Subcellular Fractionation—For preparation of digitonin-lysed cell fractions, cells were washed with PBS and suspended in PBS followed by lysis with 0.3 mg/ml digitonin for 3 min at 37 °C and centrifuged at 12,000 × *g* for 5 min at 4 °C. The pellet and supernatant fractions were used for immunoblot analysis with the pellet fraction including nuclei and heavy membranes and the supernatant fraction containing cytoplasm and light membranes.

For preparation of subcellular fractions, cells were washed with PBS and suspended in hypotonic solution (10 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 42 mM KCl, 10 μM lactacystin) for 5 min on ice. Cells were lysed using a Dounce homogenizer and centrifuged at 600 × *g* for 10 min to collect crude nuclei that were further purified as described below. The supernatant was further centrifuged at 100,000 × *g* for 90 min. The pellet and supernatant were used as membrane and cytoplasmic fractions, respectively. The crude nuclear fraction was passed through a 27-gauge needle several times, extensively washed with hypotonic solution, and centrifuged through a 2 M sucrose cushion at 150,000 × *g* for 60 min. The pellet was used as a purified nuclear fraction. Nonidet P-40-treated nuclei were prepared by washing the purified nuclei with hypotonic solution containing 0.5% Nonidet P-40 to remove contaminating membranes.

Antibodies—Anti-active caspase-3 polyclonal (2622) and monoclonal (CS-1) antibodies, which recognize caspase-3-p12, were generated (see supplemental material). Anti-caspase-3 monoclonal antibody (C31720) was obtained from Transduction Laboratories; anti-caspase-3 polyclonal antibodies (sc-1224), anti-PKC δ polyclonal antibodies (sc-937), and anti-lamin B1 polyclonal antibodies (sc-6217) were from Santa Cruz Biotechnology; anti-active caspase-3 pAb (G7481) was from Promega; anti-green fluorescent protein (GFP) monoclonal antibody (8371) from Clontech; and anti-caspase-3 polyclonal antibodies (9662) was from Cell Signaling Technology.

Fluorescence Microscopy—For immunofluorescence analysis, cells were fixed with 3.7% formaldehyde in PBS for 10 min, washed with PBS twice, permeabilized in 0.5% Triton X-100 in PBS for 10 min, and washed with PBS twice. Cells were then incubated with primary antibodies in PBS containing 1% BSA overnight at 4 °C. After washing with PBS twice, cells were incubated with Texas red (TXRD)-, fluorescein isothiocyanate-, Alexa Fluor 488-, or Cy3-labeled secondary antibodies for 10 min at room temperature and washed with PBS twice. After staining nuclei with 10 μM Hoechst 33342 (Calbiochem), cells were examined under a fluorescence microscope (Leitz Laborlux) or a confocal laser scanning microscope (Carl Zeiss).

Plasmid Constructions—The wild type procaspase-3 cDNA fragment was cloned into the EcoRI site of pUC-CAGGS (17) to generate pCAG-casp3. To construct expression plasmids for caspase fused to the N terminus of GFP, PCR was carried out using caspase-3 and caspase-7 cDNAs as templates. The fragments encoding caspase-3 or caspase-7 were cloned into the EcoRI-BamHI site of pEGFP-N1 (Clontech) to generate pcasp3-Wt-GFP or pcasp7-Wt-GFP. To generate C163S, D175A, R64E, and R207E mutations, a PCR method employing mutagenic oligonucleotide primers was used. Caspase-3 cDNAs containing these mutations were cloned into the EcoRI-BamHI site of pEGFP-N1 to generate pcasp3-C163S-GFP, pcasp3-D175A-GFP, pcasp3-R64E-GFP, and pcasp3-R207E-GFP.

RESULTS AND DISCUSSION

Although it has been widely accepted that procaspase-3 is cleaved to generate the active form in the cytoplasm, the enzymatic activity of capase-3-like proteases can be found in the nuclear fraction of apoptotic cells (18–20). It is, however, necessary to determine whether activated caspase-3 itself is present in the nuclei of apoptotic cells, as opposed to other caspases such as caspase-7 and -8 that can also cleave caspase-3 substrates (21, 22). The p17 subunit of active caspase-3 is detected not only in the cytoplasmic and mitochondrial fractions but also in the nuclear fraction of apoptotic cells (23). However, the localization of the p12 subunit of active caspase-3 remained to be determined, which is important, because caspase-3 enzymatic activity requires both p17 and p12 subunits. Therefore, we initially investigated the localization of the caspase-3-p12 subunit. HepG2 cells treated with or without an agonistic anti-Fas antibody were separated into pellet and supernatant fractions after lysis with digitonin, followed by immunoblotting (Fig. 1*A*). Although procaspase-3 was present in the supernatant fraction irrespective of induction of apoptosis, the caspase-3-p12 subunit was present in both the pellet fraction (including nuclei) and the supernatant fraction after induction of apoptosis. Subcellular fractionation was used to confirm the nuclear localization of active caspase-3 in apoptotic cells. Since active caspase-3 may be degraded by the ubiquitin-proteasome pathway (24–26), procaspase-3 was transiently overexpressed to elevate the expression levels of caspase-3, and the preparation of subcellular fractions was carried out in the presence of a proteasome inhibitor, lactacystin; PKC δ and lamin B1 were used as cytoplasmic and nuclear fraction markers, respectively. Normal non-transfected HepG2 cells (Fig. 1*B*, *left panel*) or transfected HepG2 cells treated with the anti-Fas antibody for 12 h (Fig. 1*B*, *right panel*) were fractionated into nuclear, membrane, and cytoplasmic fractions. Although the expression level of procaspase-3 in transfected cells was more than five times higher than in non-transfected cells, the cytoplasmic localization of procaspase-3 was unaffected by overexpression. Both caspase-3-p17 and caspase-3-p12 were detected not only in the cytoplasmic fraction but also in the nuclear fraction even after treatment with 0.5% Nonidet P-40 (Fig. 1*B*, *right panel*). These results strongly suggested that active caspase-3 was present in the nuclei of apoptotic cells.

