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Apoptosis by a cytosolic extract from Fas-activated cells

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Fas is a type I membrane protein and its activation by binding of the Fas ligand or an agonistic anti-Fas antibody induces apoptosis in Fas-bearing cells. In this report we prepared lysates from cells treated with anti-Fas antibody. The lysates induced apoptotic morphological changes in nuclei from normal mouse liver, accompanied by DNA degradation. The apoptosis-inducing activity was quickly generated in cells by anti-Fas antibody and was found in the soluble cytosolic fraction. Induction of the activity in cells was inhibited by a tetrapeptide, acetyl-Tyr-Val-Ala-Asp-chloromethylketone, a specific inhibitor of interleukin-1β converting enzyme. Addition of COS cell lysates containing Bcl-2 to the assay significantly inhibited the apoptotic process, indicating that the *in vitro* process reflected apoptosis that occurs in intact cells.

Keywords: apoptosis/Bcl-2/cell-free system/Fas/interleukin-1β converting enzyme

Introduction

Apoptosis is physiological cell death, which is morphologically distinguishable from necrosis (Wyllie *et al.*, 1984; Walker *et al.*, 1988). Apoptosis is accompanied by condensation of nuclei and cytoplasm, loss of microvilli and convolution of plasma membranes, as well as nuclear and cell segmentation. Programmed cell death occurs in most animal tissues to eliminate harmful cells or those produced in excess and it is mediated by apoptosis (Ellis *et al.*, 1991; Raff *et al.*, 1993). During embryological development many cells are destined to die. Immune self-tolerance is established by eliminating self-reactive cells (Cohen *et al.*, 1992). Some immune effector cells kill target cells such as virus-infected or cancerous cells by means of an apoptotic mechanism (Golstein *et al.*, 1991; Podack, 1991). Thus prevention of apoptosis sometimes causes neoplastic transformation (Korsmeyer, 1992).

A biochemical hallmark of apoptosis is extensive degradation of chromosomal DNA into nucleosome units (Compton, 1992). However, the biochemical pathway which leads to activation of nucleases is unknown. Genetic analysis in *Caenorhabditis elegans* has identified various mutants in the apoptotic pathway (Ellis *et al.*, 1991). Loss of function mutations of *ced-3* and *ced-4* prevent apoptosis (Yuan and Horvitz, 1990), whereas that of *ced-9* causes an excess of it (Hengartner *et al.*, 1992), indicating that the *ced-3* and *ced-4* products are positive regulators of apoptosis, whereas the *ced-9* product inhibits the process. Subsequent cloning of *ced-3* and *ced-4* genes has shown that *ced-3* and *ced-9* are homologs of mammalian interleukin-1β converting enzyme (ICE) and Bcl-2 respectively (Yuan *et al.*, 1993; Hengartner and Horvitz, 1994). Overexpression of Bcl-2 or a specific ICE inhibitor prevents apoptosis in mammalian cells (Korsmeyer, 1992; Gagliardini *et al.*, 1994; Reed, 1994; Enari *et al.*, 1995; Tewari and Dixit, 1995), suggesting that the apoptotic mechanism is well conserved between mammals and *C.elegans*. Many Bcl-2-like (Oltvai and Korsmeyer, 1994) and ICE-like proteins (Fernandes-Alnemri *et al.*, 1994; Kumar *et al.*, 1994; Wang *et al.*, 1994) have been identified. However, it is not clear how they regulate apoptosis.

Fas is a type I-membrane protein with an M_r of 45 kDa which belongs to the tumor necrosis factor (TNF)/nerve growth factor (NGF) receptor family (Itoh *et al.*, 1991; Oehm *et al.*, 1992). Fas ligand (FasL) is a member of the TNF family (Suda *et al.*, 1993) and has been identified as a type II membrane protein with an M_r of 40 kDa or as a soluble cytokine of M_r 26 kDa (Tanaka *et al.*, 1995). Binding of FasL or an agonistic anti-Fas antibody to Fas quickly induces apoptosis in Fas-bearing cells and kills them within hours (Itoh *et al.*, 1991; Suda *et al.*, 1993). The Fas/FasL system is involved in activation-induced suicide of T cells (Russell and Wang, 1993; Russell *et al.*, 1993), which seems to reflect down-regulation of the immune system or peripheral clonal deletion (Nagata and Golstein, 1995). In addition, FasL is expressed in activated T cells (Suda *et al.*, 1995; Vignaux *et al.*, 1995) and is an effector of cytotoxic T lymphocyte (CTL)-induced cytotoxicity (Rouvier *et al.*, 1993; Kägi *et al.*, 1994; Lowin *et al.*, 1994).

Fas-mediated cytotoxicity is typical of apoptosis, which is accompanied by morphological changes in cells and DNA degradation (Trauth *et al.*, 1989; Itoh *et al.*, 1991). Morphological changes in cells occur in the presence of inhibitors of protein and RNA synthesis (Yonehara *et al.*, 1989; Itoh *et al.*, 1991) and also in enucleated cells (Schulze-Osthoff *et al.*, 1994b), indicating that no specific gene induction is required for Fas-mediated apoptosis. In this report we prepared crude cell lysates from cells undergoing apoptosis triggered by anti-Fas antibody. The lysates induced morphological changes in nuclei prepared from normal liver. The apoptosis-inducing substance(s) was found in the soluble cytosolic fraction and its activity was significantly inhibited by Bcl-2 protein.

