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Suppression of Endoplasmic Reticulum Stress-induced Caspase Activation and Cell Death by the Overexpression of Bcl-x_L or Bcl-2

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Continuous endoplasmic reticulum (ER) stress, such as the accumulation of unfolded proteins, results in cell death and relates to the pathogenesis of some neurodegenerative diseases. Treatment of brefeldin A, an inhibitor of transport between the ER and Golgi complex, induced cell death during 24 h, which accompanied activation of caspase-2, caspase-3 and caspase-9, starting at 12 h and increasing time-dependently up to 28 h. Caspase-2 was expressed and activated in not only mitochondria and cytosol, but also in the microsomal fraction containing ER and Golgi. Of note is that overexpression of Bcl-x_L or Bcl-2 in PC12 cells markedly suppressed brefeldin A-induced activation of caspases and resulting cell death. Delivery of anti-Bcl-2 antibody into the Bcl-2-overexpressed cells again recovered apoptosis. While the brefeldin A-treatment induced the phosphorylation of both c-Jun N-terminal kinase (JNK) and p38 MAPK, overexpression of Bcl-x_L or Bcl-2 reduced the prolonged phosphorylation of JNK, but not of p38 MAPK. Pretreatment with a JNK inhibitor, SP600125, suppressed the brefeldin A-induced caspase-2 activation and cell death significantly. Thus, our results suggest that protective effects of Bcl-x_L and Bcl-2 against brefeldin A-induced cell death appear to be dependent on the regulation of JNK activation.

Key words: Bcl-2, caspase-2, cell death, endoplasmic reticulum stress, JNK.

The endoplasmic reticulum (ER) is sensitive to alterations of homoeostasis from a variety of different stimuli, such as glucose deprivation, perturbation of calcium homoeostasis and exposure to free radicals. Under such conditions, the perturbation of protein folding and accumulation of unfolded proteins occur in the ER, and this phenomenon is known as ER stress (*1*). It has been proposed that continuous ER stress results in cell death and relates to the pathogenesis of neurodegenerative diseases such as Alzheimer's and Parkinson's diseases (*2*).

Many stresses induce the activation of caspase and lead to cell death. ER stress also induces the activation of several caspases, *i.e.* caspase-7 and -12 (*3*) and caspase-4 (*4*). Ischaemic injury or amyloid β peptide may induce apoptosis through calpain-mediated caspase-12 activation (*5*, *6*). However, a functional caspase-12 is considered lost in human, since the expression of a full-length protein is inhibited by a frame-shift mutation and premature stop codon present in all splice variants (*7*). Although expression of full-length caspase-12 caused by single nucleotide polymorphism was found in people of African origin, it had no significant effect on apoptotic sensitivity (*8*). Thus, it is necessary to identify other caspases may play an important role in ER stress-induced cell death.

Recently, it has been reported that cytotoxic stress causes the activation of caspase-2, which is important to induce cell death. The transfection of small interfering RNA against caspase-2 efficiently and specifically silenced caspase-2 expression and prevented apoptosis induced by DNA-damaging agents and ultraviolet light (*9*). Caspase-2 is expressed in mitochondria and disruption of the outer mitochondrial membrane may be critical for its subcellular redistribution and activation (*10*). Interestingly, recent findings suggest that caspase-2 is also present in the nucleus and regulates mitochondrial function (*11*). Moreover, caspase-2 is expressed in the Golgi complex and cleaves goldin-160, suggesting a caspase-2-dependent transduction of apoptotic signalling through the Golgi complex (*12*). However, the correlation between the activation of caspase-2 and ER stress-induced cell death has not been elucidated.

Bcl-2 family proteins regulate mitochondria-initiated cell death pathway by modulating the mitochondrial membrane potential and permeability (*13*). Proapoptotic Bax and Bak, and Bcl-2 also localize to ER. Cells with depleted Bax and Bak, or cells with overexpressed Bcl-2, acquired resistance against cell death induced by not only mitochondrial dysfunction, but also ER stress with reduction of Ca^{2+} concentration in ER (*14*, *15*). Recently, phosphorylated Bcl-2 is localized in ER and mediates Ca^{2+} homoeostasis (*16*), and phosphatase PP2A regulates Bcl-2 phosphorylation and proteasome-mediated degradation (*17*). Although there is evidence that Bcl-2 regulates Ca^{2+} in ER, it is unknown whether and

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how Bcl-2 protects cell death triggered by unfolded protein response. Therefore, it is important to clarify the signal in both up- and down-stream of Bcl-2.

Stress-activated MAP kinases, JNK and p38, are activated by cell stress-inducing signals. Sustained activation of JNK or p38 is implicated in the induction of apoptosis (*18*). Relationship of stress-activated MAP kinases activation and Bcl-2 function is confused. The activity of Bcl-2 family proteins are regulated by posttranslational modifications, such as phosphorylation via activation of Raf-1 (*19*), mitochondria-localized pKCα (*20*), PKA (*21*) or JNK/SAPK (*22*, *23*). On the other hand, overexpression of Bcl-2 in PC12 cells blocks the activation of JNK induced by serum deprivation (*24*) and Bcl-x_L and Bcl-2 blocked thapsigargin-induced JNK activation and apoptosis (*25*). We observed that most of caspase-2 was present in cytoplasm, and partly in mitochondria and microsome. In all of the three fractions, cleaved caspase-2 and increase of caspase-2-like activity were detected by ER stress. We demonstrated here that ER stress triggers the augmentation of caspase-2 activity through the JNK activation, which is regulated by Bcl-x_L or Bcl-2.