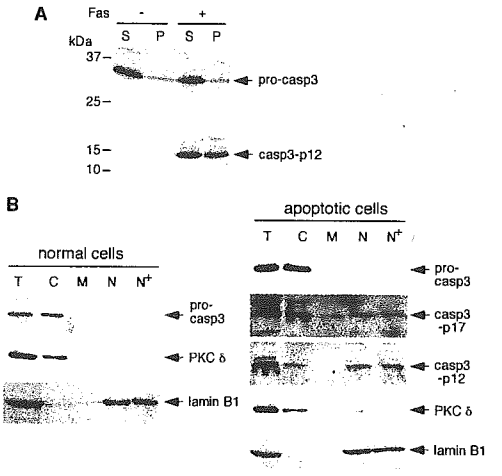

FIG. 1. **Nuclear localization of caspase-3-p17 and caspase-3-p12 subunits in apoptotic HepG2 cells.** *A*, detection of caspase-3-p12 subunit in HepG2 cells. HepG2 cells were treated with or without the anti-Fas antibody in the presence of actinomycin D for 12 h, and pellet (*P*) and supernatant (*S*) fractions were prepared after lysis with digitonin. Each fraction was subjected to SDS-PAGE and immunoblotted with anti-caspase-3 polyclonal antibodies (sc-1224 from Santa Cruz Biotechnology), which detect both procaspase-3 and caspase-3-p12. *B*, subcellular fractionation of normal (*left panel*) and apoptotic (*right panel*) HepG2 cells. Subcellular fractions from apoptotic HepG2 cells were prepared after transfection with pCAG-casp3 followed by incubation for 24 h and treatment with the anti-Fas antibody in the presence of actinomycin D for 12 h. Subcellular fractions, standardized to represent equal numbers of cells in each fraction, were subjected to SDS-PAGE and immunoblotted with anti-caspase-3 monoclonal antibody (C31720 from Transduction Laboratories), anti-caspase-3 antibody (9662 from Cell Signaling Technology), anti-caspase-3 polyclonal antibodies (sc-1224 from Santa Cruz Biotechnology), anti-PKC δ polyclonal antibodies, or anti-lamin B1 polyclonal antibodies. *T*, total cell lysate; *C*, cytoplasmic fraction; *M*, membrane fraction; *N*, purified nuclear fraction; N^+, purified nuclear fraction after treatment with 0.5% Nonidet P-40.

To directly assess the localization of active caspase-3, we prepared antibodies specific for active caspase-3-p12 subunit. Since procaspase-3 is processed at Asp^{28} and Asp^{175} sites to generate new N and C termini, we generated four affinity-purified polyclonal and three monoclonal antibodies that specifically recognize the newly exposed N-terminal region of caspase-3-p12 (see supplemental material). We also used the commercially available anti-active caspase-3 specific antibodies (G7481, Promega) that recognize the newly exposed C terminus of caspase-3-p17. Al-

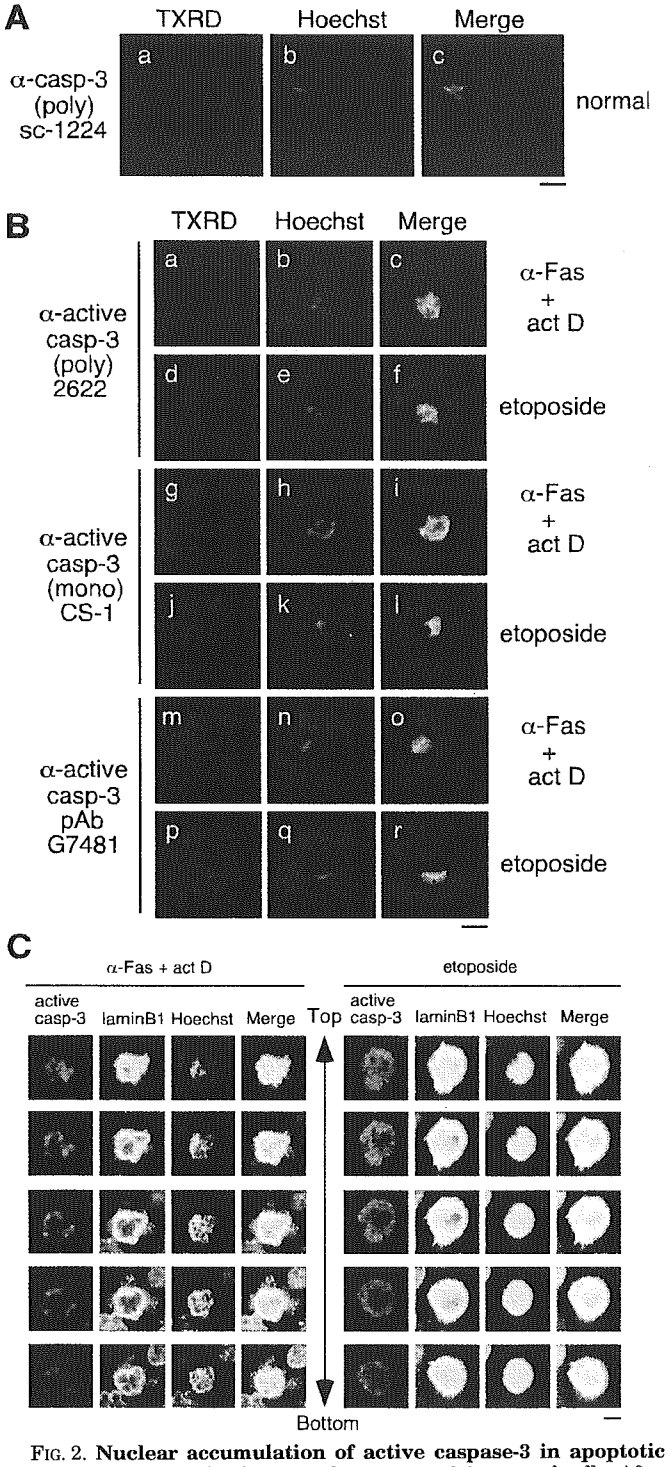

FIG. 2. **Nuclear accumulation of active caspase-3 in apoptotic cells.** *A*, cytoplasmic localization of procaspase-3 in normal cells. After fixation and permeabilization, HepG2 cells were incubated with anti-caspase-3 polyclonal antibodies (sc-1224 from Santa Cruz Biotechnology) and TXRD-labeled secondary antibodies (*panel a*), followed by staining the nuclei with Hoechst 33342 (*panel b*). Photographs were taken of the same fields in *panels a* and *b*, and merged images of TXRD and Hoechst staining are shown in *panel c*. *B*, accumulation of active caspase-3 around the apoptotic nuclei. HepG2 cells were treated with the anti-Fas antibody in the presence of actinomycin D (*act D*) for 12 h (*panels a–c, g–i,* and *m–o*) or with etoposide for 40 h (*panels d–f, j–l,* and *p–r*). After fixation and permeabilization, the cells were incubated with anti-active caspase-3 polyclonal antibodies (2622) (*panels a* and *d*), anti-active caspase-3 monoclonal antibody (CS-1) (*panels g* and *j*), anti-active caspase-3 pAb (G7481 from Promega) (*panels m* and *p*), and TXRD-labeled secondary antibodies (*panels a, d, g, j, m,* and *p*), followed by staining the nuclei with Hoechst 33342 (*panels b, e, h, k, n,* and *q*). Photographs were taken of the same fields in *panels a–c, d–f, g–i, j–l, m–o,* and *p–r*, and merged images of TXRD and Hoechst staining are shown in *panels c, f, i, l, o,* and *r*, respectively. *C*, detection of active caspase-3 in apoptotic nuclei. HepG2 cells were treated with the anti-Fas antibody in the presence of actinomycin D (*act D*) for 12 h or with etoposide for 40 h. After fixation and permeabilization, the cells were incubated with anti-active caspase-3 pAb (G7481 from Promega) and anti-lamin B1 polyclonal antibodies as a nuclear envelope marker, and Cy3- and Alexa Fluor 488-labeled secondary antibodies, followed by staining the nuclei with Hoechst 33342, and observed with confocal laser scanning microscope. The z-series of images from five sections are shown. *Bars*, 10 μm.