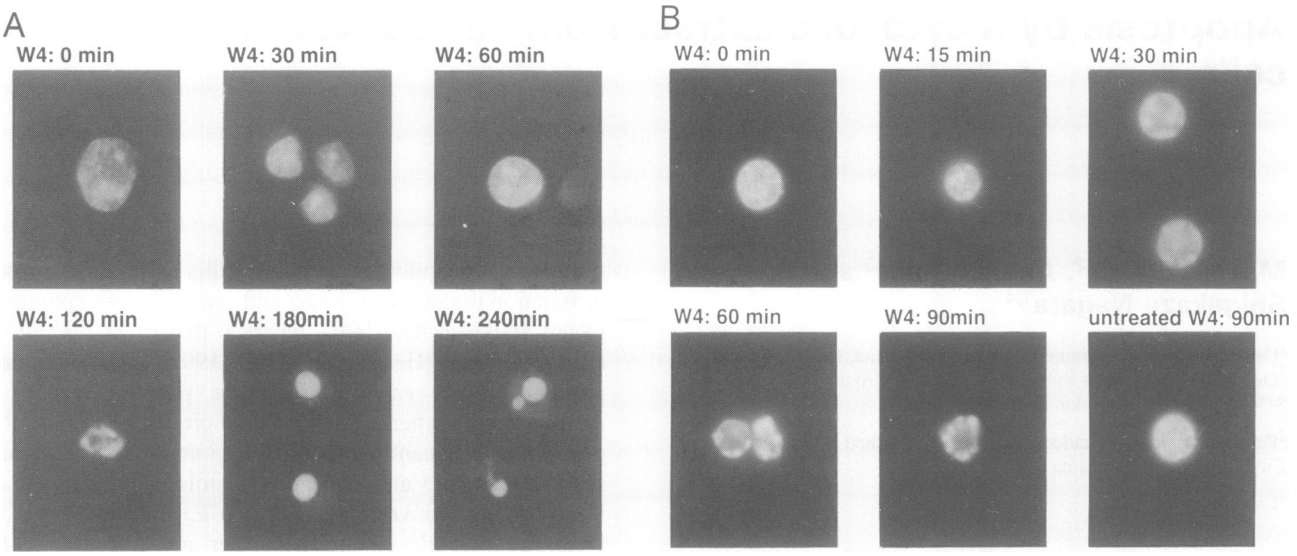

Fig. 1. Time course of morphological changes in nuclei. (**A**) Apoptosis of W4 cells induced with anti-Fas antibody. W4 cells were incubated with anti-Fas antibody and nuclei were stained with DAPI at the indicated time points. (**B**) Morphological changes in nuclei induced by Fas-activated cell lysates. Nuclei from normal mouse liver were incubated with cell lysates from W4 cells which had been exposed for 90 min to anti-Fas antibody. At the indicated times aliquots of nuclei were stained with DAPI. Nuclei were also incubated at 37°C for 90 min with lysate from untreated W4 cells and stained with DAPI.

Results

A cell-free extract from Fas-activated cells causes isolated nuclei to undergo apoptosis

When Fas-expressing WR19L transformant W4 is incubated with anti-Fas antibody, cells undergo apoptosis (Ogasawara *et al.*, 1993). As shown in Figure 1A, after a 120 min incubation with 0.5 μg/ml anti-Fas antibody nuclei became condensed and fragmented. To reconstitute this process in a cell-free system, exponentially growing W4 cells were incubated for 90 min with 0.5 μg/ml anti-Fas antibody and highly concentrated crude cell lysates were prepared as described (Lazebnik *et al.*, 1993). The morphology of normal nuclei from mouse liver incubated with the lysates quickly changed. As shown in Figure 1B, they started to shrink after 15 min at 37°C and chromatin condensed around the nuclear periphery at ~30 min. The chromatin gradually formed condensed masses and finally blebbed off from the nucleus; thus the nuclei had totally disintegrated by 90 min. These morphological changes in the nuclei proceeded in a synchronous fashion and were much quicker than those that occurred in intact cells (Figure 1A). Nuclei from HeLa cells also underwent similar morphological changes with the Fas-activated cell lysates (data not shown). On the other hand, cell lysates prepared from untreated W4 cells did not cause morphological changes in the nuclei, which remained intact after a 90 min incubation with the lysates (Figure 1B).

To confirm that the morphological changes in nuclei induced by Fas-activated cell lysates reflected apoptosis, nuclei were fixed with glutaraldehyde and examined under an transmission electron microscope. After 60 min incubation with the lysates >90% of nuclei had condensed and chromatin was fragmented in the nuclear periphery (Figure 2B). In the later stages chromatin started to bud outwards through the nuclear membrane. This morphological change in the nuclei is a typical feature of apoptosis and was similar to that observed with intact cells incubated with anti-Fas antibody (Figure 2A).

Fas-induced apoptosis in intact cells is accompanied by DNA degradation (Itoh *et al.*, 1991; Schulze-Osthoff *et al.*, 1994a). To examine the ability of Fas-activated cell lysates to cause DNA degradation *in vitro*, nuclei were incubated for various periods with the cell lysates and DNA was analyzed by agarose gel electrophoresis. As shown in Figure 3A, DNA started to degrade at 15 min and most of it was fragmented by 60 min into a ladder of 180 bp units. This DNA fragmentation was dose dependent, in that increasing the lysate concentration caused more DNA fragmentation (Figure 3B). A study of the assay component indicated that neither ATP nor an energy regenerating system was required and that non-hydrolyzable analogs of GTP or ATP did not inhibit the process (data not shown). Furthermore, inhibitors such as $ZnCl_2$ and EGTA, which inhibit apoptosis in other systems (Compton, 1992; Lazebnik *et al.*, 1993; Newmeyer *et al.*, 1994), did not inhibit the morphological changes or DNA degradation induced by Fas-activated cell lysates at concentrations of 2 and 5 mM respectively.

