An Insight Into the Mechanism of Cytotoxicity of Ricin to Hepatoma Cell: Roles of Bcl-2 Family Proteins, Caspases, Ca^{2+}-Dependent Proteases and Protein Kinase C

Rong-gui Hu, Qi-wei Zhai, Wang-yi Liu,* and Xin-yuan Liu

Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 20031, China

Abstract   The ability of ricin, a type II ribosome-inactivating protein, to induce hepatoma cell (BEL7404) to apoptosis in vitro was examined by fluorescence microscopy, flow cytometry, and DNA fragmentation assay. As a Bcl-2 lacking model, BEL7404 bore unique advantage to study the effect of over-expressing Bcl-2 on the apoptosis induced by the inhibitor of protein synthesis. By establishing a Bcl-2 over-expressing cell line (BEL7404/ Bcl-2), we found that Bcl-2 could promote the survival of the hepatoma cell against ricin insult. The ricin-induced apoptosis of BEL7404 was accompanied by increased expression of Bak and decreased levels of Bcl-xl and Bax. Caspases and PARP cleavage activity were found to be implicated in the death process. Through the inhibitor tests, our results excluded the participation of calcium-dependent proteases or protein kinase C in the apoptotic process induced by ricin, though an elevation of intracellular calcium did occur as an immediate response to ricin treatment. Cycloheximide, another protein synthesis inhibitor, did synergistically enhance rather than inhibit the cytotoxicity of ricin to hepatoma cell BEL7404. Actually, cycloheximide alone was able to induce hepatoma cell BEL7404 to death that could also be inhibited by over-expressing Bcl-2. The elevation of apoptotic protein Bak was discussed to challenge the notion that ricin exerted its cytotoxicity through nonspecific inhibition of all the de novo protein synthesis. J. Cell. Biochem. 81: 583–593, 2001. © 2001 Wiley-Liss, Inc.

Key words: apoptosis; Bcl-2; calpeptin; caspase; cycloheximide; hepatoma; ribosome-inactivating protein; ricin

Apoptosis, or programmed cell death (PCD), along with necrosis is one of the two major forms that mammalian cells take to die. Apoptosis is cell suicide mechanism that plays a central role in development, homeostasis of metazoans and pathogenesis. Apoptosis of mammalian cells can be induced by various stimuli such as tumor necrosis factor (TNF), deprival of growth factors, anticancer drugs or insults to normal cell functions, thus of therapeutic significance [Wertz and Hanley, 1996]. Cells undergo apoptosis with almost non-exceptional characteristics: cell shrinkage, membrane blebbing, chromatin condensation, and internuleosomal DNA cleavage, suggesting a highly conserved orchestration of cell to death. In mammalian cells, Bcl-2 family proteins that contain certain Bcl-2 homologous domains are believed to play a critical role in determining cell fate [Adams and Cory, 1999]. Bcl-2 family proteins can be classified into two factions according to their opposing functions: the pro-survival or anti-apoptotic Bcl-2 family proteins that include Bcl-2, Bcl-xl, Bcl-w, etc. and the pro-apoptotic proteins that encompass Bax, Bak, Bok, etc. with an essential death domain of BH3. Pro- and anti-apoptotic family members can heterodimerize and seemingly titrate one another's functions, implying that their relative concentrations may act as a rheostat for the suicide program. Increasing bodies of evidences demonstrated that a unique family of about 13 kinds of cysteine proteases functioned as activators or executors in the death program [Thornberry and Lazebnik, 1999]. Proteases of this family

have distinctive cleavage specificity for aspartic acid in the P1 position and have recently been designated as Caspases (cysteinyl aspartate-specific proteases). Interleukin-1β-converting enzyme (ICE/caspase 1) is the first member of the caspase family identified. Caspase 3 (CPP32) has been identified as one of those caspases that play the key roles in the death program. The cleavage sets of proteins by caspases include those that are of regulatory or structural function in cell life. Among them, Poly (ADP-ribose) polymerase (PARP) is one of the best described substrates for caspase 3. In response to apoptotic stimuli, the levels of intracellular death signals such as calcium ion are elevated, and the transcription factors or the other regulatory entities are then activated or suppressed to modulate the apoptotic process [Bellamy, 1997].

Ricin, a type II ribosome-inactivating protein (RIP) from the seeds of castor bean (*Ricinus communis*), has received wide attentions from researchers in both academic and practical fields since the last century [Barbieri et al., 1993]. Ricin consists of binary proteinaceous chains (A- and B-chain) that are linked via a single disulfide bond. The A-chain of ricin could act as RNA N-glycosidase that depurinated the adenosine at position 4324 of 28S RNA in rat ribosome, thus inactivating the protein-synthesis apparatus—ribosome [Endo et al., 1987]. Earlier studies on the cytotoxic mechanism of ricin were mainly focused on its internalization and intracellular routing to exert its potency [Barbieri et al., 1993]. As reported recently, ricin and other ribosome-inactivating proteins could induce the mammalian cells to death with apoptotic characters [Waring, 1990; Williams et al., 1997]. These observations have provided a new visual angle to understand the cytotoxic mechanism of these toxic proteins. An antibiotic, cycloheximide (CHX), affected not only the enzymatic or non-enzymatic translocation but also the formation of initiation complex [Hershey et al., 1996]. Ricin and CHX were known to be able to induce apoptosis of various cell lines [Geier et al., 1996], which had been employed as evidence against the postulate that protein synthesis might not be necessary for apoptosis induced by certain stimuli [Waring, 1990]. Though putatively acting on the same ribosome, the so called protein-synthesis inhibitor CHX were found to be able to antagonize cytotoxicity of ricin to cell lines such as Vero cell or MDCK [Sandvig and Deurs, 1992]. Recent studies revealed that different agents were required to suppress the cytotoxicity of CHX and ricin, suggesting that the namely protein-synthesis inhibitors perhaps induced apoptosis through distinctive pathways [Geier et al., 1996]. Though inhibition of protein synthesis was observed in every type of cells that were treated with ricin, it seemed that different cells took distinct pathways in response to ricin [Barbieri et al., 1993]. So it still remained to be elucidated how the protein synthesis inhibition could lead to apoptotic death of the cells.