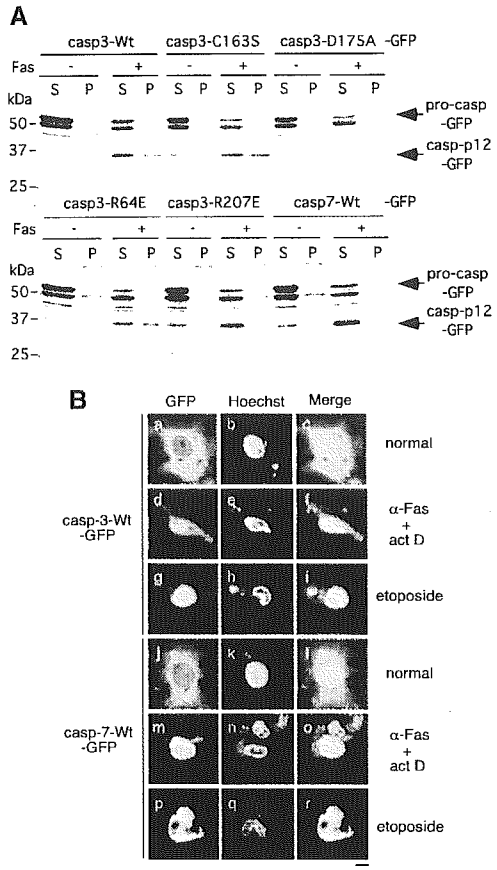

FIG. 3. **Nuclear translocation of active caspase-3 requires proteolytic activation and substrate recognition, but not catalytic activity.** *A*, localization of caspase-3- and caspase-7-GFP fusion proteins in HepG2 cells. One day before transfection, HepG2 cells were seeded at a density of 2×10^5 cells per well in 6-well dishes. In each well, 2 μg of plasmids expressing caspase-GFP fusion proteins were transfected and incubated for 24 h. After treatment with or without the anti-Fas antibody in the presence of actinomycin D (*act D*) for 12 h, cells were fractionated after lysis with digitonin and subjected to SDS-PAGE and immunoblotted with anti-GFP monoclonal antibody. *S*, supernatant fraction; *P*, pellet fraction. *B*, nuclear localization of caspase-3-, but not caspase-7-, GFP fusion protein in apoptotic cells with confocal laser scanning microscopy. One day before transfection, HepG2 cells were seeded at a density of 2×10^5 cells per 35-mm glass bottom dish. In each dish, 2 μg of pcasp3-Wt-GFP (*panels a–i*) or pcasp7-Wt-GFP (*panels j–r*) plasmids were transfected and incubated for 24 h. After treatment without (*panels a–c* and *j–l*) or with the anti-Fas antibody in the presence of actinomycin D (*act D*) for 12 h (*panels d–f* and *m–o*) or with etoposide for 40 h (*panels g–i* and *p–r*), GFP expression (*panels a, d, g, j, m,* and *p*) was observed with confocal laser scanning microscope after staining with Hoechst 33342 (*panels b, e, h, k, n,* and *q*). Photographs were taken of the same fields in *panels a–c, d–f, g–i, j–l, m–o,* and *p–r*, and merged images of GFP and Hoechst staining are shown in *panels c, f, i, l, o,* and *r*, respectively. *Bar*, 10 μm.

though procaspase-3 was detected in the cytoplasm of untreated normal cells (Fig. 2*A*), in most apoptotic cells stained with anti-active caspase-3 antibodies, which recognize the newly exposed N terminus of caspase-3-p12 (Fig. 2*B*, *panels a–l*) or the newly exposed C terminus of caspase-3-p17 (Fig. 2*B*, *panels m–r*), the signal was strongest around the condensed nucleus. To deter-

mine whether active caspase-3 is actually present in the nucleus, apoptotic cells were stained with the anti-active caspase-3 antibodies and antibodies against lamin B1, a nuclear envelope marker, and analyzed by confocal laser scanning microscopy (Fig. 2*C*). The signal for active caspase-3 was detected both in and around the condensed nuclei. Taken together, these results indicate that active caspase-3 is present not only in the cytoplasm but also in the nuclei of apoptotic cells.

To examine the molecular mechanisms governing translocation of caspase-3 from the cytoplasm into the nucleus, we constructed various procaspase-3 mutants fused with GFP. These constructs were transiently transfected into HepG2 cells. After treatment with or without the agonistic anti-Fas antibody to induce apoptosis, supernatant and pellet fractions were prepared after digitonin lysis and immunoblotted with the anti-GFP antibody (Fig. 3*A*). Although casp3-Wt-GFP was predominantly present in supernatant, like the unprocessed form before Fas treatment, proteolytically activated casp3-p12-GFP was recovered in both the supernatant and pellet fractions after Fas treatment (Fig. 3*A*). In contrast, caspase-7, another effector caspase, did not translocate to the nucleus, with both casp7-Wt-GFP and casp7-p12-GFP being detected only in the supernatant fraction irrespective of Fas treatment. Consistently, confocal laser scanning microscopy indicated that casp3-Wt-GFP was present in both cytoplasm and nuclei, whereas casp7-Wt-GFP was found only in the cytoplasm in apoptotic cells (Fig. 3*B*). These results suggested that the nuclear translocation of effector caspases is specific for caspase-3 and that the nuclear translocation of caspase-3 is an active process and not simply entail diffusion after disruption of the nuclear-cytoplasmic barrier.

To determine whether caspase-3 activation is needed for nuclear translocation, we constructed procaspase-3 mutants that cannot be activated, recognize substrates, or cleave substrates, respectively. Casp3-D175A-GFP, which is not activated due to mutation of the cleavage site between the p17 and p12 subunits, did not translocate after anti-Fas treatment, suggesting that the proteolytic activation of caspase-3 is necessary for its nuclear translocation (Fig. 3*A*). The three-dimensional structure of caspase-3 (27, 28) shows that Arg^{64} and Arg^{207} are essential for recognition of the P1 and P3 amino acids of the DXXD cleavage motif substrates, respectively. Cell fractionation analysis showed that casp3-R64E-GFP, but not casp3-R207E-GFP, translocated from cytoplasm to nuclei after Fas treatment, indicating that recognition of P3, but not P1, of a substrate(s) is necessary for nuclear translocation of active caspase-3 (Fig. 3*A*). Furthermore, casp3-C163S-GFP, in which the catalytic Cys is mutated, translocated from the cytoplasm into nuclei, indicating that substrate cleavage by caspase-3 is not essential for the translocation of active caspase-3. These results suggested that proteolytic activation and substrate recognition, but not substrate cleavage, are necessary for the nuclear translocation of active caspase-3.