Induction of the apoptosis-inducing activity by activation of Fas

As described above, cells incubated with anti-Fas antibody contained a component(s) which caused apoptosis of nuclei. This inducing activity was quickly generated in cells by anti-Fas antibody. In the experiments shown in Figure 4 W4 cells were incubated for various periods with anti-Fas antibody and nuclei were incubated with each lysate for 1 h. As shown in Figure 4, no apoptosis-inducing activity was found in lysates prepared from cells incubated for 15 min with or without anti-Fas antibody. However, activity started to accumulate in cells after 30 min and became quite concentrated after 90 min.

To characterize the molecule that induced apoptosis, the lysates were incubated for 30 min at 37°C with 100 μg/ml proteinase K or 100 μg/ml RNase A. As shown in Figure 5A, the activity was destroyed by proteinase K,

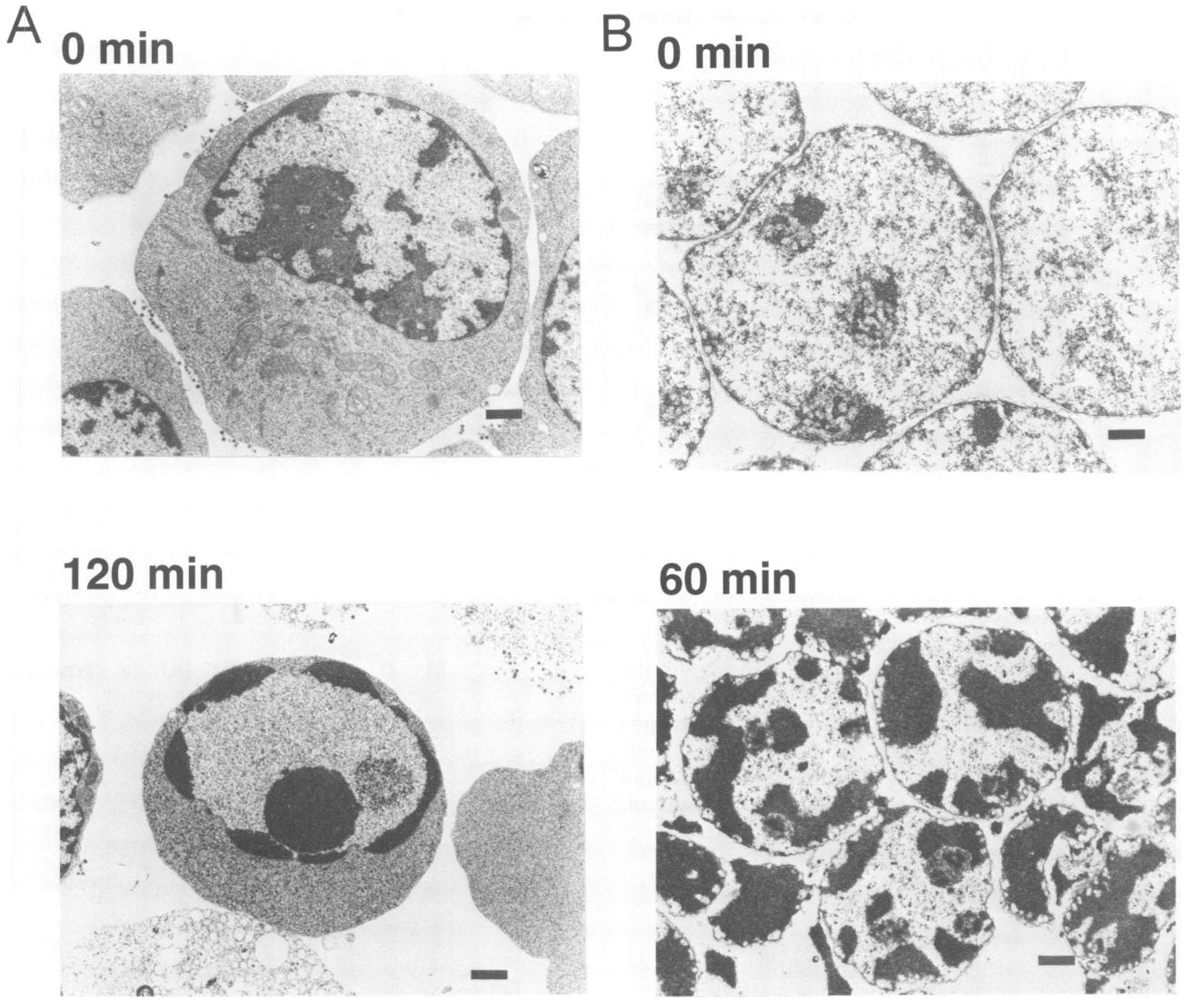

Fig. 2. Ultrastructure of mouse liver nuclei undergoing apoptosis. (**A**) Fas-induced apoptosis in intact cells. W4 cells were incubated at 37°C for 120 min with 0.5 μg/ml anti-Fas antibody and their ultrastructure was examined under a transmission electron microscope. The electron micrograph of untreated W4 cells is also shown in the upper panel. Bars 1 μm. (**B**) Apoptosis of nuclei induced by Fas-activated cell lysates. Mouse liver nuclei were incubated at 37°C for 60 min with Fas-activated cell lysates and examined under a transmission electron microscope. The upper panel shows the electron micrograph of untreated nuclei. Bars 1 μm.