In this work, hepatoma cell BEL7404 was used as a model to study the cytotoxic mechanism of ricin. Expression levels of several members of Bcl-2 family proteins were examined. Along with the observation that ricin could elicit an elevation of intracellular calcium, several specific inhibitors were employed to inquire whether calcium-dependent protease or protein kinase C were implicated in the death process induced by ricin. Cycloheximide was also found to act synergically with ricin on hepatoma cell BEL7404.

MATERIALS AND METHODS

Materials

The pcDNA3 plasmid was obtained from Clontech (Palo Alto, CA). The mouse to human primary antibody for human Bcl-2 (sc-509) and rabbit to human β-actin, Bcl-xl (sc-1599), Bak (sc-1035), Bax (sc-493-G) or ICE-1/caspase 1(sc-515), goat to human CPP32/caspase3 (sc-1226) or PARP antibodies were products from Santa Cruz Biotech (Santa Cruz, CA). Lipofectin, Dubbeco Modified Eagle's medium (DMEM) and fetal calf serum were purchased from GIBCO (Grand Island, NY). G418, Ac-YVAD-CHO and calpeptin were obtained from Calbiochem (La Jolla, CA). Enhanced chemical-luminescence (ECL) reagent was product from Amsherm-Pharmacia Biotech (Arlington Height, IL). Ricin and other regents were from Sigma Chemical Corp. (St. Louis, MO).

Cell Culture and Generation of Bcl-2 Over-Expressing Cell Line

The hepatoma cell BEL7404 was established from a patient of liver cancer [Shen et al., 1995]. The cell line was maintained at 37°C in a 5% CO_2 atmosphere in DMEM medium with 10% fetal calf serum. Human Bcl-2 was cloned into

plasmid pcDNA3 at the sites of *Kpn* I and *Xba* I. Then cell line BEL7404 were transfected with pcDNA-Bcl-2 construct or pcDNA3 only plasmid with lipofectin method as described [Zhai et al., 2000]. After 10 days screening by G418 (final concentration 100 unit/ml), several clones that stably express Bcl-2 were selected from the culture dishes. One transfectant with the highest expression level of Bcl-2, designated as BEL7404/Bcl2, along with pcDNA-only transfectant and BEL7404 were subjected to Western-blotting analyses.

Morphological Studies of the Cells With Fluorescence Microscopy and DNA Fragmentation Assay

Hepatoma cell BEL7404 and BEL7404/Bcl-2 were grown on glass coverslips and treated with or without ricin for times as indicated in the legends. The cells were washed with PBS, and then fixed with 1% glutraldehyde at room temperature for 30 min. Then the cells were stained with Hoechst 33 258 (0.15 mM) for 20 min and visualized for nuclear condensation and internucleosomal cleavage under fluorescence microscope (Olympus). Photographs were taken with Kodak color film. DNA fragmentation assay was performed as described by Zhai et al. [2000].

Cell Viability Assay, Apoptosis Assessment With FACS

Cell viability was determined by MTT staining method as indicated [Zhai et al., 2000], which was confirmed with tryptan blue exclusion. Cells that were treated with or without ricin for time indicated were collected, washed in PBS and resuspended in 100 µl of propidium iodide (PI) solution. After incubation at room temperature for 30 min, the cells were then subjected to flow cytometry analysis on a Becton Dickinson FACScan.

Determination of Intracellular Free Calcium

Cells were harvested and loaded with fluorescent calcium indicator, fura-2 (Fura-AM). The fluorochrome-loaded cells that were treated with or without indicated drugs for set times were then subjected to analysis on fluorospectrometer Hitachi F4010 as detailed by Kao [1994].

Immuno-Blotting Analyses

Immunoblotting analyses of β-actin, Bcl-2, Bcl-xl, Bak, Bax, and PARP cleavage were performed utilizing specific mono- or poly-clonal antibodies. In brief, both adherent and floating cells were harvested and lysed. The total protein concentration of cell lysate was determined by methods as indicated [Zhai et al., 2000], which was also calibrated with β-actin as internal control in certain experiments. Cell lysates from controls and those that were treated with indicated drugs were resolved by SDS-PAGE and transferred to PVDF film (Bio-Rad). Immunoblots were visualized with DAB or Enhanced Chemiluminescence (ECL) and photographed with Bio-image system. Photographs were printed with Epson Stylus Photo 700.

RESULTS

Ricin Could Induce Apoptosis of Hepatoma Cell BEL7404

Under contrast-phase microscope, large portions of cells could be observed to become global, then float and undergo lysis when treated by 10 nM ricin. Cells were then stained with Hoechst 33 258 and subjected to fluorescence microscopic analysis. While nearly all untreated hepatoma cells retained intact nuclei (Fig. 1A), approximately 40% of the ricin-treated BEL7404 cells had condense and cleaved chromosome after 15 h treatment with 10 nM ricin (Fig. 1B). DNA from all the floated and adherent cells that were incubated with 10 nM ricin were also harvested for DNA fragmentation assay. From Figure 2, it was obvious that all the ricin-treated BEL7404 had generated DNA fragments in contrast with the intact chromosomal DNA in control cell, due to internuleosomal cleavage of chromosome. All these obervations suggested that ricin could induce hepatoma cell BEL7404 to death of apoptotic features.