Nuclear pore complexes (NPCs) mediate bidirectional transport between the cytoplasm and the nucleus (29). The NPC constitutes a passive diffusion channel, which allows the diffusion of ions, metabolites, and small proteins whose relative molecular mass is less than about 40 kDa. Proteins above the size limit can enter the nucleus by energy-dependent mechanisms. Caspase-3 lacks a typical consensus nuclear localization signal, and the active caspase-3 tetramer is too big to enter the nucleus passively. Recently, Faleiro and Lazebnik (16) reported that caspase-9 inactivates nuclear transport and increases the diffusion limit of the nuclear pore, leading to the entrance of caspase-3 into nuclei by diffusion. If caspase-3 enters the nucleus by simple diffusion, procaspase-3 as well as active caspase-3 and other caspases including caspase-7 would also be detected in the nuclear fraction of apoptotic cells. However, our data showed that neither procaspase-3 nor caspase-7 translocated into nuclei after apoptosis induction. Furthermore, active nuclear transport is required for the nuclear morphological changes induced by various apoptotic stimuli (30), and nuclear translocation of active caspase-3 required proteolytic activation and recognition of substrate-like protein(s). Therefore, we propose that the nuclear translocation of active caspase-3 is dependent on active nuclear transport. Identification of the substrate-like protein(s) which function as a carrier protein to transport active caspase-3 from the cytoplasm into nucleus in apoptotic cells is needed to clear the molecular mechanisms of nuclear translocation of active caspase-3.

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BH4 peptide derivative from Bcl-xL attenuates ischemia/reperfusion injury thorough anti-apoptotic mechanism in rat hearts

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Abstract

Objective: To prevent apoptosis is thought to be promising for myocardial protection in cardiac surgery. Recently, we showed that BH4 domain of Bcl-xL is essential for the prevention of apoptosis, and that BH4 fused to HIV TAT protein (TAT-BH4) prevented apoptotic cell death. Then, we hypothesized TAT-BH4 may attenuate ischemia/reperfusion injury in rat hearts. **Methods:** The isolated rat hearts in the TAT-BH4 preconditioning group (BH4 group, $n=8$) or control group (C group, $n=8$) were subjected to warm ischemia (37 °C) for 30 min followed by 60 min of reperfusion using Langendorff perfusion system. **Results:** Left ventricular developed pressure and maximum dP/dt after reperfusion were significantly improved in the BH4 group than those in the C group ($P<0.01$). Recovery of mitochondrial respiration was significantly better in the BH4 group ($P<0.05$). Moreover, expression of caspase-3 and TUNEL-positive myocardium were decreased in the BH4 group than those in the C group. **Conclusions:** These results demonstrated that TAT-BH4 attenuates myocardial ischemia/reperfusion injury through preventing myocardial apoptosis. Thus, TAT-BH4 may be a novel therapeutic agent for myocardial protection in cardiac surgery.

Keywords: Apoptosis; Cardiomyocytes; Ischemia/reperfusion injury; Heart surgery

1. Introduction

Recent advances in myocardial protection have improved the clinical results in open-heart surgery. However, severely critical cases associated with compromised heart, such as failing heart or post-ischemic conditions, still occur, and thus, further attempts to improve myocardial protection should be addressed.

Recently, a growing body of evidence have shown that apoptosis of myocardium is one of the major contributors to ischemic/reperfusion injury in experimental models [1-5] and even in humans after open-heart surgery [6,7]. Therefore, many attempts through molecular mechanism to attenuate apoptosis of myocardium in ischemia/reperfusion injury have been reported [8-15]. However, no pharmacological strategy has been reported to attenuate apoptosis in the heart. Moreover, strategy using gene transfection during ischemia has limitations because it takes few ours to express proteins after reperfusion, which is not suitable for clinical application of myocardial protection against acute ischemic reperfusion injury.

We recently demonstrated that the biochemical role of the conserved N-terminal homology domain (BH4) of Bcl-xL is essential for the prevention of apoptosis, with respect to the regulation of mitochondrial membrane permeability and found that BH4 was required for Bcl-xL to prevent cytochrome *c* release. Using a newly developed TAT protein transduction system, which is the protein transduction domain of human immunodeficiency virus type 1 Tat protein (HIV TAT protein), we also showed that the BH4 domain fused to TAT protein (TAT-BH4), effectively prevented apoptotic cell death in vitro [16], and showed feasibility of protein transduction into cells in vivo. Chen et al. demonstrated TAT-BH4 attenuated myocardial infarction in vivo [17]. Therefore, it is expected that preconditioning of TAT-BH4 may attenuate ischemia/reperfusion injury of the myocardium during open heart surgery.

In this study, we investigated whether the preconditioning of TAT-BH4 may attenuate ischemia/reperfusion injury in isolated rat heart model as a pre-clinical trial.

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2. Method

2.1. Test compounds

TAT-BH-4 protein and TAT mutant protein were provided by Shionogi Pharmacy Co., Ltd, Osaka, Japan. The proteins were dissolved in DMSO to the concentration of 5 μg/μl before use.

2.2. Pharmacological preconditioning and rat ischemia model

Sixteen Sprague-Dawley rats (300 g, male) were used for this study. Humane animal care complied with the 'Principle of Laboratory Animal Care' formulated by the National Society for Medical Research and the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resource and published by the Institutes of Health (NIH Publication No. 86-23, revised 1985). The rats were divided into the control group (C group, $n=8$), and the TAT-BH-4 group (BH4 group, $n=8$). All rats were anesthetized by intra-peritoneal injection of sodium pentobarbital (50 mg/kg). Following 10 min of the injection and anticoagulation with heparin (200 USP units, intra-peritoneally), the hearts were quickly excised and perfused with modified Krebs-Henseleit buffer (120.0 mM NaCl, 4.5 mM KCl, 20.0 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$, and 10.0 mM glucose: gassed with 95% O_2+5% CO_2 to obtain pH 7.4 at 37 °C) at the pressure equal to 1 m H_2O by means of a Langendorff apparatus. A thin-wall latex balloon was inserted into the left ventricle through the left atrium to monitor left ventricular pressure and to control left ventricular volume. After stabilization, heart rate (HR), left ventricular developed pressure (LVDP), maximum dP/dt (max dP/dt), and coronary flow (CF) were measured with LV diastolic pressure stabilized at 10 mmHg. Then, 100 μg (20 μl) of TAT-BH4 protein (BH4 group) or TAT mutant protein (C group), diluted by 5 ml of modified Krebs-Henseleit buffer, were administered through side port of apparatus at the speed of 1 ml/min. The hearts were then subjected to global ischemia at 37 °C for 30 min, followed by 60 min of reperfusion. The balloon was deflated during ischemia. The indices of cardiac function were continuously measured after reperfusion and analyzed using Polygraph System (Nihon Kouden, Japan). After 60 min of reperfusion, frozen sections of the hearts were made and stored at −80 °C for further assessment.

2.3. The recovery of mitochondrial respiration

Mitochondria were isolated from the hearts after reperfusion in 0.3 M mannitol/10 mM potassium Hepes, pH 7.4/0.2 mM EGTA, pH 7.4/0.1% fatty acid-free BSA by centrifugation at 2500×g for 10 min. The mitochondria were washed twice with this medium without EGTA to which 5 mM potassium phosphate was added and then suspended in it. Mitochondrial respiration was measured with an O_2 electrode, and the recovery of respiration was defined as the ratio of mitochondrial respiration in the hearts after reperfusion to the hearts before ischemia.