but not by RNase A. Furthermore, the activity was completely inactivated by heating the lysates at 68°C for 10 min. These results suggest that a protein(s) was responsible for inducing DNA degradation. When the lysates were ultracentrifuged at 100 000 *g* for 90 min the supernatant exerted full activity (Figure 5B) and the membrane fraction prepared from the precipitate did not affect apoptosis (data not shown), indicating that the molecule responsible for *in vitro* apoptosis resides in the cytosol fraction.

Effect of an ICE inhibitor and Bcl-2 on apoptosis

We and others have suggested that Fas-induced apoptosis is mediated by ICE or a related enzyme (Enari *et al.*, 1995; Tewari and Dixit, 1995). ICE or ICE-related proteases are inhibited by a tetrapeptide, acetyl-Tyr-Val-Ala-Asp-chloromethylketone (YVAD·CMK), which is a non-cleavable substrate for ICE or ICE-like proteases (Thornberry *et al.*, 1992; Lazebnik *et al.*, 1994). To examine the involvement of ICE in activating the apoptosis-inducing activity, cells were treated with anti-Fas antibody for 30 min in the presence of 100 μM YVAD·CMK. The lysates prepared from these cells induced very little DNA degradation (Figure 6A) and did not cause a morphological change in nuclei (data not shown). We then examined whether the tetrapeptide can inhibit *in vitro* apoptosis induced by Fas-activated cell lysates. As shown in Figure 6B, adding up to 200 μM tetrapeptide to the cell lysates had little effect on DNA degradation induced by the cell lysates.

Bcl-2 overexpression in cells significantly inhibits Fas-mediated apoptosis (Itoh *et al.*, 1993). To examine whether or not Bcl-2 inhibits apoptosis in a cell-free system, we introduced a Bcl-2 expression plasmid into COS cells and prepared cell lysates. Western blotting of the lysates with an anti-Bcl-2 antibody showed that extracts from transfected COS cells contained large amounts of Bcl-2 (Figure 7A). The lysates were then added to the assay

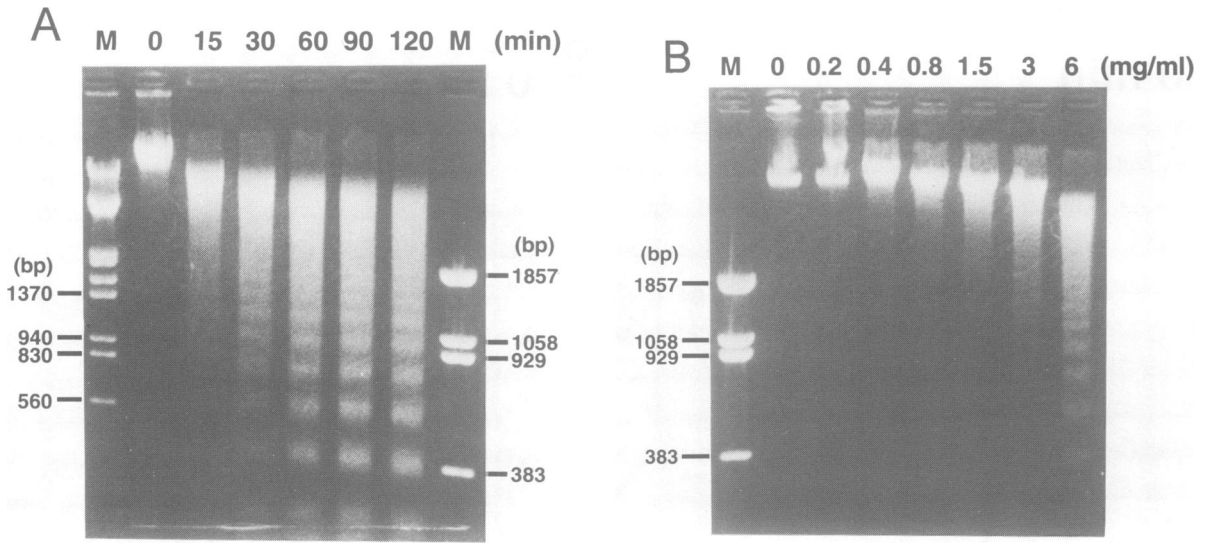

Fig. 3. DNA fragmentation induced by Fas-activated cell lysates. (**A**) Time course of DNA fragmentation. W4 cells were incubated at 37°C for 30 min with 0.5 μg/ml anti-Fas antibody and cell extracts were prepared. Nuclei from mouse normal liver were incubated at 37°C for the indicated periods with the cell extracts (6 mg/ml) and the DNA analyzed by agarose gel electrophoresis. The standard DNA fragments (lanes M) are indicated on the right and left as base pairs (bp). (**B**) Dose-dependent DNA fragmentation. Protein concentrations of the cell lysates from Fas-activated W4 cells were adjusted to the indicated concentrations. Nuclei (2×10^6) from mouse liver were added to the lysates and incubated at 37°C for 30 min and the chromosomal DNA analyzed by agarose gel electrophoresis. The standard DNA fragments (lane M) are indicated on the left in bp.

mixture for *in vitro* apoptosis. As shown in Figure 7B, the extract from untransfected COS cells had some inhibitory effect on DNA fragmentation induced by the Fas-activated cell lysates. On the other hand, the extract of COS cells transfected with the Bcl-2 expression plasmid had a more pronounced inhibitory effect, i.e. the Fas-activated cell lysates caused little DNA degradation in the presence of Bcl-2, even after 60 min (Figure 7B).

