FEBS Letters 364 (1995) 134–138 FEBS 15442

Involvement of multiple proteases during Fas-mediated apoptosis in T lymphocytes

Sek C. Chow[a,*], Marianne Weis[a], George E.N. Kass[a,**], Tim H. Holmström[b], John E. Eriksson[c], Sten Orrenius[a]

[a]*Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet, Box 210, S-171 77 Stockholm, Sweden*
[b]*Department of Biology, Åbo Akademi University, BioCity, FIN-20520, Turku, Finland*
[c]*Turku Center for Biotechnology, PB 123, FIN-20521 Turku, Finland*

Received 27 March 1995

Abstract The mechanism of Fas antigen-mediated apoptosis is at present unclear. We show here that the 100,000 × *g* supernatant from cell lysates prepared from anti-Fas-stimulated JURKAT T cells, induces chromatin fragmentation in isolated nuclei with concomitant morphological changes typically seen in apoptosis. The formation of this apoptotic nuclei promoting activity (ANPA) in JURKAT T cells after Fas antigen ligation was blocked by the serine protease inhibitors, TPCK and DCI, and by the interleukin 1-β-converting enzyme inhibitor, VAD-FMK. In addition, chromatin degradation and morphological changes mediated by the ANPA in isolated nuclei were inhibited by TPCK, but not by DCI or VAD-FMK. These results suggest that Fas-mediated apoptosis in T cells involves the activation of a cascade of proteases.

Key words: Fas antigen; T cell; Protease; Apoptosis

1. Introduction

The Fas antigen is a cell surface receptor belonging to the tumour necrosis factor/nerve growth factor super family [1], and is capable of inducing apoptosis in a variety of cell types that express the antigen [2,3]. The Fas receptor is a 48 kDa transmembrane glycoprotein [4], and its cytoplasmic domain contains a region of significant sequence homology with the tumor necrosis factor receptor p55 [1,4]. Analysis of mutant C-terminus Fas antigen suggested the presence of an inhibitory as well as a signal transduction domain in the cytoplasmic domain of the Fas antigen [5]. Molecular cloning and sequencing of the Fas ligand has revealed striking sequence homology to tumour necrosis factor (TNF)α and TNFβ [6] suggesting that TNF and Fas may utilize similar signalling mechanisms in triggering apoptosis.

The importance of the Fas antigen and its ligand in modulating T cells in the immune system is underscored by the abnormalities observed in mice homozygous for the lymphoproliferation (*lpr*) [28] and generalized lymphoproliferative disorder (*gld*) loci [7,8]. Because of the mutation in the Fas receptor and Fas ligand in *lpr* and *gld*, respectively, these mice develop an auto-immune disorder with striking similarities to systemic lupus erythematosus in human [9]. Recent studies have also suggested that the Fas antigen is involved in the generation of certain T cell-mediated cytotoxic reactions which lead to Ca^{2+}-independent T cell-mediated killing [10,11]. In spite of the large number of reports demonstrating that expression of the Fas antigen influences multiple aspects of the immune system, little is known about the signalling pathway or mechanism leading to Fas-mediated apoptosis. Using a cell-free system [12], we show in this report that Fas-mediated apoptosis may involve the activation of multiple proteases acting at different levels during the apoptotic cascade.

2. Materials and methods

2.1. Chemicals

All media and serum were from Gibco (Paisley, UK). Anti-Fas (clone CH-11) was obtained from Kamiya Biomedical Company (Thousand Oaks, USA). *N*-tosyl-L-lysine chloromethyl ketone (TPCK) and 3,4-dichloroisocoumarin (DCI) were purchased from Calbiochem (Nottingham, UK). Z-Val-Ala-Asp-fluoromethyl ketone (VAD-FMK) was from Enzyme System Products (Livermore, USA).

2.2. Cell culture and preparation of 100,000 × g supernatants

JURKAT T cells (ATCC, Maryland, U.S.A.) were maintained in suspension culture using RPMI 1640 medium supplemented with 10% heat-inactivated FCS. The cells (30×10^6 cells/ml) were stimulated with 20 ng/ml anti-human Fas or left untreated for 60 min at 37°C in RPMI 1640 (10% FCS). The cells were washed twice with ice-cold RPMI 1640 (without FCS) 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 three cycles of freezing and thawing, the cell lysates were centrifuged for 30 min at 20,000 × *g* and the pellets discarded. The cell lysates were centrifuged for a further 30 min at 100,000 × *g* and the supernatants retained. The protein concentration in the supernatants was determined using the method of Lowry et al. [13] with BSA as standard.