Over-expressing Bcl-2 Could Partially Inhibit Ricin-Induced Apoptosis of Hepatoma Cell BEL7404

Hepatoma cell line BEL7404 expressed only a non-detectable level of Bcl-2 (Fig. 3). BEL7404 was transfected with an eukaryotic expression vector (pcDNA3/Bcl-2) containing human Bcl-2 cDNA, while the cell transfected with pcDNA3 vector alone was designated as a control P and the original cell line 7404 as another control C. After drug selection, several stable G418-resistant transfectants were developed. The clones were selected and subjected to Western-blot analysis. While the control cell C and P expressed Bcl-2 at a non-detectable level, a
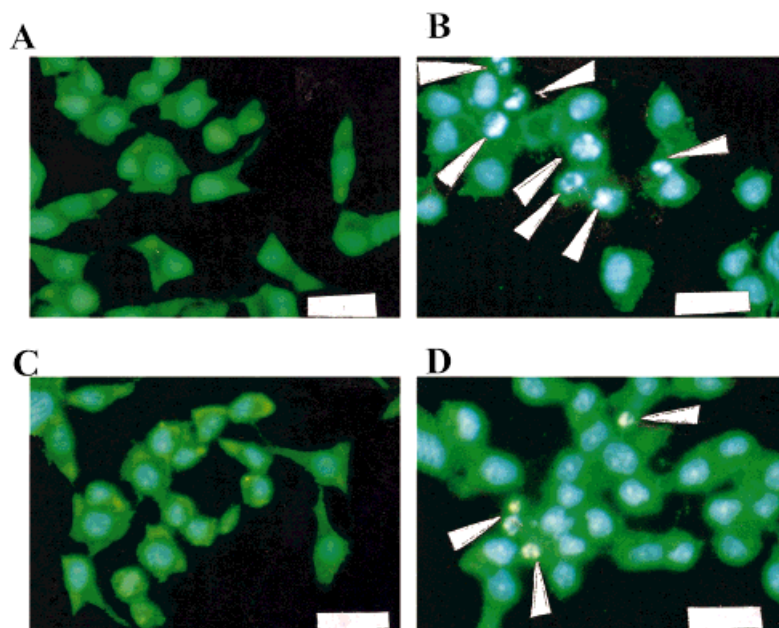
Fig. 1. Fluorescent microphotographs of hepatoma cell BEL7404 and BEL7404 /Bcl-2 treated with ricin. Hepatoma cell BEL7404 and 7404/Bcl-2 were treated with 10 nM or without ricin for 15 h, stained with Hoechst 33 258 and observed under fluoroscence microscope (Olympus). **Panels A** and **C**. Control cells of hepatoma BEL7404 and BEL7404 /Bcl-2. Most cells exhibited intact and lightly stained nuclei. **Panels B** and **D**. BEL7404 and BEL7404/Bcl-2 cells treated with 10 nM ricin for 15 h. Example cells of broken, condense and brightly-stained nuclei were indicated with arrows. Bars: (a), (b), (c), and (d) 30 μm. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

clone was found to be of the highest level of Bcl-2 expression, presenting a strong 26 kD protein band as indicated in Figure 3. The clone was designated as BEL7404/Bcl-2 and subjected to the subsequent experiments.

Only cell line 7404 was used as a control to study the effect of overexpressing Bcl-2 in the following experiments, since the cell line C and P responded to the ricin treatment in similar manners during apoptosis or cell viability assays (data not shown). The same ricin treatment was found to induce apoptosis in BEL7404 and BEL7404/Bcl-2, but obviously with quite different portions. When treated with 10 nM ricin for 15 h, about 40% of the BEL7404 cells underwent chromatin condensation or nuclear break (Fig. 1B); This percentage was found to be far less (about 10%) with the cell line BEL7404/Bcl-2 after the same treatment with ricin (Fig. 1D). After ricin treatment for the same time, less portions of chromosomal DNA were fragmented in BEL7404/Bcl-2 than in BEL7404 (Fig. 2). Analysis with flow cytometry presented the consistent results: about 27% of the BEL7404 cells become apoptotic after the toxin treatment, while the apoptotic population only amounted to 10% in BEL7404/Bcl-2 (Fig. 4A).

With MTT staining or tryptophan blue exclusion assays, approximately one fold more cells of BEL7404 /Bcl-2 than wild-type BEL7404 could survive the insult of ricin at various concentrations (as shown in Fig. 4B). This suggest that over-expressing Bcl-2 could confer the cell with the resistance against ricin only through a delay in the cytotoxic kinetics.

The Levels of Anti-Apoptotic Bcl-xl and Pro-Apoptotic Bak, Bax in Hepatoma Cell BEL 7404, and That of Bcl-2 in BEL7404/Bcl-2 Prior to or After Ricin Treatment

In order to investigate whether apoptosis of BEL7404 cells induced by ricin was due to the alterations in Bcl-2 family proteins that regulate apoptosis, we examined the expression of these proteins during the apoptotic process by immunoblotting method. BEL7404 or BEL7404 transfected with pcDNA3 alone constitutively expressed the same level of Bcl-xl, Bak, and Bax (data not shown), indicating that transfection of pcDNA3 or drug selection of G 418 did not alter the expression of these pro- or anti-apoptotic proteins.

