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Induction of *in vitro* nuclear apoptosis activity coincides with the production of 50 kDa cytosolic protein

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Abstract

Human monocytic leukemia U937 cells undergo apoptosis when cells are treated with the anticancer drug etoposide. To study the mechanism of drug-induced apoptosis, we used an *in vitro* apoptosis system with cytosol from etoposide-treated U937 cells. The cytosol from apoptotic U937 cells showed activity to induce morphologic changes and oligonucleosomal DNA fragmentation in isolated nuclei *in vitro*; both are typical features of apoptosis. We generated monoclonal antibodies to the proteins in the etoposide-treated U937 cytosol. We found that a 50 kDa protein, recognized by SN-1 monoclonal antibody, appeared in the cytosol of U937 cells, in accordance with its cell-free apoptosis activity. Z-Asp, an inhibitor of interleukin-1β converting enzyme (ICE) family proteases, inhibited the appearance of the 50 kDa protein and the emergence of the cell-free apoptosis activity in the etoposide-treated U937 cytosol. These results indicate that the 50 kDa protein is produced by the activation of ICE family protease during apoptosis and suggest some roles of the protein in the development of apoptosis.

Keywords: cell-free apoptosis; cytosol; monoclonal antibody; U937

Abbreviations: ICE, interleukin-1β converting enzyme; Z-Asp, benzyloxycarbonyl-Asp-CH_2OC(O)-2,6-dichlorobenzene; DAPI, 4′6-diamidino-2-phenylindole

Introduction

Apoptosis is a program of cellular suicide, and it has important roles in a variety of biological processes (Cohen *et al*, 1992; Duvall and Wyllie, 1986; Fisher, 1994; Thompson, 1995). Apoptosis is initiated by a number of physiological situations, such as stimulation by their ligands of death receptors, Fas and type I TNF receptor (Leist *et al*, 1995; Noguchi *et al*, 1996; Suda *et al*, 1993). In addition to physiologic stimuli, anticancer drugs can also induce apoptosis in cancer cells (Chen *et al*, 1996; Collins and Lopez Rivas, 1993; Fisher, 1994; Kaufmann, 1989; Mashima *et al*, 1995b), indicating that apoptosis plays a significant role in cancer chemotherapy.

During the development of apoptosis, cells show morphological and biochemical changes characteristic to apoptosis (Cohen *et al*, 1992; Duvall and Wyllie, 1986; Earnshaw, 1995; Fisher, 1994; Naito *et al*, 1997; Thompson, 1995; Zamzani *et al*, 1996). These include chromatin condensation, nuclear fragmentation, internucleosomal cleavage of DNA, decrease in the mitochondrial membrane potential, cell shrinkage, plasma membrane blebbing and phosphatidylserine externalization. A number of molecules have been identified to be involved in the mechanism of apoptosis. For example, interleukin-1β converting enzyme (ICE) family proteases play an important role in apoptosis development (Kroemer *et al*, 1995; Kumar, 1995; Nicholson *et al*, 1995; Steller, 1995). The activated ICE family proteases cleave several cellular proteins, such as PARP (Gu *et al*, 1995; Tewari *et al*, 1995), U1-70kD (Tewari *et al*, 1995), lamin B (Lazebnik *et al*, 1995), DNA-PK (Han *et al*, 1996), RB (An and Dou, 1996), actin (Chen *et al*, 1996; Mashima *et al*, 1995a) and PKCδ (Emoto *et al*, 1995). However, the role of the cleavage of such proteins during apoptosis has not been fully elucidated.

Bcl-2 family proteins also play a role in the control mechanism of apoptosis (Reed *et al*, 1996; Vaux, 1993). Anti-apoptotic Bcl-2 family proteins, such as Bcl-2 (Shimizu *et al*, 1995) and Bcl-XL (Boise *et al*, 1993), inhibit apoptosis initiated by a variety of stimuli. Another Bcl-2 family member, Bax (Zha *et al*, 1996) and Bad (Yang *et al*, 1995), counteract the anti-apoptotic Bcl-2 family proteins. The biochemical role of Bcl-2 protein and its relatives, however, remains unclear.