2.3. Reconstituted in vitro system

The cell-free system was essentially that described by Lazebnik et al. [12] with the exception that DTT was omited from the assays. Rat thymocyte nuclei were prepared as described previously [14] and was suspended in buffer B (5 mM $MgCl_2$, 2.1 M sucrose, 50 mM Tris, pH 7.5) at a final concentration of $4–5 \times 10^8$ nuclei/ml before used. The reaction mixture containing 10 μg of the supernatant protein and 5×10^6 nuclei were diluted to a final volume of 30 μl with buffer A (see above), supplemented with an ATP-regeneration system [12]. Samples were incubated at 37°C for the time periods indicated before analysis.

*Corresponding author. *Present address:* Centre for Mechanisms of Human Toxicity, University of Leicester, Lancaster Road, Leicester LE1 9HN, UK. Fax: (44) (116) 2525616.

**Present address:* School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH, UK

Abbreviations: TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; DCI, 3,4-dichloroisocoumarin; HMW, high molecular weight; FIGE, field inversion gel electrophoresis; ICE, interleukin 1-β-converting enzyme; VAD-FMK, Z-Val-Ala-Asp-floromethyl ketone; ANPA, apoptotic nuclei promoting activity.

SSDI 0014-5793(95)00370-3

2.4. Morphological and DNA analysis

For chromatin staining, nuclei were incubated with propidium iodide (50 μg/ml) on ice for 10 min before confocal microscopy analysis. To examine the nuclear ultrastructure, nuclei were suspended in 0.5% low melting agarose at 50°C and immediately pipetted into plug moulds. The agarose plugs were then fixed with 2.2% glutaraldehyde in PBS and processed for electron microscopy as previously described [15]. Chromatin degradation were analysed as previously reported [16,17] using conventional and field inversion gel electrophoresis (FIGE).

3. Results and discussion

To further understand the mechanisms of Fas-mediated apoptosis, we have prepared the 100,000 × *g* supernatant from cell lysates derived from anti-Fas stimulated human JURKAT T cells. When incubated with isolated rat thymocyte nuclei in a modified in vitro system [12], the supernatant induced chromatin cleavage into high molecular weight (HMW) DNA fragments ranging from > 700 to < 50 kbp (Fig. 1a), followed by the formation of oligonucleosomal length DNA fragments (Fig. 1b). These results demonstrated the precedence of HMW DNA cleavage during chromatin degradation, which is in line with recent reports on various cell types undergoing apoptosis [18–20], including JURKAT cells (Weis et al, manuscript submitted) [28]. Examination of the supernatant-treated nuclei using confocal and electron microscopy showed morphological features typical of apoptosis [21,22], i.e. chromatin abutting towards the nuclear membrane (45 min) and subsequently fragmenting into DNA-containing vesicles (60 min) (Fig. 2a and 2b). This in vitro system shares many features with JURKAT cells undergoing apoptosis after anti-Fas treatment, including (i) formation of HMW DNA fragments, (ii) generation of oligonucleosomal length DNA fragments and (iii) fragmentation of nuclei into DNA-containing vesicles (Weis et al, manuscript submitted). The apoptotic nuclei promoting activity (ANPA) was present only in the supernatant prepared from anti-Fas-treated JURKAT cells, control cell supernatant did not have this activity (Fig. 2 and Fig. 4).

The ANPA was abolished after incubating the supernatant from anti-Fas-treated JURKAT cells for 30 min at 60°C, and was inactivated by trypsin digestion and acidic pH treatment suggesting that proteins are involved. Neither the morphological changes nor the chromatin cleavage in isolated nuclei after ANPA treatment required the presence of Ca^{2+}, which is in line with several reports showing that Fas-mediated apoptosis in

Table 1
Characterization of ANPA

Treatment	Activity
Control	Yes
After overnight at room temperature	Yes
Heat inactivation 60°C, 30 min	No
Trypsin digestion*	No
0.4 M HCl for 5 min	No

Supernatant from cell lysates derived from Fas-treated JURKAT cells was subjected to the indicated treatment before incubated with isolated thymocyte nuclei. The treated nuclei were then examined for ANPA activity morphologically using confocal microscopy and chromatin cleavage into oligonucleosomal length DNA fragments by agarose gel as described in section 2.