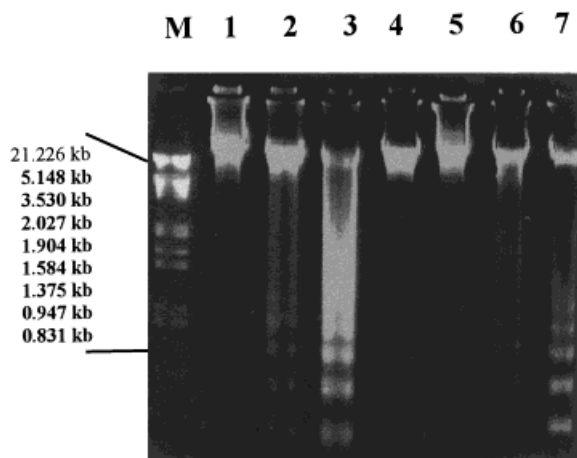Bcl-xl was expressed constitutively in BEL7404. When BEL7404 cell was exposed to

Fig. 2. DNA fragmentation analysis of hepatoma cell BEL7404 and BEL7404 /Bcl-2 treated with ricin. After exposure to 10 nM ricin for time as indicated, cells were lysed and DNA was isolated and subjected to electrophoresis in agarose gel (1.2%). BEL7404 cells were treated with 10 nM ricin for 0 h (**Lane 1**, control), 10 h (**Lane 2**) or 15 h (**Lane 3**); BEL7404/Bcl-2 cells were treated with 10 nM ricin for 0 hour (**Lane 4**), 10 h (**Lane 5**), 15 h (**Lane 6**), 20 h (**Lane 7**); Lane M, DNA Marker, (λ-DNA was digested with *EcoR* I and *Hind* III).
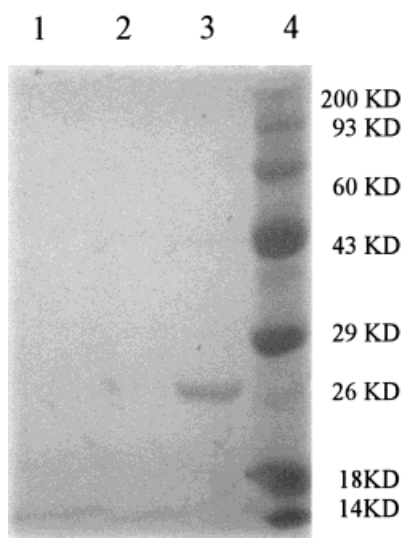

Fig. 3. Immunoblotting assays for the stable expression of human Bcl-2 in the BEL7404 clones transfected with pCDNA3/Bcl-2. Cell lysates were subjected to SDS-PAGE (12%) and electrotransferred to PVDF films. After incubation with primary antibody (mouse anti-human Bcl-2) and HRP-conjugated secondary antibody (rabbit anti-mouse), the blots were visualized with DAB. Lane 1, lysate from BEL7404; Lane 2, BEL7404 transfected with empty pCDNA3; Lane 3, one clone of BEL7404 transfected pCDNA3/Bcl-2 that was designated as BEL7404/Bcl-2. A 26 kD protein band appeared corresponding to the expressing human Bcl-2 in the cells of BEL7404/Bcl-2. Lane 4, prestained mid-range protein markers from GIBCO.
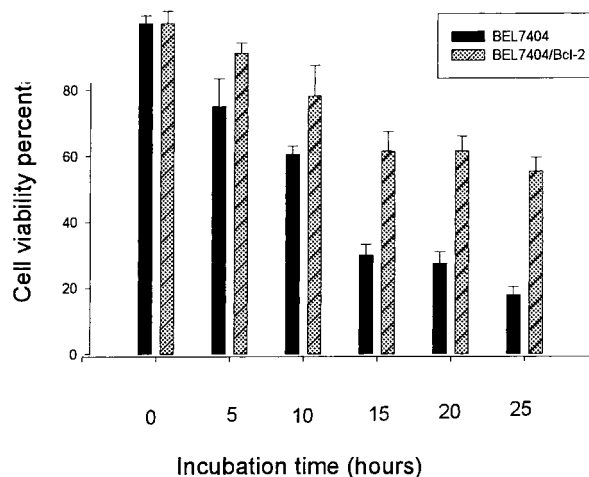
Panel A
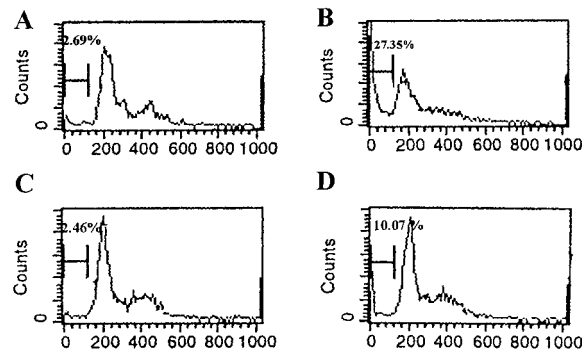
Panel B

Fig. 4. Cell viability and apoptosis assay of hepatoma cell BEL7404 and BEL7404/Bcl-2 treated with ricin. **Panel A**. Flow cytometric analyses of BEL7404 or BEL7404/Bcl-2 after continuous treatment with 10 nM ricin for 10 h. The DNA content, as measured by propidium iodide (PI) staining is expressed on the X axis versus cell number on the Y axis. Represented numbers are the percentages of apoptotic nuclei with reduced DNA content. a and c. BEL7404 and BEL7404/Bcl-2, respectively; b, d. BEL7404 and BEL7404/Bcl-2 treated with 10 nM ricin for 10 h. At least s separate experiments were carried out with results similar to the representaive data shown here. **Panel B**. Cells of BEL7404 or BEL7404/Bcl-2 were treated with 10 nM ricin for time as indicated. Cell viability of both cell lines were determined by methods of MTT—staining method, and calibrated with tryptan blue exclusion.

10 nM ricin, the expression of Bcl-xl was found to be slightly decreased along with the prolonged toxin treatment (Fig. 5A). In BEL7404/Bcl-2, the pro-survival Bcl-2 was found to undergo similar change after ricin treatment along with the apoptotic process (Fig. 5B).