To study the molecular mechanism of apoptosis, *in vitro* apoptosis systems (alternatively, cell-free apoptosis systems) were developed in several laboratories (Enari *et al*, 1996; Lazebnik *et al*, 1993; Liu *et al*, 1996; Martin *et al*, 1995; Newmeyer *et al*, 1994; Schlegel *et al*, 1995). These studies demonstrated the sequential activation of ICE family proteases and the possible role of cytochrome c in the activation of CPP32. In this study, we developed an *in vitro* apoptosis system with the cytosolic fraction prepared from etoposide-treated U937 cells. We found that a 50 kDa protein appeared in the cytosol that could induce nuclear apoptosis *in vitro*.

Results

Nuclear apoptosis induced by apoptotic U937 cytosol in a cell-free system

U937 cells underwent apoptosis when cells were treated with etoposide for 3 h. Figure 1a shows the development of

apoptosis on flow-cytometric analysis. The population of cells containing sub-G1 DNA increased drastically after 3 and 4 h of etoposide treatment, indicating the development of apoptosis. Consistent with the flow-cytometric analysis, DNA degradation into the oligonucleosomal ladder was found in U937 cells treated with etoposide for 3 and 4 h (Figure 1b).

To analyze the apoptosis mechanism *in vitro*, we prepared a cytosolic fraction from etoposide-treated U937 cells. Intact nuclei were isolated from untreated U937 cells. The nuclear DNA degraded extensively to oligonucleosome-size fragments when the nuclei were incubated in the cytosol prepared from U937 cells treated with etoposide for 3 and 4 h (Figure 1c). The nuclear DNA was not degraded by the cytosol from non-apoptotic U937 cells treated with etoposide for 2 h or less (Figure 1c).

We previously developed an apoptosis-resistant variant, UK711, from U937 cells (Kataoka *et al*, 1994). Etoposide could not induce apoptosis in the UK711 cells, but TNFα and anti-Fas antibody could (Kataoka *et al*, 1994; Naito *et al*, 1997). Figure 2 shows that the cytosols from etoposide- and TNFα-treated apoptotic U937 cells and from TNFα-treated apoptotic UK711 cells showed the activity to degrade nuclear DNA *in vitro*, whereas the cytosol from etoposide-treated non-apoptotic UK711 cells did not. These results indicate that the cytosol from apoptotic cells contains the activity to degrade nuclear DNA into oligonucleosomal ladder *in vitro*, while the cytosol from non-apoptotic cells did not show the activity.

We next examined the morphologic change of the nuclei incubated in the etoposide-treated apoptotic cytosol *in vitro*. Control nuclei showed homogenously stained chromatin, while the nuclei incubated in the apoptotic cytosol showed condensed chromatin around the shrunken nuclear periphery (Figure 3). This morphologic change in the nuclei is a typical feature of apoptosis. All of the above observations indicate that the cytosol from apoptotic U937 cells can induce nuclear apoptosis *in vitro*.