Discussion

Although apoptosis is an important physiological process which occurs at various steps during animal development (Ellis *et al.*, 1991), little is known about its molecular mechanism or the components involved in the process. Cell extracts prepared from chicken hepatoma cells sequentially synchronized at the S and M phases or extracts from *Xenopus* eggs induce morphological changes in nuclei and cause DNA fragmentation (Lazebnik *et al.*, 1993; Newmeyer *et al.*, 1994). Although the reaction observed with these extracts looks similar to apoptosis, it is not clear which parts of the apoptotic process are represented by these extracts. In this study we established a cell-free system of apoptosis using extracts from Fas-activated mouse lymphoma. The cell lysates prepared from the Fas-activated, but not from untreated, cells induced apoptotic changes in nuclei and degraded chromosomal DNA in an apoptotic fashion.

The apoptosis-inducing activity rapidly accumulated in the cells after incubation with anti-Fas antibody. Inhibitors of RNA or protein synthesis do not inhibit Fas-mediated apoptosis of cells (Yonehara *et al.*, 1989; Itoh *et al.*, 1991), suggesting that all components needed for the apoptotic process exist as latent forms in living cells. The soluble form of FasL is a trimer (Tanaka *et al.*, 1995) and binding of FasL or an agonistic antibody to Fas probably induces trimerization or oligomerization of Fas (Dhein *et al.*, 1992). The trimerized cytoplasmic region of Fas would

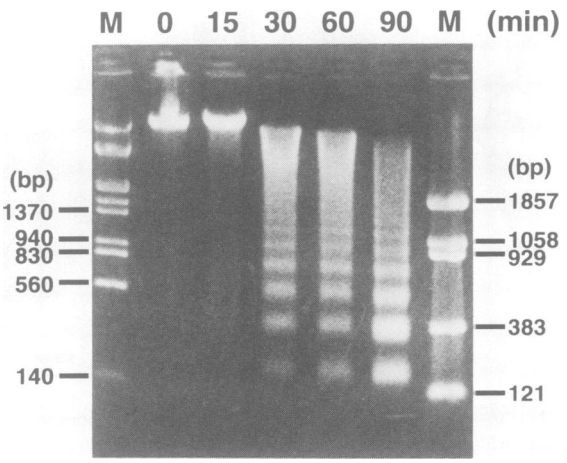

Fig. 4. Induction of apoptosis-inducing activity in cells by anti-Fas antibody. Mouse W4 cells (5×10^7 cells) were incubated with 0.5 μg/ml of anti-Fas antibody at 37°C for the indicated periods. Cell extracts were prepared as described under Materials and methods. Nuclei were added to the extracts (6 mg/ml) and incubated at 37°C for 60 min. Chromosomal DNA was then analyzed by agarose gel electrophoresis. Standard DNA fragments (lanes M) are shown on the right and left in bp.

then trigger the apoptotic process. The process could be a series of sequential reactions. The lysates prepared from cells treated with anti-Fas antibody may contain only the final product of the reaction. This molecule may simply bind to or enter the nucleus, where it induces morphological changes and causes DNA degradation. Since the functions of mitochondria (determined by MTT assay) are also quickly damaged in cells by Fas engagement (Tanaka *et al.*, 1995), it would be useful to examine whether or not molecules in Fas-activated cell lysates can also damage mitochondria. Apoptosis induced by extracts from *Xenopus* eggs requires factors in both the cytoplasmic and membrane fractions and a non-hydrolyzable GTP analog inhibits the process (Newmeyer *et al.*, 1994). In contrast,