Also presented in Figure 5A, levels of Bak were elevated along with the process of cell death triggered by ricin. Remarkably, the expression
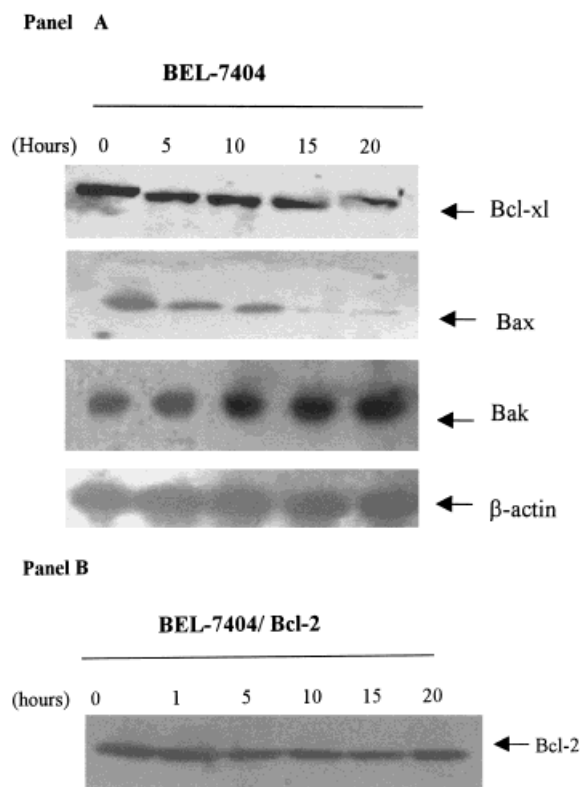
Panel A

BEL-7404

(Hours)   0   5   10   15   20

← Bcl-xl

← Bax

← Bak

← β-actin

Panel B

BEL-7404/ Bcl-2

(hours)   0   1   5   10   15   20

← Bcl-2

Fig. 5. Immunoblotting analyses of Bcl-xl, Bax, Bak and β-actin in hepatoma cell BEL-7404 or Bcl-2 in BEL7404/Bcl-2 that were treated with ricin. After treatment with 10 nM ricin for time as indicated, lysates (approximately 30 μg of total proteins in each lane) of both floated and adherent cells were run in SDS-PAGE (12%) and visualized as described in Materials and Methods. (The gel is representative of no less than 3 independent experiments.)

of Bax decreased in the early phase of apoptosis and became hardly detectable in the late phase (when treated with ricin for 15 or 20 h). Contrary to those cases where the expression levels of Bax were elevated to activate the process of cell death, our result demonstrated that Bax had underwent degradation in the death processes as published by others [Chen et al., 1997; Wood et al., 1998; Li and Dou, 2000]. Efforts by them pointed out that such a degradation of Bax could not interfere with the pro-apoptotic role of Bax in the cell death, indicating that the mechanism through which Bax activates apoptosis would be more complicated than expected [Wood et al., 1998].

Involvement of Caspase 1 and PARP Cleavage in the Ricin-Induced Apoptosis of Hepatoma Cell

Caspases could function as initiators or effectors in the lots of apoptotic processes

responding to myriad of stimuli [Thornberry and Lazebnik, 1999]. With immuno-blotting assay, it was found that expression levels of both caspase 1 and 3 changed little in the process of hepatoma cell death (data not shown). Various concentrations of caspase 1 inhibitor (Ac-YVAD-CHO) were used to test the role of caspase 1 in the death process. Consistent with the protective effect of Ac-YVAD-CHO observed from morphological study, the caspase 1 inhibitor was found to be able to inhibit the ricin-induced cell death of BEL7404 in a non-linear concentration dependent manner (Fig. 6). The inhibitory effect varied from 30% at concentration of 0.1 nM to 50% at 20 nM. Since no difference in viability was observed with control cells treated by various concentrations of Ac-YVAD-CHO or DMSO [unpublished results], the inhibitory effect of Ac-YVAD-CHO suggested that caspase-1 probably played a role in the cell death of BEL7404 (Fig. 6).

Caspases were known to participate in the apoptotic process through cascade of proteolytic events that would lead to disassembly of the cell. Among the cleaved substrates that were of regulatory or structural functions in cell life [Thornberry and Lazebnik, 1999], Poly-(ADP-ribose) polymerase was the best described
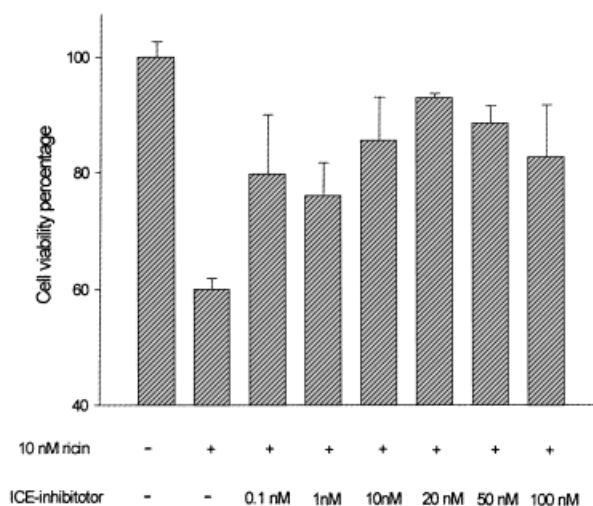

Fig. 6. Effect of Ac-YVAD-CHO, the ICE/caspase 1 inhibitor, on the ricin-induced hepatoma cell (BEL7404) death. Before BEL7404 cells were treated with 10 nM ricin, the cells were pre-incubated with Ac-YVAD-CHO for 1 h at indicated final concentrations. And 15 h later, the cells were harvested and subjected to viability assays as indicated in the Material and Methods. The results shown are the mean ±SD of five independent experiments. $P < 0.05$.

substrate for caspase 3, which could cleave 116kD PARP into 85 and 31kD fragments. Preliminary results suggested that, capase 3 was cleaved during the death process induced by ricin and zDEVD-fmk, the inhibitor of caspase 3, could partially block the cytotoxicity of ricin (data not shown). As shown in Figure 7A, PARP was found to be cleaved through the death process of BEL7404 induced by 10 nM of ricin. In the case of BEL7404/Bcl-2, the same treatment also incur the cleavage of PARP, but obviously a far less percent of the 116 kD protein was degraded (Fig. 7B). This suggested a lower caspase activity in cell death of BEL7404/Bcl-2, granted that cleaved percentage of PARP could reflect the activity of caspase 3 or its likes.