2.4. Western blotting analysis of active caspase-3

To evaluate the activation of apoptotic cascade after reperfusion, western blot analysis for detection of active caspase-3 was performed using the frozen section samples after 60 min of reperfusion. We used active caspase-3 rabbit polyclonal IgG antibody, and anti-rabbit secondary antibody conjugated to horseradish peroxidase and Phototope-HRP Western detection kit. The degree of the protein expression was semi-quantitatively evaluated with computed densitometry (Scion Image: Windows; Microsoft Corporation).

2.5. Histological analysis of apoptosis

The frozen section samples after 60 min of reperfusion were prepared for histological analysis. TUNEL staining was performed using Terminal Deoxynucleotidyle Transferase Mediated UTP-Biotin In Situ Nick-End Labeling (Tunnel Intergen Kit) according to the manufacturer's instruction. Quantitative assessment was calculated as a percentage of TUNEL-positive nuclei.

2.6. Statistical analysis

All data are expressed as mean±standard error of the means (SEM). Scores were compared using an unpaired Student's t test. A P value of less than 0.05 was considered statistically significant.

3. Results

3.1. Recovery of cardiac function after global ischemia

To evaluate the efficacy of TAT-BH4 protein, we firstly analyzed cardiac function after global warm ischemia and reperfusion. The time course of percent recovery of LVDP after global ischemia (37 °C, 30 min) was shown in Fig. 1A. In comparison with the C group, a significant improvement of the percent recovery of LVDP was observed in the BH4 group at 10, 20, 30, 40, 50, and 60 min after reperfusion. The value after 60 min of reperfusion in the C group was 47±4%, and was significantly improved to 72±4% in the BH4 group ($P<0.01$).

The time course of percent recovery of max dP/dt after global ischemia was shown in Fig. 1B. In comparison with the C group, a significant improvement of the index was observed in the BH4 group at 20, 30, 40, 50, and 60 min after reperfusion. The value after 60 min of reperfusion in the C group was 36±9%, and was significantly improved to 71±3% in the BH4 group ($P<0.01$). CF after 60 min reperfusion was also significantly higher in the BH4 group compared with the C group (13.8±0.9 vs. 8.9±1.2 ml/min; $P<0.01$) (Fig. 1C).

3.2. Mitochondrial function by the recovery of respiration

Consistently, mitochondrial function assessed by the recovery of respiration markedly increased (C; 30±5, B; 77±10%, $P<0.05$) (Fig. 2).

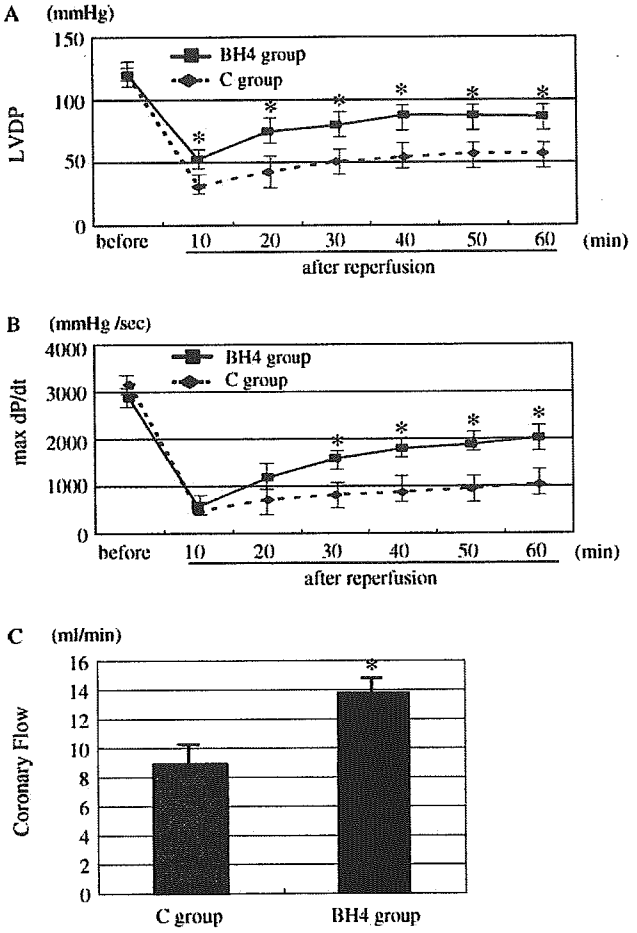

Fig. 1. Cardiac function after reperfusion. The isolated hearts from the two groups were subjected to 30 min of normothermic global ikschemia followed by 60 min of reperfusion. Better recovery of LVDP (A) and maximum dP/dt (B) after ischemia was shown in the BH4 group than in the C group. Data are expressed as percentage of basal value before ischemia. $^{**}P<0.01$, $^{*}P<0.05$. The coronary flow after reperfusion (C): the coronary flow 60 min after reperfusion was measured. $^{*}P<0.05$. $n=8$ in each group. All values are expressed as mean±SEM.

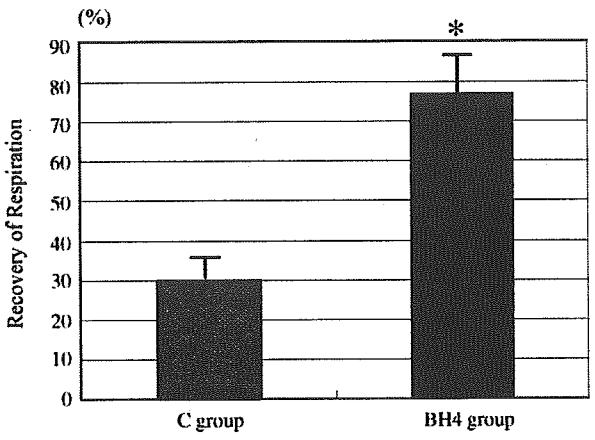

Fig. 2. The recovery of respiration: the recovery of mitochondrial respiration 60 min after reperfusion was measured. $^{*}P<0.05$. $n=8$. All values are expressed as mean±SEM.

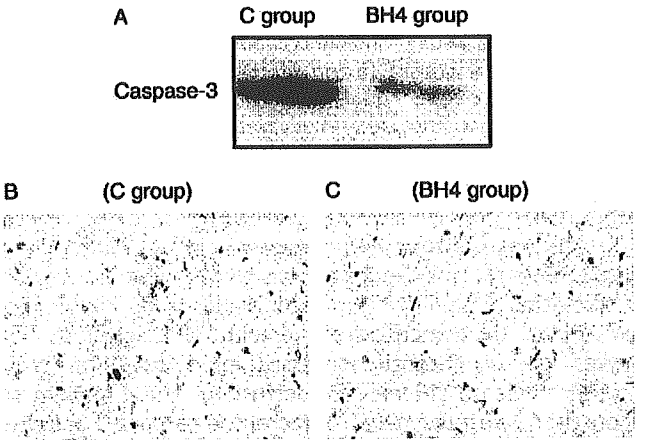

Fig. 3. Apoptosis of the myocardium after reperfusion. The myocardial expression of caspase-3 was significantly lower in the BH4 group than in the C group (A). The TUNEL-positive cardiomyocytes has detected in the control (B), but not in the BH4 group (C).