MATERIALS AND METHODS

Cell Culture—PC12 cells were obtained from RIKEN BRC (RCB009; Tsukuba, Ibaragi) and cultured in Dulbecco's modified Eagle's medium supplemented with 5% heat-inactivated foetal calf serum, 5% heat-inactivated horse serum, 100 μg/ml streptomycin and 100 units/ml penicillin G at 37°C in an atmosphere of 5% CO_2/95% air. Cells were plated on collagen-coated dishes the day before at a density of 6.5×10^4 cells/cm^2. Plasmids, pEF–BOS, pEF–BOS–Bcl-x_L and pEF–BOS–Bcl-2, were constructed as described elsewhere (*26*). Each plasmid was cotransfected with pST-neo into cells by electroporation using the Bio-Rad Gene Pulsar apparatus at 250 μF, 0.33 kV. Selection was done using 0.8 mg/ml Geneticin® (G418, GIBCO, Rockville, MD, USA).

Antibodies (Ab) and Reagents—The antibodies used in this study were: anti-caspase-2 Ab from Pharmingen Becton Dickinson Biosciences (San Diego, CA, USA) and from Alexis Biochemicals (Lausen, Switzerland); anti-caspase-9, Bcl-2, extracellular signal-regulated kinase (ERK), JNK, p-JNK and p38 Abs from Cell Signaling (Beverly, MA); anti-GRP78, actin, Bcl-x_L,GM-130 and FITC-labelled Bcl-2 Abs from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-phospho-p38 Ab from New England Biolabs (Boston, MA); anti-caspase-3 Ab from Upstate Biotechnology (Lake Placid, NY, USA); and anti-cytochrome *c* oxidase complex IV Ab from Molecular Probes, Inc. (Eugene, Ore., USA). Ac-VDVAD-4-Methyl-Coumaryl-7-Amide (MAC), Ac-DEVD-MAC and Ac-LEHD-MAC, which were used as substrates for the determination of caspase-2-, caspase-3- and caspase-9-activity, respectively, were purchased from Peptide Institute, Inc. (Suita-shi, Osaka). z-VAD-fluoromethyl ketone (fmk), z-DEVD-fmk and z-VDVAD-fmk were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). We used BioPorter® reagent which was obtained from Gene Therapy Systems, Inc. (San Diego, CA, USA) for delivery of antibody into cells and CanGetSignal® which was obtained from Toyobo Co., Ltd. (Osaka) for antigen–antibody reaction in immunoblotting.

Preparation of Cell Lysate and Immunoblotting—Cells were lysed with lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.5% Triton X-100, 1% Nonidet P-40, 0.25% sodium deoxycholate, 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 50 μg/ml antipain and 10 μg/ml chymostatin) and centrifuged at $10{,}000 \times g$ for 10 min. When we prepared cell lysate to detection of phosphorylation, we used adding Phosphatase Inhibitor Cocktail (Nacalai Tesque, Inc., Kyoto) into lysis buffer. The supernatant was used as whole cell lysate. For the preparation of a subcellular fraction, cells were lysed in buffer A (20 mM Hepes–KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.1 mM PMSF and 250 mM sucrose) and centrifuged at $900 \times g$ for 10 min to remove nuclear material and unlysed cells. The supernatant was further centrifuged at $12{,}000 \times g$ for 15 min. The pellet was dissolved in 1% Triton X-100 lysis buffer and used as the mitochondrial fraction (mt). The supernatant was further centrifuged at $105{,}000 \times g$ for 1 h. Then, the supernatant was used as the cytosolic fraction (cy) and the pellet was dissolved in 1% Triton X-100 lysis buffer and used as the microsomal fraction (ms).

Samples were separated by SDS–polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes as described previously (*27*). We used Tris-buffered saline containing 0.5% skimmed milk or CanGetSignal® reagent for antigen–antibody reaction, immunodetection was performed using an enhanced chemiluminescence reagent, ECL (Amersham, Tokyo).

Measurement *of Caspase Activity*—The measurement of Ac-VDVAD-MAC (for caspase-2), Ac-DEVD-MAC (for caspase-3) or Ac-LEHD-MAC (for caspase-9) cleavage activities was performed as described elsewhere (*27*, *28*) with a minor modification. Cleavage of the fluorogenic peptidic substrates was monitored by AMC liberation using 380 nm excitation and 460 nm emission wavelength. Fluorescence units were converted to nmol of AMC using a standard curve generated with free AMC.

Lactate Dehydrogenase (LDH) Assay—The LDH activity released into the medium and in cells was detected using the LDH-Cytotoxic Test (Wako Pure Chemical Inc., Osaka). Procedures were followed by the manufacturer's instructions. The LDH activity of whole cell lysate dissolved by Triton X-100 was expressed as 100%. Data were collected from three independent experiments and were presented as the mean ± S.E.

Cells Staining with Hoechst Dye—Cells were fixed in 1% glutaraldehyde for 30 min at room temperature. After washing by PBS, Hoechst 33342 (Sigma-Aldrich Inc., St Louis, MO, USA) was added and monitored with a blue filter in the fluorescence microscope. Cells with fragmented nuclei were estimated as apoptotic cells.

Delivery of Antibody into Cells—Procedures were followed by the manufacturer's instructions. In brief, cells were seeded in a 24-well plate and incubated

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overnight. BioPorter® reagent was dissolved in methanol and aliquot in eppendorf tube. After methanol was evaporated, each diluted antibody solution and serum-free medium was added and incubated for 3–5 min at room temperature. Cells were washed by serum-free medium and incubated with the above mixture of reagent plus antibody for 3–4 h. We added serum with or without brefeldin A and incubated for a day.