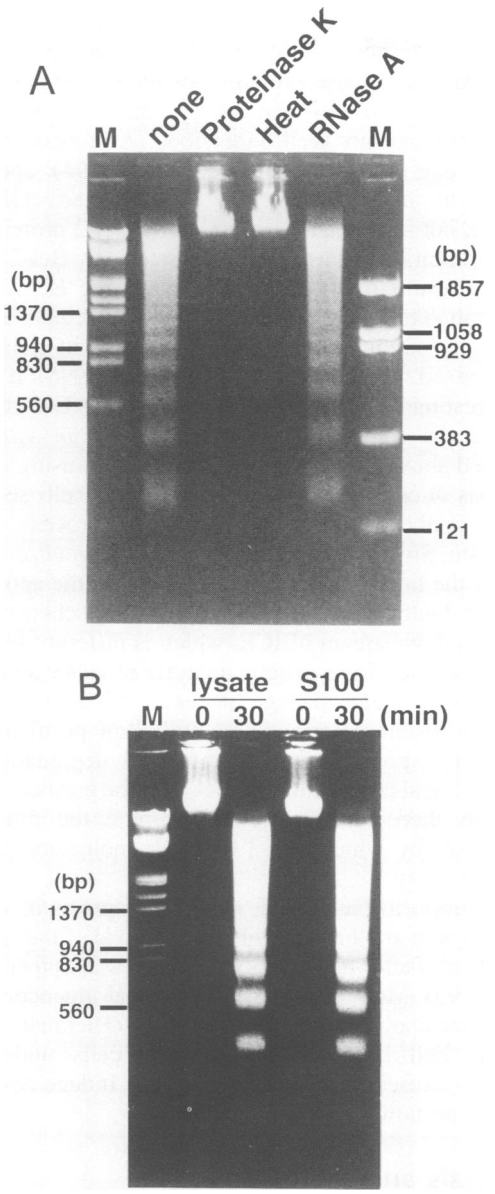

Fig. 5. A protein(s) in the cytosolic fraction is responsible for inducing apoptosis *in vitro*. (**A**) W4 cells were incubated at 37°C for 30 min with anti-Fas antibody and cell lysates were prepared as described under Materials and methods. The lysates (6 mg/ml) were then incubated at 37°C for 90 min with 100 μg/ml proteinase K or RNase A or at 68°C for 10 min (Heat). Nuclei (2×10^6) were added to the treated lysates and incubated at 37°C for 60 min. Chromosomal DNA was extracted and analyzed by agarose gel electrophoresis. The standard DNA fragments (lanes M) are shown on the right and left in bp. (**B**) Lysates from Fas-activated W4 cells were prepared as above, centrifuged at 100 000 *g* for 90 min and then the supernatants were used as an S100 fraction. Nuclei (2×10^6) from mouse liver were incubated at 37°C for 30 min with the crude lysate (6 mg/ml) or S100 fraction (6 mg/ml) and chromosomal DNA was analyzed as above.

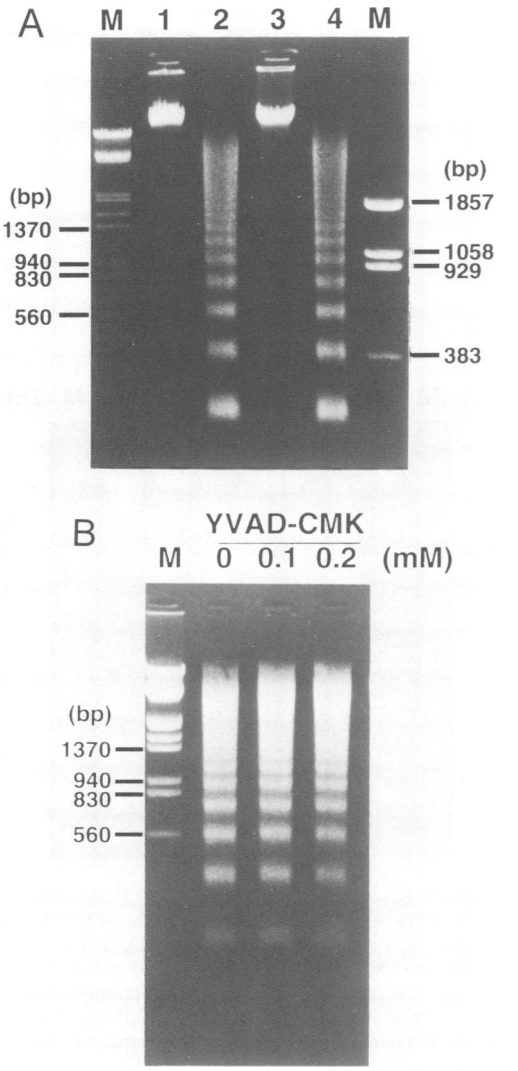

Fig. 6. Effect of YVAD·CMK tetrapeptide on Fas-mediated apoptosis. (**A**) Effect of YVAD·CMK on apoptosis-inducing activity in cells. W4 cells (5×10^7) cells were incubated with 0.5 μg/ml anti-Fas antibody in the presence (lanes 1 and 3) or absence (lanes 2 and 4) of 100 μM YVAD·CMK. Nuclei were incubated with the cell lysates (6 mg/ml) at 37°C for 30 (lanes 1 and 2) or 60 min (lanes 3 and 4) and chromosomal DNA was analyzed by agarose gel electrophoresis. The standard DNA fragments (lanes M) are shown in bp. (**B**) The effect of YVAD·CMK on *in vitro* apoptosis. W4 cells were incubated at 37°C for 30 min with 0.5 μg/ml anti-Fas antibody. The lysates (6 mg/ml) were preincubated in the absence or presence of 100 or 200 μM YVAD·CMK for 30 min at room temperature. Nuclei (2×10^6) were then added to the cell lysates and incubated at 37°C for 15 min. After incubation chromosomal DNA was analyzed by agarose gel electrophoresis.

we found full apoptosis-inducing activity in the cytosolic fraction of Fas-activated cells and a GTP analog did not inhibit the process. It is possible that the apoptotic process was arrested at an intermediate stage in *Xenopus* eggs and that a GTP binding protein and/or proteins in the membrane fraction were required to complete the process. The lysates from Fas-activated cells seemed to contain a factor which has passed this stage.