3.3. Evaluation of apoptosis by active caspase-3 expression and TUNEL staining

Then, we evaluated the apoptosis of the myocardium by Western blotting of active caspase-3 and TUNEL staining of the myocardium 60 min after reperfusion. The western blotting analysis for caspase-3 showed lower expression of active caspase-3 in the BH4 group compared with the C group (Fig. 3A). According to semi-quantitative analysis with computed densitometry, the BH4 group showed 1/3-1/4 less caspase-3 expression than the C group. The TUNEL staining of the heart section showed none of TUNEL-positive cells in the BH4 group (Fig. 3C), but significantly positive in the C group (Fig. 3B). The percent TUNEL-positive cells in the C group was 2.2±0.3% of all cardiomyocytes, whereas TUNEL-positive cells could not be seen in BH4 group ($P<0.01$).

4. Discussion

In the present report, we showed cardioprotective effect of TAT-BH4; a novel linkage of the protein transduction domain of HIV TAT to the functional domain of Bcl-xL. The recovery of cardiac function, mitochondrial respiration of the myocardium after ischemia/reperfusion was significantly better by TAT-BH4 administration. Moreover, TAT-BH4 attenuated caspase-3 expression and reduced apoptosis of cardiomyocytes.

Apoptosis is an actively regulated process of cellular self-destruction, thereby distinct from necrosis. It encloses mitochondrial changes with characteristic release of substances promoting apoptosis like cytochrome *c*. Downstream in the apoptotic program the caspase cascade such as caspase-3 is activated, followed by cytoskeletal alterations, chromatin condensation, and DNA fragmentation, culminating in cell death. Previous experimental studies have well shown that apoptosis of cardiomyocytes is induced from early stage of ischemia/reperfusion, playing an important role for the cardiac dysfunction [1-5]. Using isolated rat

heart model, Scarabelli et al. [4] demonstrated that apoptosis (caspase-3 and TUNEL-positive nuclei) is seen in the very early stages (5 min of reperfusion) of ischemia/reperfusion in both endothelial cells and cardiomyocytes. Previous clinical studies also demonstrated apoptosis is evident early after ischemia/reperfusion during open-heart surgery. Schmitt et al. [6] showed that cytochrome *c* release and TUNEL-positive myocytes were increased even at the time of weaning from extracorporeal circulation (40 min of reperfusion), and apoptotic index showed a negative correlation with left ventricular function during surgery. Wu et al. [7] demonstrated percent TUNEL-positive myocytes was significantly increased even just after cross-claming release (10 min of reperfusion). These findings are consistent with our result of increased caspase-3 activities and TUNEL-positive myocytes 60 min after reperfusion.

There are many reports to attenuate ischemia/reperfusion injury through inhibiting apoptosis [8-15]. Several reports demonstrated the effects of gene transfer of anti-apoptotic gene [8-12]. Huang et al. [11] showed the effects of Bcl-xL gene transfer in rat model, but gene transfection needs a couple of days to gene expression, so this method is not suitable clinically for cardio-protection during open heart surgery. Other reports [13-15] showed the effects of anti-apoptotic protein administration such as caspase inhibitor [13], but this method needs frequent or continuous administration of relatively high dose of drugs during ischemia and reperfusion, which might induce deleterious side effects in other organs. Compared with the previous methods, TAT-BH4 administration before ischemia has some advantages over previous approach, because administration of essential domain for Bcl-xL by TAT system enables effective protein transduction into the target cells and potentiate immediately after reperfusion.

Our results showed that BH4 administration suppressed apoptotic cascade, resulting in improvement of cardiac function from early stage of reperfusion. Caspase-3 expression, an important molecule in the cellular suicide cascade, was minimal and no TUNEL-positive cells were seen in the BH4 group. The mechanism that BH4 inhibits apoptosis has already been demonstrated in our previous report [16]. BH4 of Bcl-xL is essential for inhibition of apoptosis with respect to the regulation of mitochondrial membrane permeability and is able to inhibit both voltage-dependent anion channel activity even in the presence of Bax and apoptotic mitochondrial membrane permeability loss. The mechanisms of this rapid effect for cardiac function by inhibiting apoptosis is remain to be addressed. Cheng et al. [18] reported that even such a small number of cells affected by apoptosis may have a significant impact on cardiac contractility, because single-cell death impinges upon the force-generating ability of neighboring cells that are still viable but stunned. Relatively few apoptotic cells may substantially impair side-to-side slippage of myocytes, resulting in a disproportionate and much more severe cardiac dysfunction. As a result, TAT-BH4 administration before ischemia inhibited almost completely apoptotic cascade during ischemia/reperfusion, contributing improvement of cardiac function from the early stage of reperfusion.

The HIV TAT protein: the amino-acid transduction domain of TAT contains a domain that facilitates protein transduction across cellular membranes. Although the exact mechanism of protein transduction across cellular membranes remains unknown, TAT-mediated protein transduction has been shown to occur even at 4 °C and is receptor independent [19]. These characteristics of TAT protein are also thought to be promising for protein transduction during open heart surgery.

These well known mechanisms of TAT-BH4 preventing mitochondrial function and apoptosis characterizes the promising possibility for clinical appreciation of this agent in cardiac surgery. Our data have strongly supported the importance of further investigation for clinical appreciation of this agent in future.

In conclusion, we obtained evidence that TAT-BH4 attenuates myocardial ischemia/reperfusion injury via inhibition of apoptosis of the myocardium. Thus, TAT-BH4 may be a novel therapeutic strategy for the protection of post-operative cardiac dysfunction in cardiovascular surgery.

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Two distinct Fas-activated signaling pathways revealed by an antitumor drug D609

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During the process of death receptor-mediated apoptosis, Bid is cleaved by activated caspase-8, and then cleaved Bid conveys apoptotic signals to the mitochondria by activating Bax/Bak. In the present study, we found that D609 (an antitumor drug with multiple activities) blocks Fas-induced apoptosis. D609 did not interfere with activation of caspase-8 and cleavage of Bid, whereas it blocked cytochrome *c* release from the mitochondria by inhibiting the activation of Bax and Bak. D609 had no protective effect against apoptosis of SKW6.4 cells, which are typical type I cells. Studies using permeabilized cells revealed that in addition to activation of caspase-8, Fas activated a distinct and D609-sensitive signaling pathway that transmitted signal(s) sensitizing the mitochondria to apoptotic stimuli, and that D609 itself promoted mitochondrial resistance to apoptotic stimuli.
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Introduction