RESULTS

Localization of Transfected Bcl-x_L and Bcl-2—PC12 cells, harbouring the human Bcl-x_L or Bcl-2 gene, were selected using geneticin and expressed proteins were detected by immunoblotting. While it has been reported that Bcl-x_L or Bcl-2 protein is expressed in the membrane of cell organelles, especially mitochondria and ER (*29*), we determined exactly where of mitochondria (mt), microsome (ms) or cytosol (cy), the transfected Bcl-x_L and Bcl-2 genes' products were located. We found that the two proteins were expressed equally in the mitochondria and microsome but not in the cytosol (Fig. 1A). Since the anti-Bcl-2 Ab used in this experiment detects only Bcl-2 of human origin, detected bands were presumed to originate from the human Bcl-2 gene. In contrast, anti-Bcl-x_L Ab recognized Bcl-x_L protein not only of human, but also of rat origin. While weak Bcl-x_L band of rat origin was induced in the Bcl-2 transfection (Fig. 1A, Bcl-2 lanes), the mechanism of its induction remains to be unknown. The marker proteins in each fraction were shown in the lower lanes; cytochrome *c* oxidase subunit IV (for mitochondria), GRP78 and GM-130 (for ER and Golgi, namely microsome), and ERK (for cytosol), which confirm the separation of these organelle. Among the transfected cells, no differences were detected in the expression of other proteins related to cell death, *i.e*. Bax or Akt (data not shown).

Protection Against Brefeldin A-induced Cytotoxicity by Bcl-x_L and Bcl-2—To evaluate the effect of brefeldin A-induced cell death, the level of LDH activity in the medium was determined (Fig. 2A). When cells were treated with 2.5 μg/ml brefeldin A for 24 h, LDH activity accounted for 60% of total LDH activity in vector-transfected cells. Given that the level of spontaneously released LDH activity was ~10%, the incidence of brefeldin A-induced cell death was estimated to be 50%. In both Bcl-x_L and Bcl-2-transfected cells, brefeldin A-induced cell death was suppressed by more than 50%. Notably, Bcl-2 reduced the cytotoxicity by brefeldin A at doses of 1–5 μg/ml. Under the same conditions, we estimated the degree of apoptotic cell death by staining with Hoechst 33342. The percentage of nucleus fragmentated cells was about 25% at 24 h after treatment with brefeldin A (2.5 μg/ml) in vector-transfected cells, but <3% in Bcl-x_L or Bcl-2-transfected cells (Fig. 2B).

In order to confirm the preventive effect of Bcl-x_L or Bcl-2 on the ER stress-induced cell death, we examined the cell death induced by thapsigargin, an inhibitor of ER-associated Ca^{2+}-ATPase. While thapsigargin (1 μg/ml) induced death in 60% of cells at 48 h, its cytotoxicity was suppressed to <20% in Bcl-x_L or Bcl-2-transfected cells (data not shown).

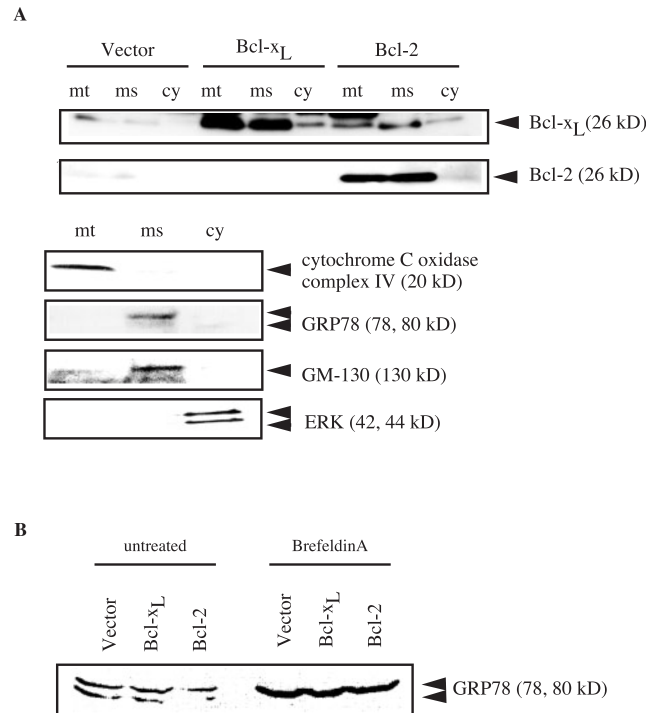

Fig. 1. **Overexpression of Bcl-x_L or Bcl-2 in PC12 cells.** (A) Transfected human Bcl-x_L or Bcl-2 gene was expressed stably in PC12 cells. Overexpressed Bcl-x_L or Bcl-2 protein was detected in mitochondria (mt), in microsome (ms) and partly in cytosol (cy). Each fraction (40 μg protein) was subjected to SDS–PAGE and examined for the presence of marker proteins, cytochrome *c* oxidase subunit IV (for mitochondria), GRP78 and GM-130 (for ER and Golgi, namely microsome) and ERK (for cytosol). (B) Whole cell lysate (20 μg protein) was prepared after 18 h of incubation with (+) or without (–) 2.5 μg/ml brefeldin A. GRP78 protein was detected by the immunoblotting. Experiments were performed three times, and the representative data was shown.

When cells are exposed to ER stress, several stress-responsive proteins are produced (*1*). As shown in Fig. 1B, brefeldin A-induced expression of GRP78, a major chaperone protein (*30*), not only in vector-transfected cells, but also in Bcl-x_L or Bcl-2-transfected cells. Therefore, the anti-apoptotic action of Bcl-x_L or Bcl-2 seems to target an area downstream of the chaperone protein's induction.