Fas-mediated apoptosis can be inhibited by ICE inhibitors, such as the viral protein Crm A and the non-cleavable ICE substrate YVAD·CMK (Enari *et al.*, 1995; Tewari and Dixit, 1995). Thymocytes from ICE-deficient mice showed resistance to Fas-mediated apoptosis (Kuida *et al.*, 1995), suggesting that ICE-dependent proteolysis is a key step in the Fas-mediated apoptotic pathway. In this report cell lysates prepared from cells treated with anti-Fas antibody in the presence of 100 μM YVAD·CMK did not show apoptosis-inducing activity *in vitro*. On the other hand, when the tetrapeptide was added to the cell lysates it had little effect on DNA degradation. This result suggests that one step of apoptosis in which ICE or ICE-

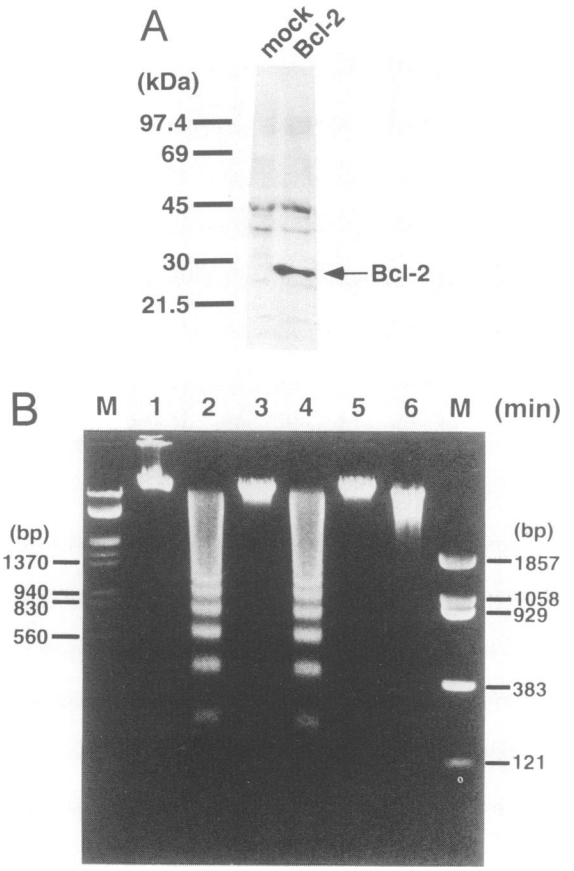

Fig. 7. Effect of Bcl-2 on *in vitro* apoptosis. **(A)** Production of Bcl-2 in COS cells. COS cells were transfected with the pEF-BOS vector (mock) or pEF-BOS carrying human Bcl-2 cDNA (Bcl-2). At 72 h after transfection COS cell lysates were prepared and an aliquot of the lysates (60 μg) was resolved by electrophoresis through a 4–20% gradient polyacrylamide gel in the presence of 0.1% SDS. Proteins were blotted onto nitrocellulose (Millipore) and analyzed by Western blotting using anti-human Bcl-2 antibody (Pezella *et al.*, 1990). The proteins were then detected using the ECL system (Amersham) after staining with horseradish peroxidase-conjugated goat anti-mouse IgG antibody. Molecular weight standards (Rainbow Marker; Amersham) were electrophoresed in parallel. The standard proteins are shown in kDa. **(B)** Effect of Bcl-2 on *in vitro* apoptosis induced by lysates activated by Fas. Lysates were prepared from W4 cells incubated at 37°C for 30 min with 0.5 μg/ml anti-Fas antibody. The lysates (6 mg/ml) were incubated at room temperature for 30 min without (lanes 1 and 2) or with an extract (18 μg) from mock-transfected COS cells (lanes 3 and 4) or with an extract (18 μg) from COS cells transfected with the Bcl-2 expression plasmid (lanes 5 and 6). Nuclei (2×10^6) were then added to the lysates and incubated at 37°C for 0 (lane 1), 30 (lanes 2, 3 and 5) or 60 min (lanes 4 and 6). Fragmentation of chromosomal DNA was examined by agarose gel electrophoresis. The standard DNA fragments are shown in bp.

like proteases are involved occurred in the cells during incubation with anti-Fas antibody. This result is different from that obtained using an extract from chicken hepatoma (Lazebnik *et al.*, 1994), in which the tetrapeptide specifically inhibited the *in vitro* apoptotic process. The extract from the hepatoma was prepared after sequential synchronization at the M and S phases, where cells do not show apoptosis. Apoptosis occurs in this cell line after one more cell cycle (Lazebnik *et al.*, 1993). On the other hand, the lysates in the present experiment were prepared from Fas-activated cells that actively undergo apoptosis.

Bcl-2 is a protein localized in membrane fractions of mitochondria, nuclei and endoplasmic reticulum (Reed, 1994). Many Bcl-2-related molecules and their binding proteins have been identified (Oltvai and Korsmeyer, 1994). Although these proteins positively and negatively regulate apoptosis in various systems, the molecular mechanisms are not well understood. As found with the *Xenopus* egg extract (Newmeyer *et al.*, 1994), apoptosis induced by cell lysates from Fas-activated cells was partially, but significantly, inhibited by Bcl-2 protein. The partial inhibition of Fas-induced apoptosis by Bcl-2 agrees with the results obtained on overexpressing Bcl-2 in the cells (Itoh *et al.*, 1993). The Bcl-2 binding protein BAG-1 completely inhibits Fas-mediated apoptosis in cells when co-expressed with Bcl-2 (Takayama *et al.*, 1995). It would be interesting to examine whether or not BAG-1 can also enhance the inhibitory activity of Bcl-2 *in vitro*. As discussed above, the factor responsible for causing *in vitro* apoptosis in cell lysates from Fas-activated cells seems to be the final product of the apoptotic process in the cytoplasm. Since Bcl-2 inhibited apoptosis *in vitro*, it may do so at the final stage, such as transport of the apoptosis-inducing factor into the nucleus. In this scheme Bcl-2 functions downstream of ICE, which is different from the model proposed from genetic analysis of *ced-9* and *ced-3* mutants in *C.elegans* (Hengartner *et al.*, 1992). The *in vitro* apoptosis system described here will allow purification of the factor responsible for causing DNA degradation and morphological changes in nuclei. Using the purified protein it would then be possible to examine the molecular mechanism by which Bcl-2 or other molecules prevent apoptosis.