Stimulation of death receptors such as Fas and TNFα triggers an apoptotic cascade, initially by recruiting a death receptor, adaptor protein Fas-associated death domain protein, and procaspase-8, to form the death-inducing signaling complex (DISC), after which procaspase-8 is cleaved and activated (Kischkel *et al.*, 1995; Boldin *et al.*, 1996; Wallach *et al.*, 1999). Two distinct Fas-induced apoptotic signaling pathways, the choice of which mainly depends on the extent of caspase-8 activated at the DISC, have been described (Scaffidi *et al.*, 1998). In type I cells, there is extensive activation of caspase-8, which is sufficient to activate downstream execution caspases such as caspase-3 or -7 that commit the cell to death. In type II cells, however, the DISC causes little activation of caspase-8; therefore, mitochondria-mediated amplification of apoptotic signals is necessary (Scaffidi *et al.*, 1998). In type II cells, activated caspase-8 cleaves and activates a BH3-only protein, Bid (Li *et al.*, 1998a; Luo *et al.*, 1998), which conveys apoptotic signals to the mitochondria by activating proapoptotic molecules Bax or Bak to induce mitochondrial release of cytochrome *c* (Desagher *et al.*, 1999; Eskes *et al.*, 2000; Wei *et al.*, 2000). After it is released into the cytoplasm, cytochrome *c* binds to apoptotic protease-activating factor 1 (Apaf1), which recruits and activates procaspase-9 to trigger activation of the caspase cascade and commit the cell to apoptotic death (Liu *et al.*, 1996; Li *et al.*, 1997).

In addition to the caspase-8/Bid signaling pathway, Fas has also been reported to stimulate other signaling pathways, leading to the activation of extracellular signal-regulated kinase (ERK) (Shinohara *et al.*, 2000; Desbarats *et al.*, 2003; Lambert *et al.*, 2003), c-Jun N-terminal kinase (JNK) (Yang *et al.*, 1997), tyrosine kinase (Eischen *et al.*, 1994), acidic sphingomyelinase (aSMase) (Cifone *et al.*, 1995), or phosphatidylcholine-specific phospholipase C (PC-PLC) (Cifone *et al.*, 1995). However, little is known about the role of these pathways in the regulation of apoptosis. Our previous study has revealed that Fas stimulates a caspase-8-independent signaling pathway, and although this pathway may not be able to induce directly apoptosis, it sensitizes the mitochondria to apoptotic stimuli by causing exposure of the N-terminus of Bak (Zhang *et al.*, 2004). These results suggested that in addition to the caspase-8/Bid pathway, Fas stimulates other signaling pathway(s) to regulate apoptosis, although the precise mechanisms are still to be determined.

The xanthogenate tricyclodecan-9-yl (D609) was originally developed as a selective antitumor drug (Amtmann and Sauer, 1987), and now is also known as an antioxidant (Zhou *et al.*, 2001) and inhibitor of PC-PLC and SMase (Schutze *et al.*, 1992; Wiegmann *et al.*, 1994; Amtmann, 1996). It has been reported that D609 impairs TNF-mediated cytotoxicity in murine fibrosarcoma L929 cells and WEHI164 cells, protects mice against TNF-, LPS-, or SEB-mediated lethal shock (Machleidt *et al.*, 1996), and inhibits glutamate-induced nerve cell death (Li *et al.*, 1998b). In the present study, we showed that D609 protected cells from Fas-induced apoptosis, not by interfering caspase-8/Bid activation

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but by inhibiting the activation of Bak and Bax through blockage of caspase-8/Bid-independent apoptotic signaling and by increasing mitochondrial resistance to apoptotic signals.

Results

D609 inhibits Fas-induced apoptosis in Hela and Jurkat cells

To develop a better understanding about Fas-induced apoptosis, we examined the influence of a variety of inhibitors on apoptosis induced by an agonistic anti-Fas antibody CH11 in Hela cells. We found that D609 inhibited CH11-induced apoptosis in a dose-dependent manner, as assayed by Annexin-V staining (Figure 1a and b), nuclear morphology (Figure 1c), and activation of caspase-3 (Figure 1d). D609 also protected Jurkat cells from CH11-induced apoptosis (Figure 1e). In contrast, D609 did not have any protective effect against CH11-induced apoptosis of SKW6.4 cells, which are typical type I cells (Figure 2a and b). These results suggested that D609 might prevent Fas-induced apoptosis by blocking a mitochondria-mediated apoptotic signaling pathway.

D609 inhibits activation of Bak/Bax and cytochrome c release

Next, we attempted to determine the mechanism by which D609 inhibited apoptosis. First, we examined whether D609 had any effect on an initial step in Fas-induced apoptosis, namely activation of caspase-8 and cleavage of Bid. D609 failed to inhibit the cleavage of procaspase-8 (Figure 3a, upper panel) in Hela cells. Cleavage of Bid was slightly delayed (Figure 3a, middle panel), possibly due to inhibition of a caspase feedback pathway, since D609 blocked mitochondria-mediated amplification of apoptosis signaling (see below). To address this issue further, we investigated CH11-induced cleavage of Bid in the presence or absence of D609 in Bcl-2 overexpressing Hela cells. After stimulation by CH11, cleavage of Bid showed the same kinetics