Non-Commitment of Calcium-Dependent Proteases or Protein Kinase C in Ricin-Induced Apoptosis of Hepatoma Cell

In the literatures of ricin-induced apoptosis, dideoxyforskolin, an inhibitor of plasma Ca^{2+}

Fig. 7. Immunoblotting analysis of PARP cleavage in BEL7404 and BEL7404/Bcl-2 treated with ricin. Cells were treated with 10 nM ricin for time as indicated, then lysed and run in SDS-PAGE (9%) and subject to immunoblotting analyses, using a polyclonal antibody that recognized the terminus of PARP. The 116 kD intact PARP and its 89 kD fragments were visualized according to described in Material and Methods. **Panel A**. Immunoblotting analysis of PARP cleavage in BEL-7404 treated with 10 nM ricin for time as indicated. **Lane 1–6**, cells were treated with ricin for 0, 5, 10, 15, 20, 25 h respectively. **Panel B**. Immunoblotting analysis of PARP cleavage in BEL-7404 /Bcl-2 treated with 10 nM ricin. Lane 1–6, cells were treated with ricin for 0, 5, 10, 15, 20, 25 h respectively.

ion-channel, was found to be able to partially inhibit the cytotoxicity of ricin on MDCK cell [Oda et al., 1997]. So efforts were made to test whether intracellular concentration of calcium ion ($[Ca^{2+}]_i$) was elevated in response to ricin. In our serial assays with fluorospectrometer, drastic increases in intracellular Ca^{2+} were observed immediately after the addition of ricin to the cell suspension. The qualitative appearances of the time drive curves were not quite different in both cell lines. Data of fluorescence density were further processed to quantitate $[Ca^{2+}]_i$ before and after the treatment of the cell lines with ricin. $[Ca^{2+}]_i$ was elevated from approximately 100 nM, peaked at 220 nM, and then dropped slowly (Fig. 8A), corresponding to a slope in the trace figures (data not shown). So over-expressed Bcl-2 probably did not chang the way in which hepatoma cell responded to the stimulation of ricin.

Since $[Ca^{2+}]_i$ was elevated by the exposure to ricin, we want to know whether the alteration in $[Ca^{2+}]_i$ would lead to a subsequent activation of calcium-dependent proteases or protein kinase C which could modulate cell to apoptosis [Dowd, 1995]. So calpeptin, GF 109203X or calphostin C, inhibitors for calcium-dependent proteases and protein kinase C [Banik et al., 1998; Capiati et al., 2000] respectively, were employed to probe the commitment of these enzymes. As shown in Figure 8B, the cell pretreated with or without calpeptin, GF109203X or calphostin C did not behave distinctly in their viability against ricin treatment, suggesting that activation of calcium-dependent proteases, at least calpains, or protein kinase C was not responsible for hepatoma cell death incurred by ricin.
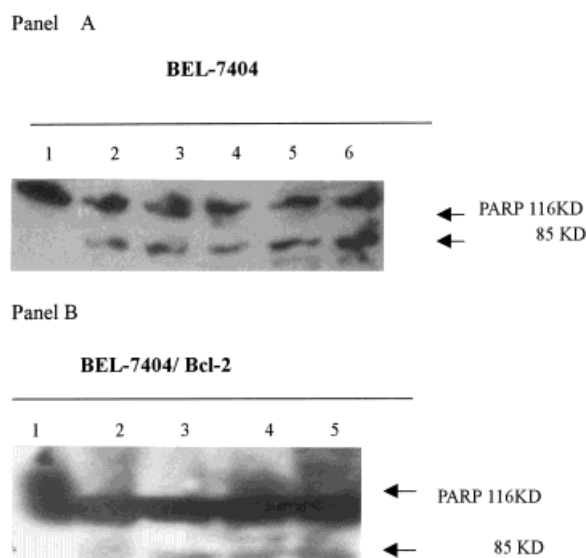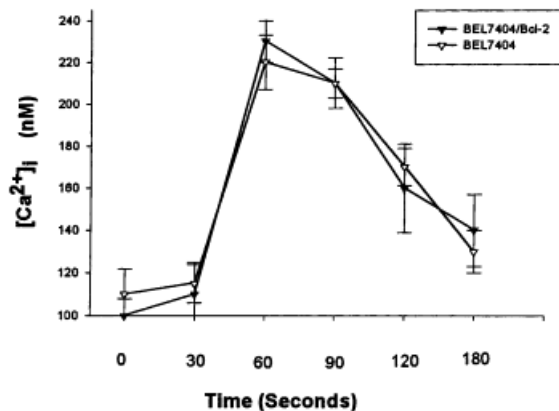
Influence of Cycloheximide on Cell Death of BEL7404 Induced by Ricin

Cycloheximide was one of the best known antibiotics that could interfere with protein biosynthesis [Hershey et al., 1996]. Moreover, it was reported that CHX could antagonize the cytotoxicity of ricin on many kinds of mammalian cells [Sandvig and Deurs, 1992]. Here we want to ask whether CHX could also influence the cytotoxicity of ricin on hepatoma cell BEL7404. From Figure 9, CHX at various concentrations could not inhibit but synergistically enhance the cytotoxicity of ricin on cell BEL7404. It was also found that CHX alone could induce the hepatoma cellBEL7404 to