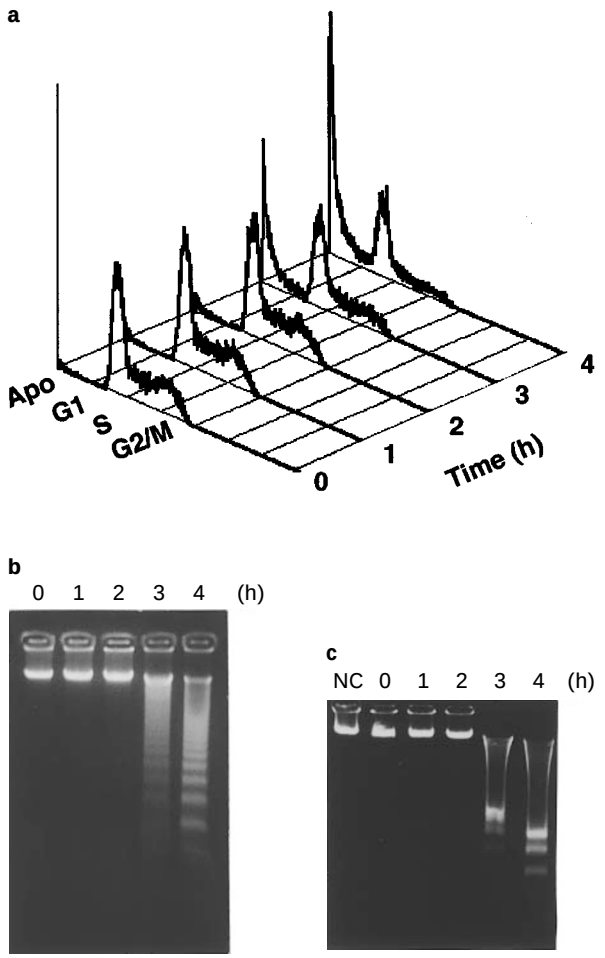

Figure 1 Development of apoptosis and emergence of cell-free apoptosis activity in U937 cytosol. (**a, b**) U937 cells were treated with 10 μg/ml of etoposide for the indicated times, and the development of apoptosis was examined by (**a**) flow-cytometric analysis or (**b**) DNA fragmentation analysis. (**c**) Cytosols were prepared from the U937 cells treated with etoposide for the indicated times, and incubated with isolated U937 nuclei for 1 h. DNA fragmentation was examined as described in Materials and Methods. NC: no cytosol

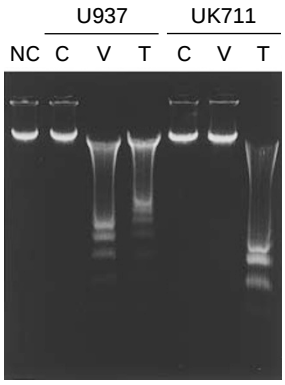

Figure 2 Cell-free apoptosis activity in the cytosol from apoptosis-resistant variant cells. U937 cells and their variant, UK711, showing resistance to etoposide-induced apoptosis were treated with (C) or with 10 μg/ml etoposide for 4 h (V) and 1 ng/ml TNFα in the presence of 1 μg/ml cycloheximide for 2 h (T). Then, cytosol was prepared and incubated with isolated U937 nuclei. DNA fragmentation was examined as described in Materials and Methods. NC: no cytosol

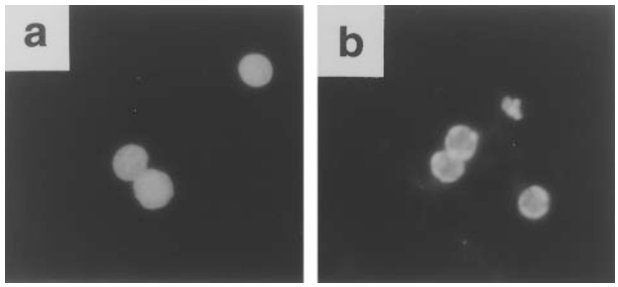

Figure 3 Morphologic changes in the nuclei induced by etoposide-treated U937 cytosol. Nuclei from normal U937 cells were incubated with cytosol from etoposide-treated U937 cells. After 0 (**a**) or 1 h (**b**), the nuclei were stained with DAPI

Appearance of a 50 kDa protein in the apoptotic cytosol

To isolate an active factor responsible for the cell-free apoptosis activity, we fractionated the active cytosol by ultracentrifugation at 125 000 *g* for 3 h. The majority of the cell-free apoptosis activity was recovered in the precipitated fraction (Figure 4a). We tried to purify further the active factor by various chromatographies, but all of our attempts were unsuccessful due to the instability of the active factor (data not shown). Then, by using the precipitated fraction for immunization, we developed monoclonal antibodies that could bind to proteins in the active cytosol in our cell-free apoptosis system. One of the antibodies, named SN-1, specifically recognized a 50 kDa protein in the active cytosol (Figure 4b). The 50 kDa protein was enriched in the precipitated fraction that showed cell-free apoptosis activity (Figure 4a and b). On the other hand, the protein was eliminated from the supernatant fraction that showed minimal activity (Figure 4a and b).