Suppression of Brefeldin A-induced Activation of Caspases-2, 3, 9 by the Overexpression of Bcl-x_L and Bcl-2—As brefeldin A-induced cell death, we examined whether it induces activation of caspases. We measured the activation of caspase-2, -3 and -9 as the cleaving capacity of the substrates, employing Ac-VDVAD-MAC for caspase-2, Ac-DEVD-MAC for caspase-3 and Ac-LEHD-MAC for caspase-9, respectively (Fig. 3A). Brefeldin A-treatment induced caspase-2, -3 and -9 activities in the vector-transfected cells with similar kinetics; their activities appearing at 12 to 18 h and increasing time-dependently up to 32 h. In the Bcl-x_L cells and Bcl-2 transfected cell, however, only marginal levels of caspase-2 and -3 activity were induced by the brefeldin A treatment. Of note, no significant activation of caspases was induced in the Bcl-2 cells during the

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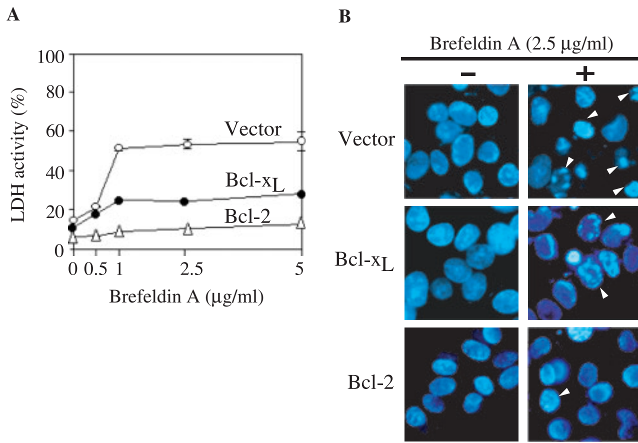

Fig. 2. **Effects of overexpression of Bcl-x_L or Bcl-2 on brefeldin A-induced cell death in PC12 cells.** (A) After cells were incubated with brefeldin A at the indicated concentrations for 24 h, cell death was evaluated by measuring the percentage of LDH activity released into the culture medium from cells. Data are shown as the mean ± S.E. ($n = 3$). (B) Cells were incubated with or without 2.5 μg/ml brefeldin A for 24 h and stained with Hoechst dye. Arrow heads show apoptotic cells. Experiments were performed three times.

incubation period. This observation was confirmed by immunoblotting. Namely, brefeldin A treatment cleaved pro-caspase-2 (48 kDa) and generated the intermediate cleaved fragment (35 kDa) and finally active form of caspase-2 (17 and 15 kDa) at 18 h in vector-transfected cells. However, these cleavage products were minimally observed in the Bcl-x_L-transfectd cells and not observed in the Bcl-2-transfected cells (Fig. 3B). Similarly, the processing of pro-caspase-3 (32 kDa) and pro-caspase-9 (51 kDa) into their cleaved forms were observed in vector-transfected cells, but not in Bcl-x_L or Bcl-2-transfected cells.

We then investigated the release of cytochrome *c* in vector- and Bcl-x_L- or Bcl-2-transfected cells after treatment with brefeldin A. Cytochrome *c* release into cytosol was induced by the brefeldin A in vector-transfected cells in a time-dependent manner, which coincided well with the decrease of cytochrome *c* in the mitochondria (Fig. 4). However, no significant, if any, release of cytochrome *c* was released in Bcl-x_L cells and Bcl-2 cells. These findings indicate that brefeldin A causes ER stress, which induces the activation of caspases and subsequent cell death concomitant with the release of cytochrome *c* into the cytosol. These events were attenuated by the overexpression of Bcl-x_L and Bcl-2.