*The ANPA containing supernatant was incubated with 60 μg/ml trypsin for 60 min at 37 °C, followed by the addition of 156 μg/ml soybean trypsin inhibitor. Parallel incubation in the absence of trypsin had no effect on ANPA-induced chromatin degradation in isolated nuclei.

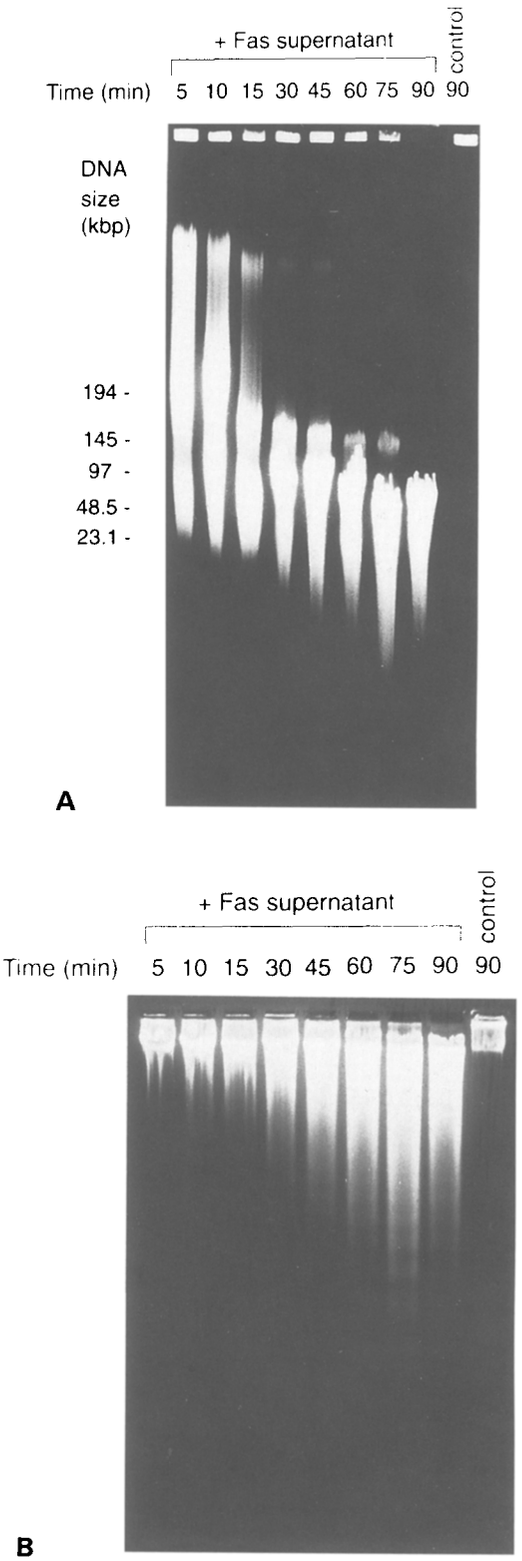


Fig. 1. Time course of the induction of chromatin degradation in isolated rat thymocyte nuclei by the supernatant (Fas supernatant) prepared from cell lysate derived from anti-Fas stimulated JURKAT cells. (A) HMW DNA fragments. (B) Oligonucleosomal length DNA fragments. Isolated nuclei (5×10^6) were incubated with the supernatant at 37°C as described in section 2.