In addition to FasL, TNF induces apoptosis in various cells (Beyaert and Fiers, 1994) and removal of the survival factor from factor-dependent cells causes apoptosis (Raff *et al.*, 1993). Many anti-cancer drugs and glucocorticoids also induce apoptosis in various systems (Hickman, 1992). Whether cell lysates prepared from cells undergoing apoptosis caused by these reagents can induce apoptosis *in vitro* can now be investigated.

Materials and methods

Preparation of the cell lysates

The parental WR19L and its transformant expressing mouse Fas were maintained in RPMI1640 medium containing 10% fetal calf serum (FCS). Cells were incubated with the anti-Fas antibody (Jo-2) (Ogasawara *et al.*, 1993) and cell lysates were prepared essentially as described (Lazebnik *et al.*, 1993). In brief, cells were washed with an extraction buffer consisting of 50 mM PIPES, pH 7.4, 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 20 μM cytochalasin B and a mixture of protease inhibitors [1 mM phenylmethylsulfonyl flouride (PMSF), 1 μg/ml leupeptin, 1 μg/ml pepstatin, 5 μg/ml antipain and 1 μg/ml chymopapain] and then collected in a small glass Dounce homogenizer. Cells were disrupted by five cycles of freezing and thawing, which was accompanied by grinding with the pestle during each thawing cycle. Crude extracts were obtained by centrifugation at 10 000 *g* for 12 min at 4°C. Sometimes the cell lysates were further centrifuged at 100 000 *g* for 90 min in a Beckman TLS-55 rotor and the supernatant used as the cytosolic fraction.

Preparation of COS cell lysates containing Bcl-2

The expression plasmid for Bcl-2 has been described previously (Itoh *et al.*, 1993). Monkey COS cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% FCS. COS cells were transfected by electroporation at 230 V with a capacitance of 960 μF as described (Suda *et al.*, 1993). After transfection the cells were cultured for 3 days in DMEM containing 10% FCS and crude cell lysates were prepared as described above, except the homogenization buffer contained 2% CHAPS.

Preparation of nuclei

Nuclei were prepared from mouse liver as described (Newmeyer and Wilson, 1991). In brief, minced mouse liver was homogenized in homogenization buffer (15 mM PIPES–NaOH, pH 7.4, 80 mM KCl, 15 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.5 mM spermidine, 0.2 mM spermine and 1 mM PMSF) containing 250 mM sucrose in a Dounce homogenizer. After filtration through four layers of cheesecloth, an equal volume of homogenization buffer containing 2.3 M sucrose was added and mixed thoroughly. Homogenates were then layered over 5 ml homogenization buffer containing 2.3 M sucrose in a Beckman SW28 centrifuge tube and centrifuged at 22 000 r.p.m. for 90 min at 4°C. The pellet was resuspended in homogenization buffer at a concentration of $1–2\times10^6$ nuclei/μl and stored at –80°C.

Assay of in vitro apoptosis

The reaction mixture contained 10 mM HEPES, pH 7.0, 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT, 2 mM ATP, 10 mM creatine phosphate, 50 μg/ml creatine kinase, various amounts of the crude cell lysates and ~10^6 nuclei in a final volume of 30 μl. The mixture was incubated at 37°C and 6 μl aliquots were stained with 10 μg/ml 4′,6-diamidino-2-phenylindole (DAPI; Sigma) in 200 mM sucrose, 5 mM $MgCl_2$, 15 mM PIPES, pH 7.4, 80 mM KCl, 15 mM NaCl, 5 mM EDTA and 3.7% (v/v) formaldehyde. The nuclei were observed under a fluorescence microscope equipped with filter combination UV-2A (Nikon OPTIPHOT).

To examine DNA degradation, the reaction mixture containing 2×10^6 nuclei was incubated at 37°C for various periods. Nuclei were collected by centrifugation for 5 min at 10 000 r.p.m., then resuspended in 500 μl 100 mM Tris–HCl, pH 8.5, 5 mM EDTA, 0.2 M NaCl, 0.2% SDS, 0.1 mg/ml proteinase K. After an overnight incubation at 37°C, DNA was precipitated by adding an equal volume of isopropanol, dissolved in 20 μl Tris–HCl, pH 8.0, containing 1 mM EDTA and 0.1 mg/ml RNase A and incubated at 37°C for 30 min. The DNA was then analyzed by gel electrophoresis using a 2% agarose gel in the presence of 0.5 μg/ml ethidium bromide.

For electron microscopy 3×10^7 nuclei were incubated with crude cell lysates as above and fixed with 3% glutaraldehyde in phosphate-buffered saline (PBS). Nuclei were washed with PBS overnight, fixed with 1% OsO_4 and stained with 5% uranyl acetate. Nuclei were then dehydrated in graded ethanol and embedded in Glycid ether 100 (Serva). Thin sections were prepared, placed on copper grids and then stained with lead citrate. Transmission electron micrographs were then taken using a JEM-1200ExII (JEOL).

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