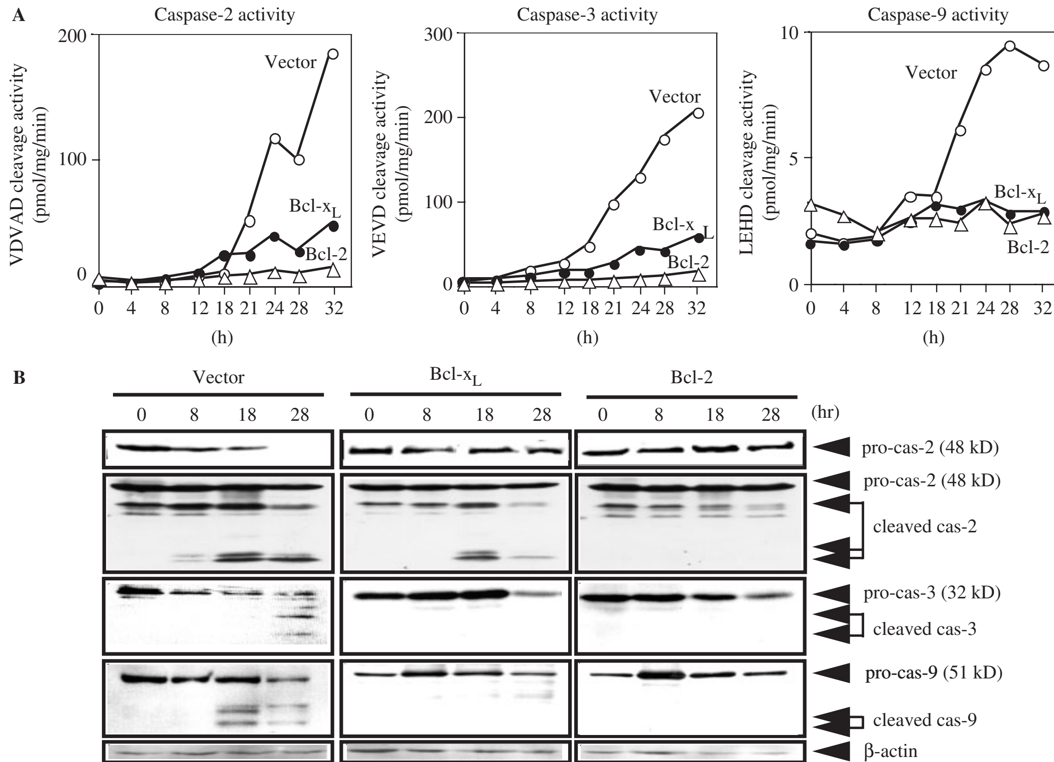

Fig. 3. **Caspase activity in PC12 cells treated with brefeldin A.** (A) Time course of caspase activities in lysate from vector-transfected cells (open circles), Bcl-x_L-transfected cells (closed circles) and Bcl-2-transfected cells (open triangles) after treatment with 2.5 μg/ml brefeldin A. Caspase activities were measured by the cleavage of substrates, Ac-VDVAD-MAC (for caspase-2), Ac-DEVD-MAC (for caspase-3) or Ac-LEHD-MAC (for caspase-9), respectively. Experiments were performed four times. (B) After treatment with 2.5 μg/ml brefeldin A, whole cell lysate (25 μg protein) was subjected to SDS–PAGE and detected procaspase-2. Also procaspase-3 or procaspase-9 and their cleaved fragments were detected by immunoblotting. To detect the cleaved fragments of caspase-2, 40 μg protein of whole cell lysate was applied and anti-caspase-2 Ab (Alexis Biochemicals) was used. Experiments were performed three times.

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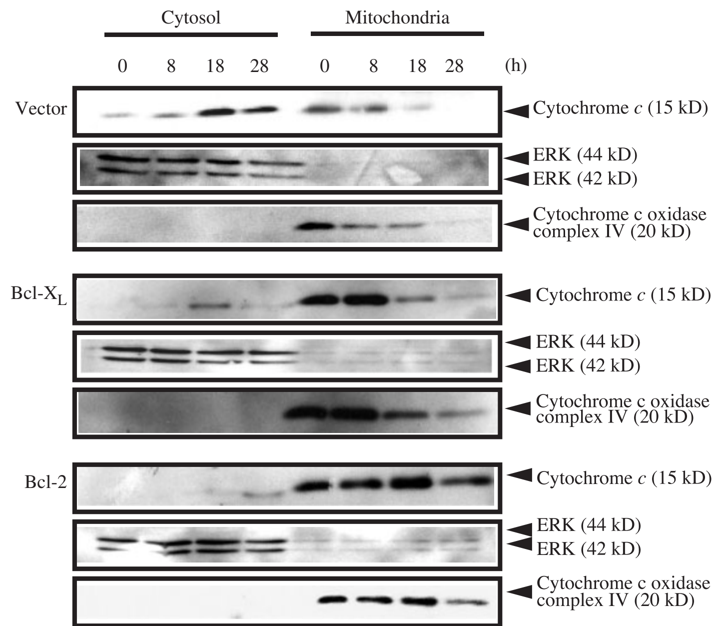

Fig. 4. **Release of cytochrome *c* in the cytosolic fraction of cells treated with brefeldin A.** Time course of cytochrome *c* release in cells treated with 2.5 μg/ml brefeldin A. After cells were separated into a mitochondrial fraction (30 μg protein used) and cytosolic fraction (50 μg protein used), we detected cytochrome *c* by immunoblotting. Cytochrome *c* oxidase (subunit IV, 20 kDa) and ERK (42, 44 kDa) were used as marker proteins of each fraction. Experiments were performed four times.

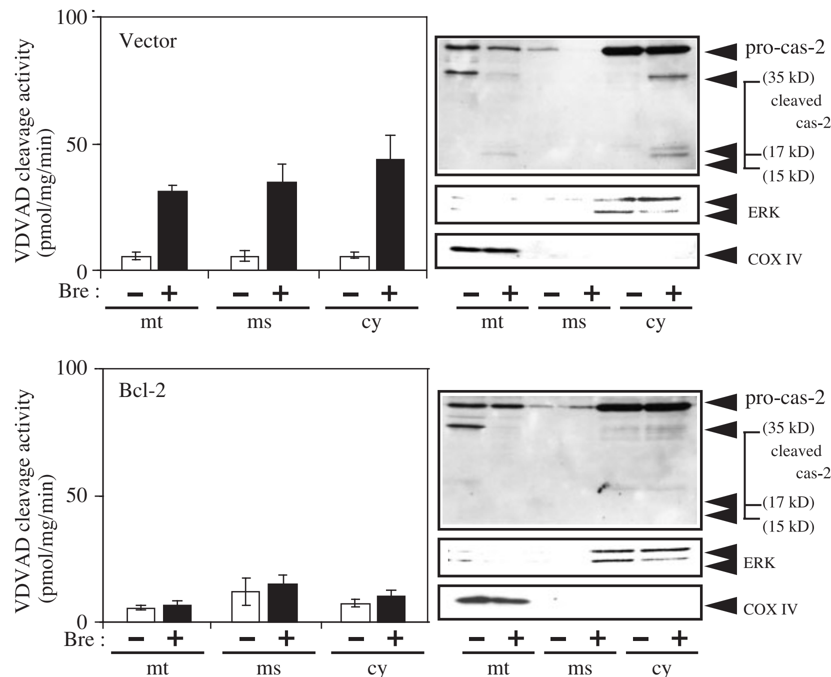

Fig. 5. **Caspase-2 activation induced by brefeldin A.** Caspase-2 activity was determined in mitochondria (mt), microsome (ms) and cytosol (cy), prepared from cells treated with 2.5 μg/ml brefeldin A (close column) or DMSO (open column) for 24 h. Data are shown as the mean ± S.E. ($n = 3$). $*p < 0.01$ *vs* DMSO. Each fraction was subjected to SDS–PAGE and detected procaspase-2 and cleaved. ERK and cytochrome *c* oxidase subunit IV (COX IV) were used as marker proteins of cytosolic and mitochondria fraction, respectively.