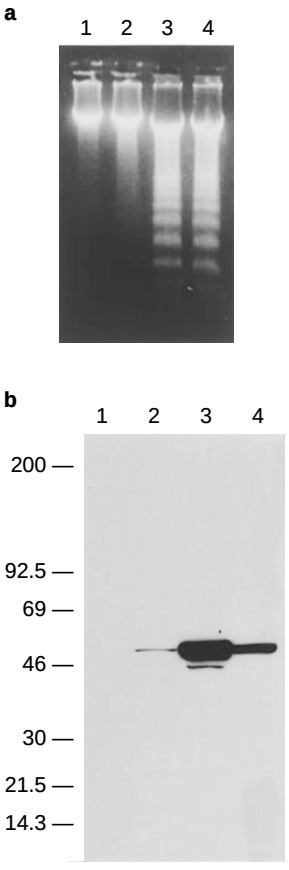

Figure 4 Elimination of the 50 kDa protein and cell-free apoptosis activity from supernatant fraction. Cytosol from etoposide-treated U937 cells was ultracentrifuged for 3 h. The supernatant and precipitated fractions were examined for cell-free apoptosis activity at 4 mg/ml (**a**) and 50 kDa protein presence by Western blot analysis (**b**: 40 μg protein/lane). Lane 1: untreated U937 cytosol; 2: supernatant fraction of the etoposide-treated U937 cytosol; 3: precipitated fraction of the etoposide-treated U937 cytosol: 4: etoposide-treated U937 cytosol

We next examined the time-dependent appearance of the 50 kDa protein in the cytosol prepared from etoposide-treated U937 cells (Figure 5). The cytosol from U937 cells treated with etoposide for 3 and 4 h contained the 50 kDa protein, while the cytosol from the cells treated for 2 h or less did not, showing a good agreement with the emergence of cell-free apoptosis activity in the cytosol (Figure 1c).

The 50 kDa protein band also appeared in U937 cytosol when cells were induced to undergo apoptosis by etoposide, CPT-11, TNFα and staurosporine (Figure 6). In addition, the 50 kDa protein band appeared in the TNFα-treated UK711 cytosol but not in the etoposide-treated UK711 cytosol. The amount of the 50 kDa protein correlates with the extent of cell death (data not shown). Since all apoptotic cytosol with the 50 kDa protein showed the cell-free apoptosis activity (Figures 1 and 2), the 50 kDa protein could play a role in the development of nuclear apoptosis.

Effect of ICE family protease inhibitor on the appearance of a 50 kDa protein and cell-free apoptosis activity

ICE family proteases play an important role in the mechanism of apoptosis (Kroemer *et al*, 1995; Kumar, 1995; Nicholson *et al*, 1995; Steller, 1995). Therefore, we examined the effect of Z-Asp, an inhibitor of ICE family proteases (Mashima *et al*, 1995b), on the appearance of the 50 kDa protein in U937 cytosol and on the emergence of the cell-free apoptosis activity. As shown in Figure 7a, Z-Asp completely inhibited the appearance of the 50 kDa protein in the etoposide-treated U937 cytosol. This cytosol did not show the activity to degrade nuclear DNA (Figure 7b). These results indicate that the appearance of the 50 kDa protein and the emergence of the

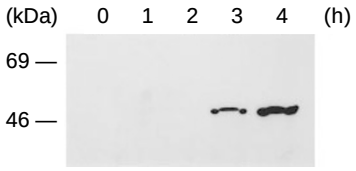

Figure 5 Time-dependent appearance of the 50 kDa protein in etoposide-treated U937 cytosol. U937 cells were treated with 10 μg/ml of etoposide for the indicated times, and the cytosols were prepared. The appearance of the 50 kDa protein band in the cytosol was examined by Western blot analysis

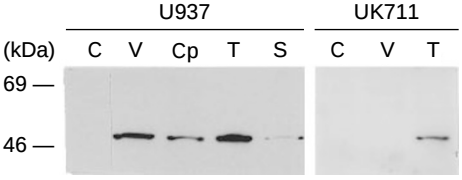

Figure 6 Appearance of the 50 kDa protein in cytosol from U937 and UK711 cells. Cytosols were prepared from the cells treated without (C) or with 10 μg/ml etoposide (V), 10 μg/ml CPT-11 (Cp), 1 ng/ml TNFα plus 1 μg/ml cycloheximide (T) and 1 μM staurosporine (S)

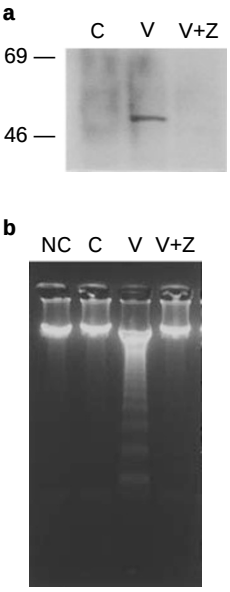

Figure 7 Effect of Z-Asp on the appearance of the 50 kDa protein (**a**) and the cell-free apoptosis activity (**b**). Cytosols were prepared from U937 cells treated without (C) or with 10 μg/ml etoposide in the absence (V) or presence of 50 μg/ml Z-Asp (V+D). NC: no cytosol

cell-free apoptosis activity in the cytosol required activation of ICE family proteases during apoptosis of U937 cells.