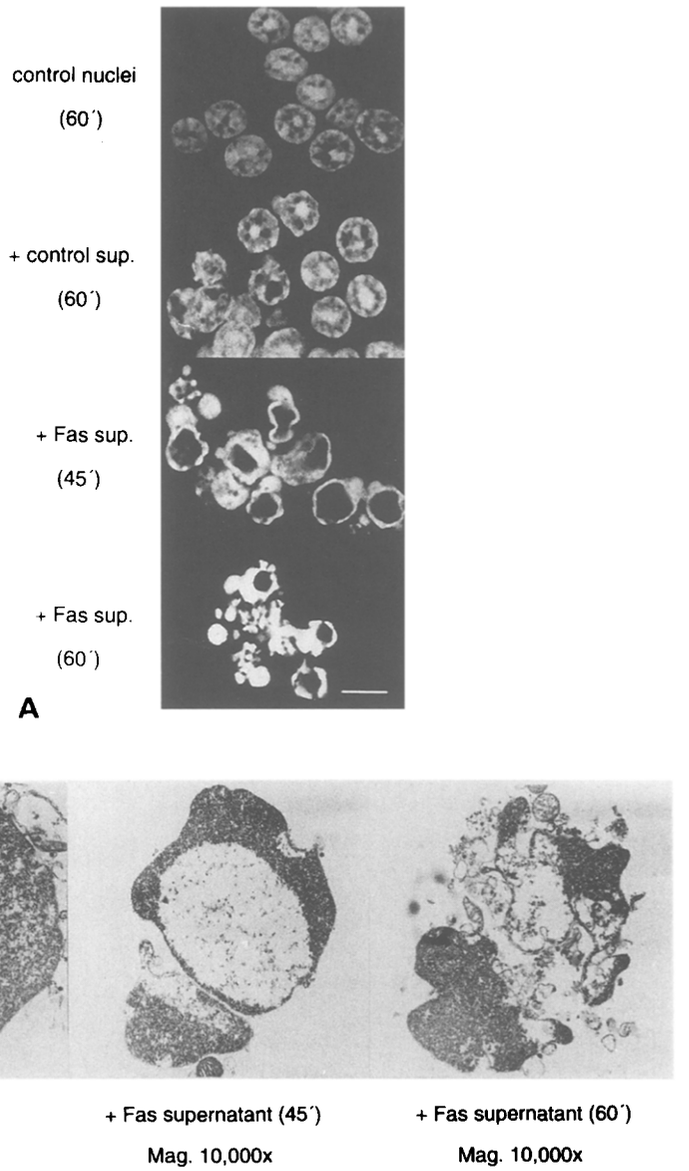
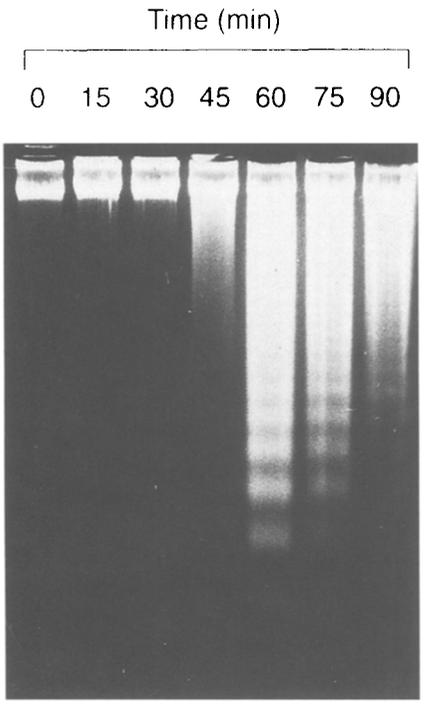


Fig. 2. Morphological changes in isolated thymocyte nuclei resulting from incubation with the supernatant (Fas supernatant) from anti-Fas treated JURKAT cells. (A) Propidium iodide staining of nuclear chromatin in nuclei exposed to ANPA. Bar represents 5 μm. (B) Ultrastructural changes in nuclei after incubation with the supernatant containing ANPA. Isolated thymocyte nuclei (5×10^6) were incubated with the supernatant at 37°C, and at the time points indicated the reactions were terminated on ice and samples prepared for morphological examinations as described in section 2.

various cell types was Ca^{2+} independent [10,11]. However, the removal of Mg^{2+} from the cell-free system or the addition of Zn^{2+} or aurintricarboxylic acid, effectively prevented the ANPA-induced chromatin cleavage and morphological changes in isolated nuclei (results not shown). This presumably was due to inactivation of constitutive endonuclease(s) required for chromatin degradation in the isolated nuclei.