Localization of Caspase-2—While it has been reported that caspase-2 is distributed not only in the cytosol and mitochondria (*10*), but also in the nucleus (*11*) and Golgi complex (*12*), we determined where caspase-2 is localized and activated by brefeldin A treatment. As shown in Fig. 5, caspase-2 activity was observed ubiquitously in the mitochondria, microsome and cytosol in vector-transfected cells but not in Bcl-2-transfected cells.

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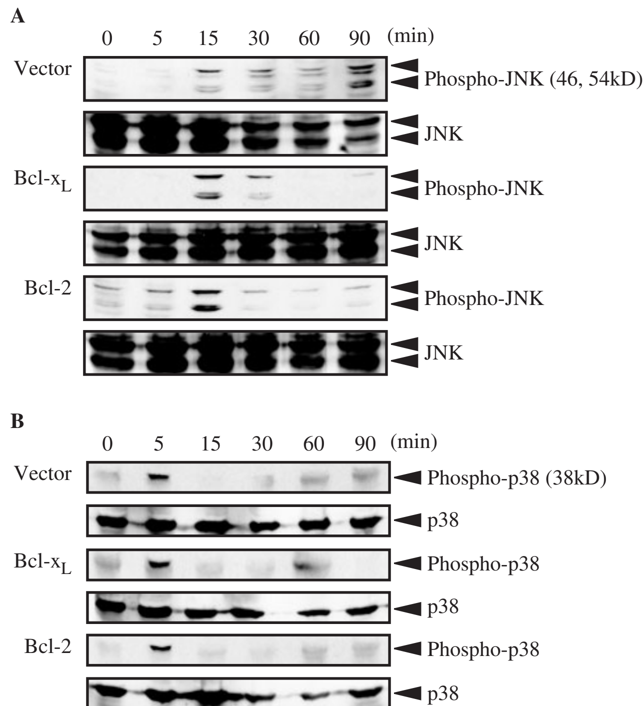

Fig. 6. **ER stress-induced phosphorylation of JNKs was inhibited in cells overexpessing Bcl-x_L or Bcl-2.** Vector-cells or cells overexpressing Bcl-xL or Bcl-2 were treated with brefeldin A (2.5 μg/ml) for the indicated time. Whole cell lysates (80 μg protein) were prepared from cells untreated or treated with brefeldin A, and the phosphorylation of JNK or p38MAPK were detected by immunoblotting. Experiments were performed three times.

By immunoblot analysis, amounts of pro-caspase-2 decreased in all three fractions and cleaved active form of caspase-2 was detected but faintly in mitochondria and cytosol fractions. In the Bcl-2 cells, no changes were observed in both levels of pro-caspase-2 and active form, except for ~35 kDa fragment detected in mitochondria. Similar observation was obtained in Bcl-x_L cells (data not shown).

Regulation of Caspase-2 by JNK—Finally, we questioned how the activation of caspase-2 occurs and is regulated. Troy *et al*. (*31*) indicated that both caspase-2 and caspsae-3 are activated downstream of JNK's activation. In addition, ER stress-inducers including thapsigargin and tunicamycin are known to activate JNK via ASK1 and also induces the activation of p38 MAPK (*32*). Therefore, we examined whether brefeldin A activates JNK or p38 MAPK. As shown in Fig. 6A, brefeldin A-induced marked phosphorylation of JNK at 15 min and its phosphorylation was prolonged up to 90 min in vector-transfected cells. In Bcl-x_L- or Bcl-2-transfected cells, brefeldin A-induced JNK phosphorylation was also detected at 15 min, while the prolonged phosphorylation was suppressed. Transient phosphorylation of p38 MAPK was observed at 5 min and re-phosphorylation was detected slightly from 60 to 90 min, in vector-transfected cells. In Bcl-x_L or Bcl-2-transfected cells, however, the brefeldin A-induced phosphorylation of p38 MAPK was not much suppressed (Fig. 6). These results that the phosphorylation of JNK caused by ER stress well paralleled with the activation of caspase-2 and caspase-3 as shown in Fig. 3.

Then, we investigated the effect of SP600125, a selective JNK inhibitor (*33*), on the activation of caspase-2 or caspase-3 caused by brefeldin A. Pretreatment with SP600125 (3 μM) reduced both the capase-2 and caspase-3 activities induced by brefeldin A by more than 60–70%, while a p38MAPK inhibitor, SB203580 failed to suppress the activation of these caspase activities (Fig. 7A). In addition, SP600125 reduced brefeldin A-induced cell death, more potently than SB203580.

Thus, these results indicate that brefeldin A-induced activation of caspase-2 and caspase-3 requires JNK activation but not p38 MAPK. Protection of cell death by a JNK inhibitor indicates that the brefeldin A-induced cell death is dependent on the JNK pathway rather than p38 MAPK.

Finally, we examined whether protection against brefeldin A-induced cell death in Bcl-2-transfected cell is dependent on the overexpression of Bcl-2 protein of human origin (Fig. 8). We used BioPorter reagent in order to deliver anti-Bcl-2 antibody into cells as described in the section 'Materials and Methods'. It has been reported that 50% of the input fluorescence is in the cytosol/membrane fraction when FITC-labelled IgG is added onto cultured cells with this reagent (*34*). When cells were incubated with BioPorter reagent plus FITC-labelled anti-Bcl-2 antibody, fluorescence was observed markedly in cytosol, but not in nuclear. Delivery of 2 μg anti-Bcl-2 antibody, which reacts with human origin but not rodent, again induced apoptosis in Bcl-2-transfected cells. This result shows that overexpressed Bcl-2 is involved in brefeldin A-induced cell death.