Discussion

In this study, we demonstrated that the cytosolic fraction prepared from apoptotic U937 cells shows the activity to induce nuclear apoptosis *in vitro*. The apoptotic cytosol contains activated ICE family proteases (An and Dou, 1996; Chen *et al*, 1996; Emoto *et al*, 1995; Enari *et al*, 1996; Han *et al*, 1996; Kumar, 1995; Mashima *et al*, 1995a, 1997; Naito *et al*, 1997; Noguchi *et al*, 1996; Tewari *et al*, 1995a), and a family member , CPP32, was reported to show the activity to induce DNA fragmentation in isolated nuclei *in vitro* (Nicholson *et al*, 1995). These observations suggest a role of CPP32 in the induction of nuclear apoptosis *in vitro*. However, CPP32 is not likely to play an important role in our system, because YVAD-CHO and DEVD-CHO, inhibitors of ICE family proteases, did not inhibit the cell-free apoptosis activity of the cytosol in our system (data not shown). The emergence of the cell-free apoptosis activity in the etoposide-treated U937 cytosol was inhibited by co-treatment with Z-Asp (Figure 7). This result indicates that the cell-free apoptosis activity in our system arises after activation of ICE family proteases.

To study the factor that induced nuclear apoptosis in our cell-free system, we developed a monoclonal antibody that can recognize a 50 kDa protein in active cytosol in our cell-free system. So far, all of our results showed that the appearance of the 50 kDa protein in the cytosol closely correlates with the emergence of the cell-free apoptosis activity. In addition, the 50 kDa protein was enriched in the precipitated fraction where the cell-free apoptosis activity was recovered, but the protein was eliminiated from the supernatant fraction, where minimal activity was detected (Figure 4). These observations suggest that the 50 kDa protein could play a role in the induction of nuclear apoptosis in our cell-free system. Because immunodepletion of the 50 kDa protein from cytosol was not successfully performed with SN-1 antibody, we could not demonstrate further the role of this protein in the nuclear apoptosis *in vitro*.

The appearance of the 50 kDa protein was abrogated when apoptosis was inhibited by an ICE family protease inhibitor, Z-Asp. This observation suggests that the 50 kDa protein could be produced by proteolytic processing of a precursor protein, presumably by a member of the ICE family proteases during apoptosis. The 50 kDa protein was not detected in total cell lysate from untreated cells (data not shown). However, it is not likely that the 50 kDa protein is newly synthesized during apoptosis because it was detected even when the cells were treated with TNFα in the presence of cycloheximide (Figure 6). Several proteins have been reported to be cleaved by the ICE family proteases during apoptosis. These include PARP (Gu *et al*, 1995; Tewari *et al*, 1995b), U1-70kD (Tewari *et al*, 1995a), lamin B (Lazebnik *et al*, 1995), DNA-PK (Han *et al*, 1996), RB (An and Dou, 1996), actin (Chen *et al*, 1996; Mashima *et al*, 1995) and PKCδ (Emoto *et al*, 1995). However, such proteins are not degraded to generate a 50 kDa fragment during apoptosis, suggesting that the 50 kDa protein recognized by the SN-1 monoclonal antibody was different from a fragment of such proteins. Recently, mitochondrial 50 kDa protein was purified to induce nuclear apoptosis (Sucin *et al*, 1996). But this protein could be different from our cytosolic 50 kDa protein, because SN-1 antibody did not recognize mitochondrial proteins (data not shown). Further studies are needed to identify the 50 kDa protein and to elucidate its role in apoptosis.