Panel A

Panel B

Fig. 9. Cell viability assay of BEL7404 and BEL7404/Bcl-2 treated with various doses of cycloheximide (CHX) alone or together with 10 nM ricin for 15 h. Cells of BEL7404 or BEL7404/Bcl-2 are treated with indicated drugs for 15 h, and then collected for viability assays with MTT staining method. The data shown are the mean of at least 3 independent experiments.
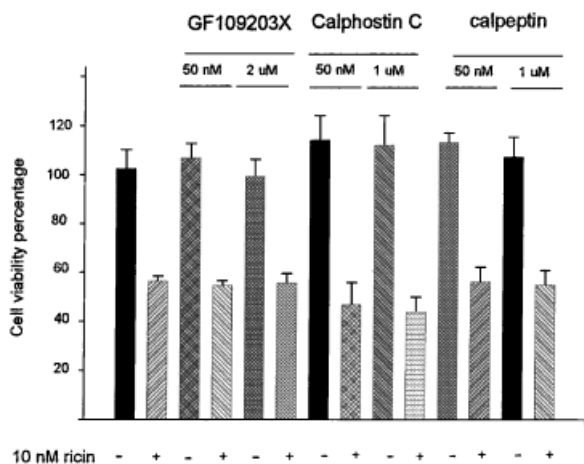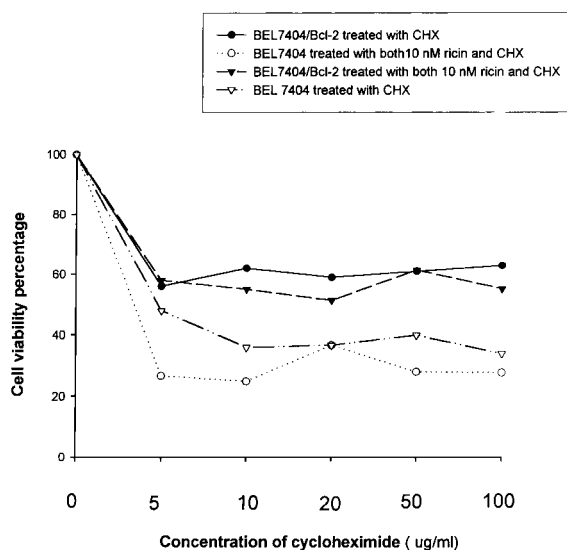
Fig. 8. The change of intracellular [Ca^{2+}] and the roles of calcium-dependent proteases and protein kinases. **Panel A**. The change of intracellular [Ca^{2+}] in BEL7404 and BEL7404/Bcl-2 cells induced by 10 nM ricin after Fura-2 AM loading, approximately 1×10^6 cells per sample were treated with 10 nM ricin and monitored with fluorospectrometer (Hitachi F 4010). Fluorescent density (Fi) of Fura-2 were recorded with Ex = 340 nm and Em = 505 nm. Width of slice band = 5 nm. Fi were then converted to [Ca^{2+}] according to formula: [Ca^{2+}] = K$_n$ ×[F$_i$-F$_{min}$]/[F$_{max}$-F$_I$] (K$_n$ = 224 nM), F$_{max}$ and F$_{min}$, were determined by adding Triton X-100 to the final concentration (0.5%), and the subsequent addition of EGTA to final concentration of 5mM. The pH value was adjusted to 7.2 with 0.1 M NaOH. The data shown are the mean ±SD of no less than 3 independent experiments. $P < 0.01$. **Panel B**. Effects of inhibitors of calcium-dependent proteases or protein kinase C on the cytotoxicity of ricin to BEL7404. After the cells were incubated with GF109203X, Calphostin C or calpeptin of concentrations indicated for 1 h, the cells were then treated with or without 10 nM ricin and subjected to cell viability assays. The results shown are the mean ±SD of at least 3 independent experiments. $P < 0.05$.

death in a nonlinear dose-dependent manner. Since over-expression of Bcl-2 could inhibit the ricin-induced cell death as described above, it remained to be elucidated whether over-expressed Bcl-2 could inhibit the cytocidal effect of CHX. Obviously, over-expression of Bcl-2 was able to inhibit cytotoxicity of CHX to the same degree as it did on that of ricin (Fig. 9). Interestingly, cell BEL7404/Bcl-2 could survive in the presence of both ricin and CHX with the same viability as treated with ricin or CHX respectively.

DISCUSSIONS

The observations that ricin can induce mammalian cells to apoptosis have led to further investigation into how this process occurs. It is established that Bcl-2 family proteins can function as arbitrators to determine the fate of cell and a cascade of proteolytic events take place in executing the death process [Thornberry and Lazebnik, 1999]. So what role do Bcl-2 family proteins play and whether caspases or other proteases are involved in the ricin-induced cell death?

With hepatoma cell BEL7404 as a Bcl-2-lacking model, we found that over-expressing

Bcl-2 could partially prevent the cell death induced by ricin. Accumulating evidences have suggested that Bcl-2 can prevent the cell death induced by various stimuli through heterodimerizing with Bax or other pathways [Mahajan et al., 1998; Adams and Cory, 1999]. Because our unpublished results rule out the implication of reactive oxidative species (ROS), over-expressing Bcl-2 can probably inhibit ricin-induced death of hepatoma cell through titrating the function of its pro-apoptotic homologues, such as Bax, that modulate the toxin-induced death process.

What seemed to be a paradox to this hypothesis was that Bax was found to be degraded in the apoptotic process (Fig. 5A). Degradations of both pro- and anti-apoptotic proteins by proteases such as caspases were found to exist in apoptosis of various systems [Chen et al., 1997; Wood et al., 1998; Breitschopf et al., 2000; Li and Dou, 2000]. Actually ubiquitin-related proteosome or calpains have been reported to be involved in the cleavage of Bax in yeast [Li and Dou, 2000] or HL60 cell [Wood et al., 1998]. Wood and his colleagues found that inhibitors of calpain could inhibit the degradation of Bax but not the cleavage of PARP or cell death. They postulated that the degradation of Bax might be an event distinct from the caspases-mediated cell apoptosis. Since calpeptin, calpain inhibitor, could not inhibit the degradation of Bax in our system (data not shown), it remained to be elucidated how the degradation of Bax occurred and to what event it was related to the possible role in modulating the apoptotic process.