DISCUSSION

We reported here that brefeldin A treatment resulted in cell death, which accompanied the activation of caspase-2 and -3 via JNK pathway. In addition, overexpression of Bcl-x_L or Bcl-2 suppressed the activation of caspases, protecting cell death possibly by reducing JNK activity. Caspase-2 is activated in many cell types in response to various apoptotic stimuli, including growth factor withdrawal (*35*), DNA-damaging agents (*9*, *36*), TNF-α (*37*), Fas ligation (*38*) and ischaemia (*39*). Pro-caspase-2 has characteristics of an initiator caspase, since it contains a large N-terminal prodomain with a caspase-associated recruitment domain (CARD) and the ability to interact through this CARD with the TNF-R1-associated adaptor protein CRADD (*40*). Thus, recent reports postulated that caspase-2 is the initiator caspase acting upstream of the effecter caspases including caspase-3. We attempted to clarify the relationship between caspase-2 and caspase-3. However, we could not conclude that caspase-2 is located upstream of caspase-3 at least by the kinetics, since activation of the two caspases started almost simultaneously and was hard to separate (Fig. 3). In addition, a caspase-2

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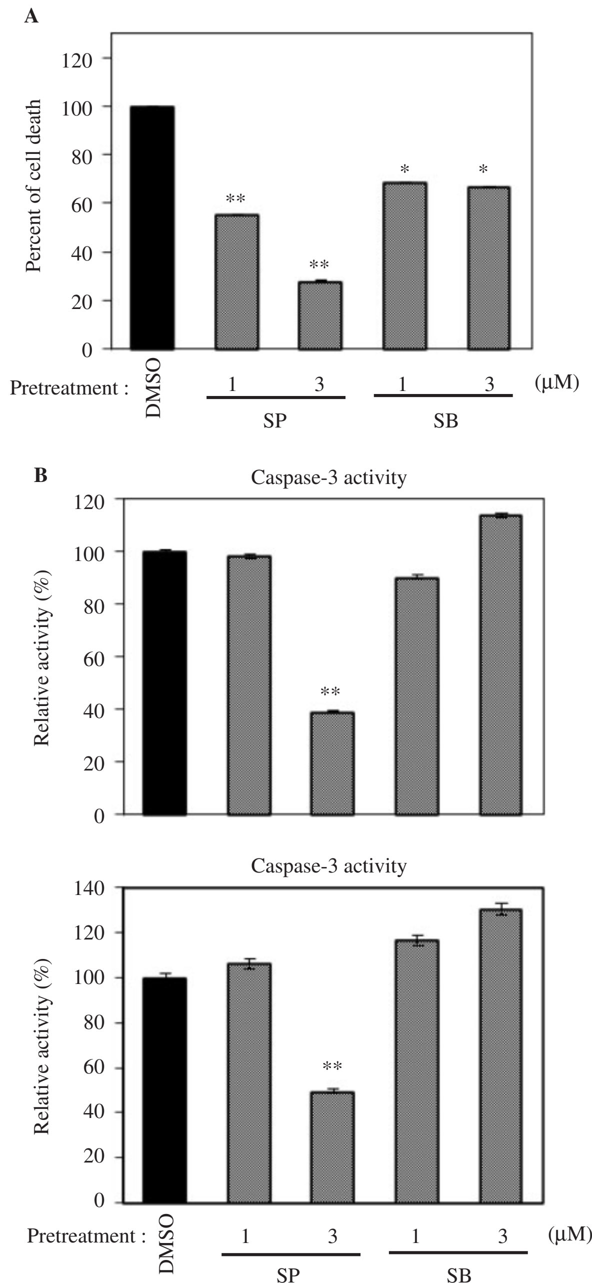

Fig. 7. **Effects of kinase inhibitors on brefeldin A-induced cell death and caspase activation.** Cells were pretreated with SP600125 (1, 3 μM) or SB203580 (1, 3 μM) for 4 h, and the cells were incubated with brefeldin A for a further 24 h. In the absence of inhibitor (shown as 'DMSO'), (A) percentage of cell death or (B) caspase activity induced by brefeldin A was set as 100%. Data were calculated as the relative activity (%) and are shown as the mean ± S.E. ($n = 3$). $^{**}p < 0.01$ *vs* DMSO, $^{*}p < 0.05$ *vs* DMSO.

inhibitor (z-VDVAD-fmk) did not reduce caspase-3 activation and *vice versa* (data not shown). So, we assumed that the activation of caspase-2 and caspase-3 occurred simultaneously and independently.

It has been demonstrated that ER stress induces the expression of caspase-12 and also leads to the translocation of cytosolic caspase-7 to the ER surface. Caspase-7 associates with procaspase-12 and cleaves its prodomain to generate an active caspase-12 (*3*). Caspase-12 cleaves pro-caspase-9 at the processing site for activation (*41*). We also observed the cleavage of pro-caspase-12 and increase of caspase-7 activity (DQTD cleavage activity) induced by brefeldin A in vector cells (data not shown). Caspase-7 activity was increased about 2-fold at 8 h, 7-fold at 18 h and 8-fold at 24 h after brefeldin A treatment (at 0 time was 8–10 pmol/mg/min). However, the relationship among the activation of caspase-7, -9, -12 and caspase-2 is largely unknown.

