apoptosis in this experimental model and that other proteases can also catalyze lamin breakdown in apoptosis. Etoposide, which blocks the religation of chromatin by topoisomerase II, may initiate apoptotic chromatin fragmentation by the activation of this enzyme which would introduce the first nick in the chromatin and drastically change chromatin structure. Since topoisomerase II molecules occur with a periodicity of 300 kbp along the chromatin [44], this mechanism would explain the formation of 300-kbp fragments, and multiples thereof, during the early phase of etoposide-induced apoptosis. This would be in accordance with the observation that increased topoisomerase II activity is found at sites of active transcription, particularly in interphase resting cells. This hypothesis is also strongly supported by the recent finding that treatment of isolated thymocyte nuclei with etoposide results in the accumulation of 300-kbp fragments without any indication of further chromatin degradation or appearance of apoptotic nuclear morphology (S. Nobel, A. Slater, and S. Orrenius, unpublished).

Recent studies have suggested that there exists a "cytoplasmic regulator" of apoptosis and that the apoptotic process can proceed also in the absence of a nucleus when induced by either a protein kinase C inhibitor, staurosporine [7], or by ligation of the Fas/APO-1 cell surface antigen [8]. Our observation that VADcmk blocked apoptosis in both MPS- and etoposide-treated cells when present during the initiation of the process suggests that ICE-like protease(s) may serve this function in thymocytes. However, in contrast to the apoptotic models employed in the studies with cytoplasts it

appears that in MPS- and etoposide-treated thymocytes the apoptotic signal originates in the nucleus since, in both cases, the process is known to be blocked by inhibitors of mRNA and protein synthesis [45, 46]. In the case of etoposide-induced apoptosis, it is an interesting possibility that the formation of the 300-kbp fragments may serve as a signal to initiate apoptosis by the activation of cytoplasmic ICE-like protease(s). In MPS-treated thymocytes, apoptosis was blocked also by the CRP inhibitor AAPFcmk, suggesting that this enzyme is involved in the proteolytic cascade initiated by MPS treatment. CRP activity is strictly Ca^{2+}-regulated [21], and it seems plausible that its activation is the result of the elevation of intracellular free Ca^{2+} concentration which is associated with MPS-induced apoptosis in thymocytes [46]. Since intracellular Ca^{2+}-regulated serine protease activity has been found to be associated with both the nuclear scaffold and the endoplasmic reticulum [21, 47], it seems possible that CRP activity may be involved not only in lamin proteolysis but also in the degradation of cytoskeletal and/or other cytoplasmic proteins during MPS-induced apoptosis. However, further work is required to define the critical protease substrates and to elucidate the interaction between the various proteases in thymocyte apoptosis.

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