Materials and Methods

Cell lines and cell culture

Human monocytic leukemia U937 cells were obtained from the Japanese Cancer Research Resources Bank, Tokyo, Japan. UK711, an apoptosis-resistant variant of U937, was isolated in our laboratory (Kataoka *et al*, 1994). The cells were grown in RPMI 1640 medium (Nissui Co. Ltd., Tokyo, Japan) supplemented with 10% heat inactivated fetal bovine serum and 100 μg/ml kanamycin in a humidified atmosphere of 5% CO_2 and 95% air at 37°C.

Measurement of apoptosis by flow cytometer

U937 cells (5×10^5 cells/ml) were treated with 10 μg/ml of etoposide for the indicated times. After incubation, 5×10^5 cells were harvested, washed in ice-cold phosphate-buffered saline (PBS), and fixed in 70% ethanol for 30 min on ice. Then, the cells were washed, treated with 1 mg/ml RNase A for 1 h at 37°C, and stained in propidium iodide solution (50 μg/ml in 0.1% sodium citrate, 0.1% NP-40). The stained cells were analyzed in a FACScan flow cytometer (Becton Dickinson, Braintree, MA).

DNA fragmentation analysis

DNA fragmentation of cells and isolated nuclei were analyzed, as described previously (Fujita *et al*, 1993; Kataoka *et al*, 1993). Briefly, the treated cells or nuclei were precipitated by centrifugation and sequentially digested by 0.5 mg/ml proteinase K for 1 h at 50°C and by 0.2 mg/ml RNase A for 1 h at 50°C. After the samples were electrophoresed in 2% agarose gels, the DNA was stained by ethidium bromide and photographed on a UV illuminator.

Induction of nuclear apoptosis in a cell-free system

U937 cells were treated with 10 μg/ml etoposide for the indicated times. In some cases, benzyloxycarbonyl-Asp-CH_2OC(O)-2,6-dichlorobenzene (Z-Asp) (Funakoshi, Tokyo), an ICE family protease inhibitor (Mashima *et al*, 1995), was added to the culture together with etoposide. The cells were harvested, washed with PBS, resuspended in extract preparation buffer (50 mM PIPES (pH 7.0), 50 mM KCl, 5 mM EGTA, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethyl sulfonyl fluoride, 1 μg/ml chymostatin, 1 μg/ml leupeptin, 1 μg/ml antipain, 1 μg/ml pepstatin A) and pelleted by centrifugation. The pelleted cells were quickly frozen at -80°C and thawed on ice-water three times. The samples were centrifuged (100 000 *g* for 1 h at 4°C), and the supernatant was collected as cytosol. Nuclei were prepared from exponentially growing U937 cells, as described (Lazebnik *et al*, 1993). The isolated nuclei (10^6/assay) were incubated in 20 μl of cell-free apoptosis buffer (10 mM HEPES (pH 7.0), 40 mM β-glycerophosphate, 50 mM NaCl, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mM dithiothreitol, 2 mM ATP, 10 mM creatine phosphate and 50 μg/ml creatine kinase) containing 4 mg/ml of the cytosolic protein for 1 h at 37°C. After the incubation, nuclear DNA fragmentation was analyzed as described above. When the nuclear morphology was examined, the incubated nuclei were stained in 1 μg/ml of 4′,6-diamidino-2-phenylindole (DAPI) for 10 min and observed under a fluorescent microscope.

Generation of monoclonal antibodies

The etoposide-treated U937 cytosol was further centrifuged at 125 000 *g* for 3 h at 4°C. The cell-free apoptosis activity was recovered in the precipitated fraction. Female Balb/c mice were intraperitoneally immunized with the precipitated proteins (50 μg protein/mouse) emulsified with Freund complete adjuvant (DIFCO, Detroit, MI). Two weeks later, the second immunizations were given to the mice by intrasplenic injection of the precipitated proteins adsorbed to nitrocellulose membrane. Then, weekly *i.v.* reimmunization were given 6 times or more. The spleen cells were fused with mouse myeloma P3U1 cells using polyethylene glycol (MW 4000), and hybridomas were grown in hypoxanthine-aminopterin-thymidine selection media. The culture supernatants were screened by enzyme-linked immunoabsorbent assay for differential binding to the cytosol from etoposide-treated and untreated U937 cells. A hybridoma, named SN-1, proudced an IgM antibody that could bind to the cytosol able to induce nuclear apoptosis *in vitro*, but could not bind to the control cytosol. Western blot analysis was carried out with the hybridoma supernatant as the first antibody.

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