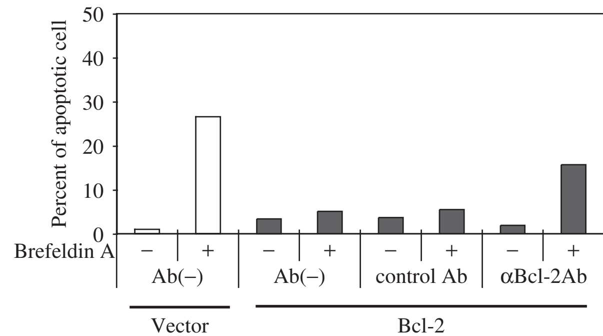

Fig. 8. **Effect of antibody introduction into cells on brefeldin A-induced cell death.** Delivery of antibody was performed as described in the section 'MATERIALS AND METHODS'. Cells were treated with or without brefeldin A (2.5 μg/ml) for 24 h following incubation with BioPorter reagent and/or antibodies. Control Ab (mouse Ig; 2 μg) or FITC-labelled anti-human Bcl-2 antibody (2 μg) was used. After brefeldin A treatment, cells were fixed and stained with Hoechst dye. Percent of apoptotic cell was estimated by counting 200–300 cells. Experiments were performed three times and representative data was shown.

Two alternatively spliced isoforms, caspase-2L and caspase-2S, with opposing effects on cell death have been identified (*42*), a short form (2S) as antiapoptotic and a long form (2L) as proapoptotic (*43*). We found that a long form is degraded into active fragments and the degradation is almost suppressed by the Bcl-x_L and particularly by Bcl-2 (Fig. 3B). As for the substrate specificity, VDVAD is defined and widely used as an optimal substrate for caspase-2, yet this substrate is not specific to caspase-2 alone but is cleaved by caspase-3 and -7 (*44*). So, we provided alternative data on the cleavage by western blot analysis. On the subcellular localization of caspase-2, study using multiple antibodies indicates its localization in nuclei, cytotosol and Golgi, without much evidence in mitochondria, while another study shows evidence that caspase-2 and -9 are released from mitochondria (*43*). These differences may be because of the variation of the cells observed.

Cytotoxic stress causes the activation of caspase-2, which is required for the permeabilization of mitochondria and induces the release of cytochrome *c* (*11*, *36*, *45*). But cleavage of caspases and poly (ADP-ribose) polymerase was observed in cell-free extract lacking cytochrome *c* that was isolated from brefeldin A-treated cells (*3*). In addition, caspase-2 is spontaneously recruited to a large protein complex independent

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of cytochrome *c* and Apaf-1. Since this recruitment of caspase-2 is sufficient to mediate its activation, caspase-2 is activated by oligomerization independent of the Apaf-1 apoptosome (*46*). Therefore, the machinery for the activation of caspase-2 comprises two signalling pathways, *i.e*. one dependent on apoptosomes via mitochondria and other based on the formation of oligomers. In our experiment, brefeldin A-induced caspase-2 activation seems to be dependent on the mitochondrial pathway, as an apparent release of cytochrome *c* is observed. Moreover, recent reports suggest that activation of initiator caspases (caspase-8, -9 and -2) was not dependent on proteolytic cleavage (*47–49*). Exactly, pro-caspase-2 is able to stimulate mitochondrial release of cytochrome *c* (*48*); however, this effect is not suppressed by Bcl-2. In another report (*49*), pro-caspase-2 is trapped using biotinylated VAD-fmk during heat-shock-induced apoptosis, but not by ER stress. And in this case, Bcl-2 blocks heat-shock-induced apoptosis, but not activation of pro-caspase-2. We detected increase of caspase-2 activity connect to its cleavage, and Bcl-2 overexpression suppressed clearly its cleavage and increase of activity. Then, under our experimental conditions, we thought caspase-2 activation was occurred dependent on its cleavage.

Stress in the ER leads to the accumulation of unfolded proteins in that compartment, and activates ER transmembrane signal transducers, *i.e*. RNA-dependent protein kinase-like ER eIF2a kinase, inositol requiring ER transmembrane RNase a isoform (IRE1α) and activating transcription factor 6 (*50–52*). ER stress induced the activation of JNKs through formation of an IRE1-TRAF2-apoptosis signal-regulating kinase (ASK1) complex and cell death (*53*, *54*). Recently it was reported that machinery downstream of JNKs activates caspases via the translocation of Bax to mitochondria from the cytosol (*55*, *56*). The translocated Bax induces an increase of mitochondrial membrane permeabilization and cytochrome *c* release. We observed that brefeldin A induced the release of cytochrome *c* into cytosol and overexpression of Bcl-2 or Bcl-x_L inhibited its release. Our notion is that Bcl-2 inhibits the release of cytochrome *c* and then prevents cell death. Furthermore, our results suggest that the suppression of caspase activation by Bcl-2 resulted from the inhibition of JNK activation, which is an earlier event than the release of cytochrome *c*. Similar results have been reported previously; overexpression of Bcl-2 in PC12 cells blocked the activation of JNK (at 2–8 h) induced by serum deprivation (*24*) and Bcl-x_L and Bcl-2 blocked thapsigargin-induced JNK activity and apoptosis (*25*). Further study is needed to clear the causality of two phenomena, suppression of JNK activity, especially at late phase, and resistance against cell death, in Bcl-2 overexpressing cells. While no direct interaction of Bcl-x_L or Bcl-2 with JNKs has been demonstrated, Bcl-x_L, Bcl-2 or their associated proteins may interfere with the upstream signals leading to the JNK activation. This notion remains to be tested.

Interestingly, exposure to β-amyloid also causes a rapid activation of the JNK pathway in PC12 cells, and caspase-2 and caspase-3 are activated downstream of JNK (*31*, *57*). Therefore, unfolded proteins including β-amyloid can cause the activation of caspase-2 and subsequent cell death. Our findings on the protective effect of Bcl-x_L or Bcl-2 provide new insights into the ER stress-induced activation of JNKs and caspase and cell damage.

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