The ANPA was detected in the cell lysate prepared from JURKAT cells after 45 min of anti-Fas stimulation and maximal activity was observed within 60 min (Fig. 3). The supernatant containing ANPA was unable to digest λ and plasmid DNA (not shown), thus, excluding the involvement of nuclease activity. It had no effect on intact cells (rat thymocytes and human JURKAT cells), but readily induced DNA fragmentation in nuclei isolated from both JURKAT cells and rat liver (results not shown). This finding suggests the existence of a common target and initiation process leading to nuclear chromatin cleavage in cells undergoing Fas-mediated apoptosis.

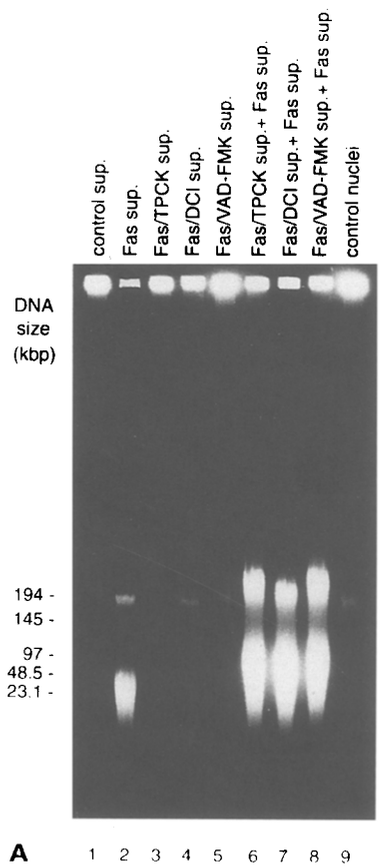
Because proteases have been implicated in apoptosis in various cell types [19,20,23] and recently shown to directly promote chromatin degradation in isolated nuclei [24,25], the possibility that the formation of ANPA, as well as its manifestations, involved proteolysis was examined. Indeed, our recent studies (Weis et al, manuscript submitted) showed that TPCK and DCI [26] prevented Fas-mediated apoptosis in JURKAT cells, suggesting that proteolysis may play an important role in this model system. First, to determine whether the formation of ANPA involves proteolysis, JURKAT cells were pre-treated with TPCK, DCI or the interleukin 1-β-converting enzyme (ICE) inhibitor, VAD-FMK [27] for 10 min prior to anti-Fas stimulation. All three protease inhibitors were found to efficiently blocked chromatin cleavage into HMW and oligonucle-

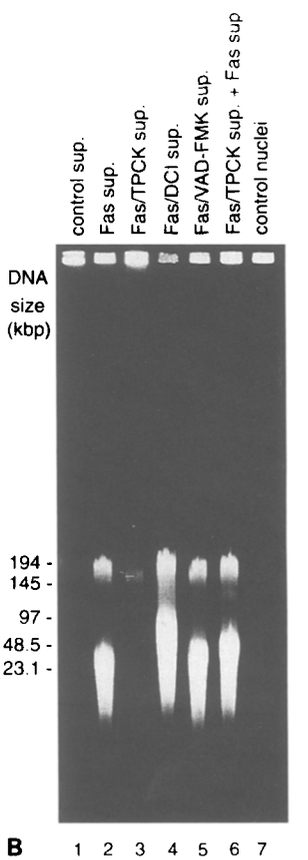


←
Fig. 3. Time-dependent increase in ANPA in JURKAT cells after anti-Fas stimulation. JURKAT cells (30×10^6/ml) were treated with anti-Fas (20 ng/ml) at 37°C. At the time points indicated, an aliquot of 1 ml cell suspension was taken and supernatant prepared as outlined in section 2. Equivalent amounts of protein from each supernatant were incubated with isolated nuclei for 60 min at 37°C, and the formation of oligonucleosomal length DNA fragments analyzed using agarose gel electrophoresis.

osomal length DNA fragments in anti-Fas-treated JURKAT cells (result not shown). Thus, Fas-mediated apoptosis in T cells appears to involve both serine, and ICE-like protease activities. Supernatants prepared from these cells (non-apoptotic) did not induce chromatin cleavage into HMW DNA fragments (Fig. 4a) or oligonucleosomal length DNA fragments (not shown) when incubated with thymocyte nuclei. However, the addition of supernatant containing ANPA to these assays induced chromatin cleavage, indicating that the potential carry over of protease inhibitors in the supernatants from treated cells, which might have inhibited endogenous proteases involved in chromatin degradation in isolated nuclei [19,20], was negligible (Fig. 4a). It can be concluded from these findings that the generation of ANPA involves proteolytic activity.