Cascades of proteolytic events were believed to take place in executing the death program [Thornberry and Lazebnik, 1999]. Caspases or other proteases such as calcium-dependent proteases (calpains) were activated to trigger a cascade of cleavage events. Studies pointed out that caspase 3 and caspase 6, but not caspase 1 were directly involved in apoptosis of MDCK cell induced by ricin and viscumin [Komatsu et al., 1998]. Similar results were also obtained with apoptosis of HL 60 induced by immunotoxin, DT388 GM-CSF [Kim et al., 1999; Frankel et al., 2000] or MCF 37 cell induced by B3(Fv)-PE38 [Keppler-Hafkemeyer et al., 1998], where caspase 3 was found to be implicated in the death process. In this paper, we found that caspase 1 and caspase 3 were involved in ricin-induced apoptosis of hepa-

toma cell BEL7404. A tempting explanation to the protective effect of overexpressing Bcl-2 was that overexpressing Bcl-2 suppressed the activity of caspase 3 and inhibited the cytotoxicity of ricin on cell BEL7404.

Calpains might not be responsible for cell death of BEL7404 induced by ricin. Calcium-dependent protein kinases, such as protein kinase C (PKC), were probably also not implicated in the death process, since their inhibitors, calphostin C or GF 109203X, could not interfere with cytotoxicity of ricin on the cell. So calcium might not function directly as an intracellular signal in ricin-induced cell death of BEL7404, though ricin did incur an elevation of intracellular concentration of calcium in a Bcl-2-not-affecting manner.

Though CHX could antagonize the cytotoxicity of ricin to cell lines such as Vero cell or MDCK [Sandvig and Deurs, 1992], CHX alone was toxic to cell BEL7404 and could act synergistically with ricin. As shown in Figure 9, overexpressing Bcl-2 were found to be able to inhibit the hepatoma cell death induced by CHX. Interestingly, the cell survivability conferred by over-expressing Bcl-2 amounted to the same level in presence of ricin alone or both ricin and CHX. So there probably lay a Bcl-2-repressible pathway that was committed to apoptosis of cell BEL7404 induced by ricin and CHX.

In the field of apoptotic studies, CHX has been a classic probe to inquire whether an apoptotic process needs the de novo synthesis of protein [Wyllie et al., 1984]. If one apoptotic process could be inhibited by CHX, then apoptosis was taken as one that needs novel protein-synthesis, vice versa. Obviously, the validity of this notion was totally based on an, untested in most cases, assumption that inhibition of all the novel protein-synthesis was the only in vivo action mechanism of CHX as it did in cell-free translation system. This had been challenged by more and more reports that in vivo CHX was actually a pleiotropic antibiotic. Studies revealed that CHX could interfere with DNA replication [Sherwood et al., 1988], RNA synthesis [Ohh and Takei, 1996], and even enhance the transcription of cytochrome-P450 genes, *CYP2H1* and *CYP2H2* in embryo hepatocytes [Hamilton et al., 1992]. CHX could also abrogate autophagy, a major process for degradation of cytoplasmic proteins and organelles [Sandvig and Deurs, 1992]. To our current knowledge, CHX

was toxic to cell lines such as MAD 231 [Geier et al., 1996], lymphcyte T blasts [Williams et al., 1997], breast cancer cell MCF-7 cell [Keppler-Hafkemeyer et al., 1998] and hepatoma cell BEL7404 (in this paper), but bore no harm to cell types such as Vero or MDCK [Sandvig and Deurs, 1992]. All these suggested that the in vivo action mechanism of CHX could be more complicated than granted from any single case. Therefore, caution should be taken to investigate whether novel synthesis of protein was necessary for an apoptotic process, using CHX as a probe.

As in in vitro translation systems, ricin, Diphtheria toxin (DT) or immunotoxins such as B3(Fv)-PE38 could potently inhibit protein synthesis in nearly all the cell types examined [Sandvig and Deurs, 1992; Keppler-Hafkemeyer et al., 1998], which was proposed to be the general mechanism of their cytotoxicities. But this notion was challenged by findings that caspases activation and arrest of protein synthesis were distinct pathways in apoptosis of MCF 7 induced by immunotoxin, B3(Fv)-PE38 [Keppler-Hafkemeyer et al., 1998]. Furthermore, ricin has been reported to induce the expression of TNF or other cytokines [Licastro et al., 1993]. Similar results have also been obtained with other ribosome-inactivating proteins such as Shiga toxin [Thorpe et al., 1999] or their derivatives [Kim et al., 1999]. Bussing et al. [1999] reported that ricin could trigger the expression of a mitochondrial protein, Apo2.7 [Bussing et al., 1999]. In our experiment with hepatoma cell BEL7404, we found that treatment by ricin could increase the expression of pro-apoptotic protein, Bak, though it was still unknown about the relationships between the elevation of Bak and apoptosis in our system. The fact that the so called protein-synthesis inhibiting toxins could induce the expression of Apo 2.7 (by ricin and viscotoxins), Bak (by ricin) or FAS-ligand (by DT388-GM-CSF) suggested that the de novo synthesis of protein did occur in the apoptotic process triggered by these toxins. So taken into account the results of other researchers, our work may lead to the postulate that ribosome-inactivating proteins, like ricin, can induce cell to death through mechanism that is a little different from their in vitro function as inhibitors of protein-synthesis. In other words, these toxins did not inhibit all the de novo the protein synthesis non-specifically as it did in in vitro translation systems.

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