To examine the possibility that the degradation of chromatin in isolated nuclei induced by ANPA involves proteolysis, the following approach was undertaken. JURKAT cells were first stimulated for 60 min with anti-Fas to generate maximal level





of ANPA (Fig. 3), before TPCK, DCI or VAD-FMK were added to the cells which were incubated for a further 15 min. This enabled us to discriminate between the effects of the protease inhibitors on the ANPA per se from those on its release mechanisms. As expected, JURKAT cells stimulated with anti-Fas for 60 min prior to treatment with protease inhibitors were undergoing apoptosis with their chromatin being cleaved into HMW and oligonucleosomal length DNA fragments (results not shown). Supernatants were prepared from these cells and their ANPA examined. The supernatant from JURKAT cells treated with anti-Fas for 60 min prior to addition of TPCK, was unable to induce chromatin cleavage into HMW DNA fragments (Fig. 4b) or oligonucleosomal length DNA fragments (not shown) when incubated with isolated nuclei, and chromatin degradation occurred only when supernatant containing ANPA was included in the assay (Fig. 4b). In contrast, supernatants from cells stimulated with anti-Fas prior to addition of DCI or VAD-FMK, readily induced chromatin degradation in isolated nuclei, suggesting that these two inhibitors had no effect on the ANPA per se (Fig. 4b).

Taken together, our results suggest that the formation of ANPA in JURKAT cells after ligation of the Fas antigen involves the activation of both serine and ICE-like proteases. In addition, the manifestation of nuclear chromatin cleavage and apoptotic morphology by the ANPA appears to involve proteolysis. Since VAD-FMK had no effect on the ANPA itself, the possible proteinase activity associated with the ANPA is likely to be different from the ICE-like protease activity in mitotic extracts [25] reported earlier. In summary, our studies suggest that multiple proteases are activated at different levels during an apoptotic cascade in T cells after Fas antigen stimulation.

Acknowledgements: This work was funded by grants from the Swedish Medical Research Council to S.O., Swedish Work Environmental Fund to S.C.C. and S.O., and a fellowship from the Swedish Medical Research Council to G.E.N.K..We thank I. Peters and A. Gahm for technical assistance.

References

[1] Itoh, N., Yonehara, S., Ishii, A., Yonehara, M., Mizushima, S., Sameshima, M., Hase, A., Seto, Y. and Nagata, S. (1991) Cell 66, 233–243.
[2] Yonehara, S., Ishii, A. and Yonehara, M. (1989) J. Exp. Med. 169, 1747–1756.
[3] Trauth, B.C., Klas, C., Peters, A.M., Matzku, S., Möller, P., Falk, W., Debatim, K.-M. and Krammer, P.H. (1989) Science 245, 301–305.
[4] Oehm, A., Behrmann, I., Falk, W., Pawlita, M., Maier, G., Klaus, C., Li-Weber, M., Richard, S., Dhein, J., Trauth, B.C., Ponsting, H. and Krammer, P.H. (1992) J. Biol. Chem. 267, 10709–10715.
[5] Itoh, N. and Nagata, S. (1993) J. Biol. Chem. 268, 10932–10937.
[6] Suda, T., Takahashi, T., Golstein, P. and Nagata, S. (1993) Cell 75, 1169–1178.
[7] Watanabe-Fukunaga, R., Brannan, C.I., Copeland, N.G., Jenkins, N.A. and Nagata, S. (1992) Nature 356, 314–317.
[8] Takahashi, T., Tanaka, M., Brannan, C.I., Jenkins, N.A., Copeland, N.G., Suda, T. and Nagata, S. (1994) Cell 77, 969–976.
[9] Cohen, P.L. and Eisenberg, R.A. (1992) Immunol. Today 13, 427–428.
[10] Rovier, E., Luciani, M.F. and Golstein, P. (1993) J. Exp. Med. 177, 195–200.
[11] Kagi, D., Vignaux, F., Ledermann, B., Burki, K., Depraetere, V., Nagata, S., Hengartner, H. and Golstein, P. (1994) Science 265, 528–530.
[12] Lazebnik, Y.A., Cole, S., Cooke, C.A., Nelson, G.W. and Earnshaw, W.C. (1993) J. Cell Biol. 123, 7–22.
[13] Lowry, C.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[14] Alnemri, E.S. and Litwack, G.J. (1990) J. Biol. Chem. 265, 17323–17333.
[15] Eriksson, J.E., Paatero, G.I.L., Meriluoto, J.A.O., Codd, G.A., Kass, G.E.N., Nicotera, P. and Orrenius, S. (1989) Exp. Cell. Res. 185, 86–100.
[16] Sorenson, C.M., Barry, M.A. and Eastman, A.J. (1990) J. Natl. Cancer Inst. 82, 749–755.
[17] Brown, D.G., Sun, X.-M. and Cohen, G.M. (1993) J. Biol. Chem. 268, 3037–3039.
[18] Oberhammer, F., Wilson, J.W., Dive, C., Morris, I.D., Hickamnn, J.A., Wakeling, A.E., Walker, P.R. and Sikorska, M. (1993) EMBO J. 12, 3679–3684.
[19] Weaver, V.M., Lach, B., Walker, P.R. and Sikorska, M. (1993) Biochem. Cell. Biol. 71, 488–500.
[20] Walker, P.R., Weaver, V.M., Boleslaw, L., LeBlanc, J. and Sikorska, M. (1994) Exp. Cell. Res. 213, 100–106.
[21] Wyllie, A.H., Kerr, J.F. and Currie, A.R. (1980) Int. Rev. Cytol. 68, 251–305.
[22] Arends, M.J., Morris, R.G. and Wyllie, A.H. (1990) Am. J. Pathol. 136, 593–608.
[23] Gadliardini, V., Fernandez, P.-A., Lee, R.K.K., Drexler, H.C.A., Rotello, R.J., Fishman, M.C. and Yuan, J. (1994) Science 263, 826–828.
[24] Wright, S.C., Wei, Q.S., Zhong, J., Zheng, H., Kinder, D.H. and Larrick, J.W. (1994) J. Exp. Med. 180, 2113–2123.
[25] Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.C. (1994) Nature 371, 346–347.
[26] Harper, J.W., Hemmi, K. and Powers, J.C. (1985) Biochemistry 24, 1831–1841.
[27] Dolle, R.E., Hoyer, D., Prasad, C.V.C., Schmidt, S.J., Helaszek, C.T., Miller, R.E. and Ator, M.A. (1994) J. Med. Chem. 37, 563–564.
[28] Weis, M., Kass, G.E.N., Holmström, T.H., Eriksson, J., Orrenius, S. and Chow, S.C., Cellular events in Fas/APO-1-mediated apoptosis in JURKAT T lymphocytes (manuscript submitted).

←
Fig. 4. Involvement of proteolysis in the formation of the ANPA and its manifestation in isolated thymocyte nuclei. (A) Effect of protease inhibitors on the formation of ANPA in anti-Fas-treated JURKAT cells. Supernatants (sup.) from JURKAT cells pretreated for 10 min with TPCK (100 μM), DCI (100 μM) or VAD-FMK (10 μM), followed by anti-Fas stimulation for 75 min were examined for the presence of ANPA (lanes 3, 4 and 5, respectively) by FIGE analysis of chromatin degradation in nuclei incubated with the supernatants. The same supernatants plus supernatant containing ANPA (Fas sup.) were added to nuclei (lanes 6, 7 and 8). (B) Effect of the protease inhibitors on the ANPA per se. Supernatants from JURKAT cells stimulated with anti- Fas for 75 min, with TPCK, DCI or VAD-FMK added during the last 15 min of incubation, were examined for the ANPA (lanes 3, 4 and 5) by FIGE analysis of treated nuclei. Since no ANPA was detected in the supernatant from cells treated with anti-Fas prior to TPCK (lane 3), supernatant containing ANPA was added to the assay (lane 6).