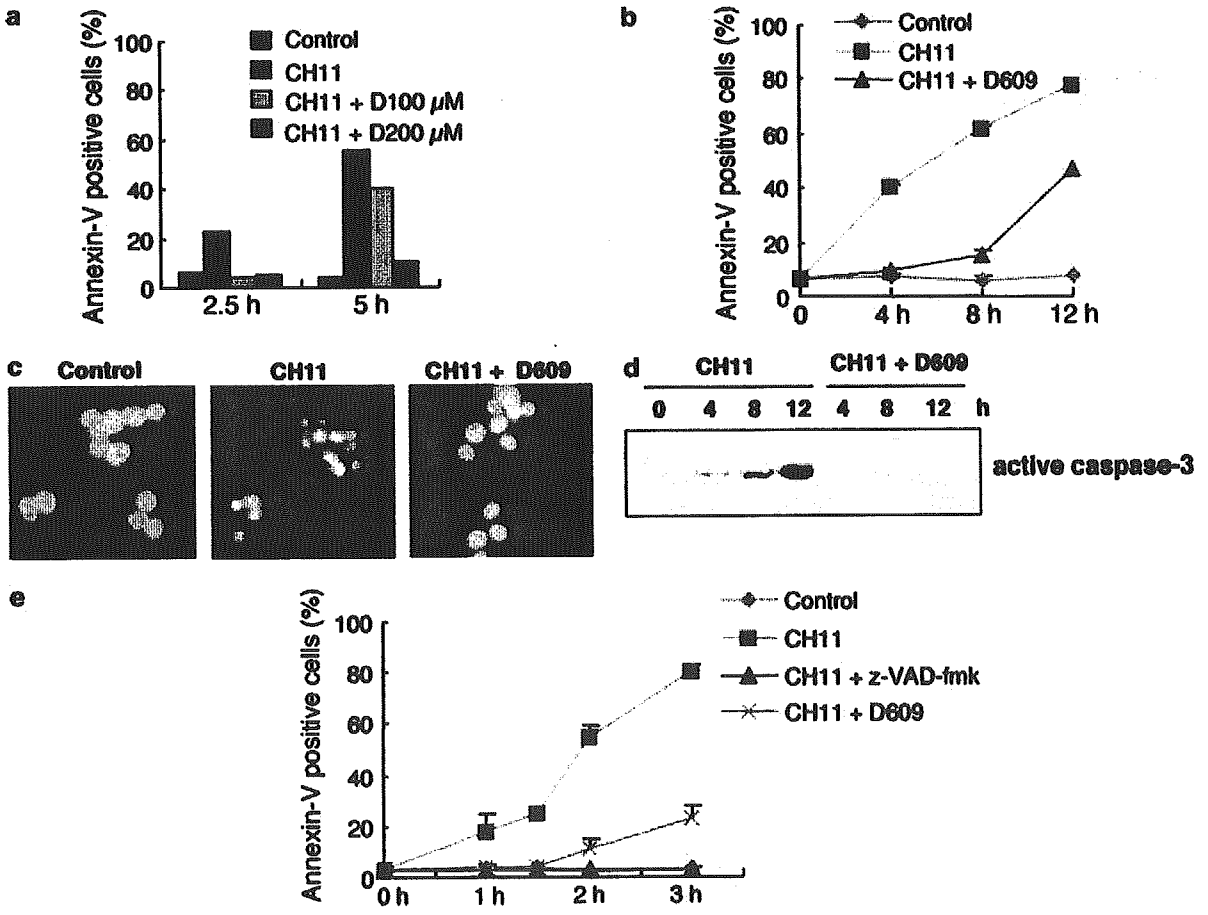

Figure 1 D609 prevents Fas-induced apoptosis. (**a**) Hela cells were incubated with or without D609 at the indicated concentrations for 30 min, and then stimulated with or without 0.25 μg/ml CH11 for the indicated times. Apoptotic cells were detected by Annexin-V staining. (**b** and **c**) Hela cells were incubated with or without 0.25 μg/ml CH11 in the presence or absence of 200 μM D609 for the indicated times (**b**), or for 8 h (**c**). Apoptotic cells were detected by Annexin-V staining (**b**) or by staining with Hoechst 33342 (**c**). (**d**) Hela cells were treated as in (**b**) for the indicated times. After lysis, cell extracts were subjected to Western blotting with an antibody to anti-active caspase-3. (**e**) Jurkat cells were treated with or without 0.25 μg/ml CH11 in the presence or absence of 100 μM z-VAD-fmk or 200 μM D609 for the indicated times. Apoptotic cells were detected by Annexin-V staining

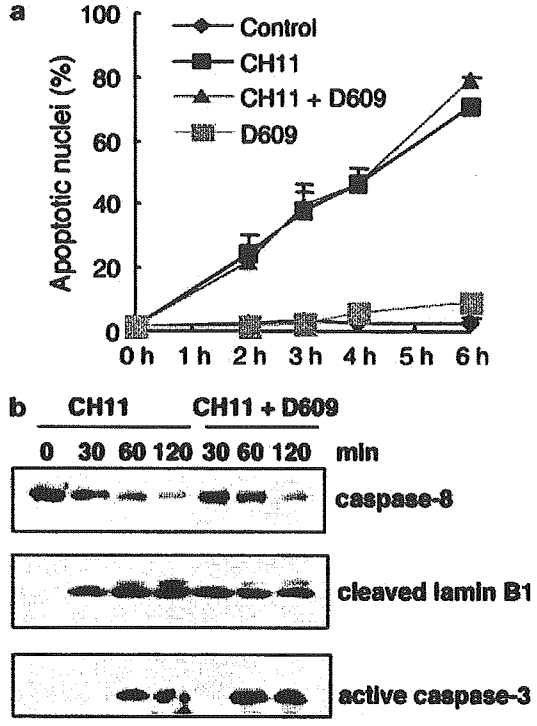

Figure 2 D609 is unable to prevent Fas-induced apoptosis in type I cells. (**a**) SKW6.4 cells were treated with or without 0.25 μg/ml CH11 in the presence or absence of 200 μM D609 for the indicated times. Apoptotic nuclei were detected by Hoechst 33342 staining. (**b**) SKW6.4 cells were treated as indicated and lysed, followed by Western blot analysis with antibodies to caspase-8, lamin B1, and active caspase-3

irrespective of the presence of D609 (Figure 3a, lower panel). These results are consistent with the finding that D609 could not inhibit CH11-induced apoptosis of SKW6.4 cell (Figure 2), suggesting that it prevented Fas-induced apoptosis without interfering with the activation of caspase-8/Bid.

To investigate whether D609 inhibited apoptosis upstream of the mitochondria, we examined a series of mitochondrial events occurring during apoptosis. D609 inhibited the release of cytochrome *c* from the mitochondria in CH11-stimulated Hela cells (Figure 3b and c). Since Bak/Bax is thought to control the mitochondrial release of cytochrome *c* (Wei *et al.*, 2001), we investigated whether D609 had any effect on the activation of Bak or Bax. It has been reported that a conformational change and homo-oligomerization of Bax/Bak are closely associated with the activation of these proteins (Gross *et al.*, 1998; Hsu and Youle, 1998; Griffiths *et al.*, 1999; Antonsson *et al.*, 2000; Wei *et al.*, 2000; Cheng *et al.*, 2003); therefore, we examined the occurrence of these events in the presence or absence of D609 after CH11 stimulation. Employing specific antibodies that only recognized activated Bax/Bak that had undergone a conformational change, we found that D609 inhibited the CH11-induced conformational change of Bak and Bax (Figure 3b and d). D609 also inhibited translocation of Bax to the mitochondria (Figure 3c), and inhibited the oligomerization of Bak and Bax in CH11-treated cells (Figure 3e and f). These results suggested that D609 inhibited apoptosis at the mitochondrial level or further upstream.

D609 confers mitochondrial resistance to Bid

Inhibition of Bax/Bak activation and cytochrome *c* release by D609 suggested that it inhibited apoptotic signaling pathways leading to the mitochondria and/or acted at the mitochondrial level. We examined both possibilities. First, to examine the latter possibility, we employed an *in vitro* assay using Hela cells permeabilized by digitonin. To examine whether D609 inhibits apoptotic signaling at the mitochondria, Hela cells were incubated with D609 for 30 min or 2 h, and then permeabilized, followed by incubating with various concentrations of recombinant human Bid (rBid) or with isotonic buffer as a negative control. Intriguingly, when Hela cells were incubated with D609 for 2 h, rBid only induced little cytochrome *c* release in these cells (Figure 4), whereas incubation with D609 for a shorter period (30 min) could not inhibit rBid-induced cytochrome *c* release (Figure 5b). These results suggested that D609 could increase mitochondrial resistance to apoptotic stimulation.

D609 inhibits caspase-8-independent apoptotic signaling activated by Fas stimulation

To examine the possibility that D609 also inhibited apoptotic signals to the mitochondria, we also employed a permeabilized cell system. The lysate of cells treated with CH11 for 30 min was used. At 30 min, release of cytochrome *c* was not detected (data not shown). As shown in Figure 5a, the lysate derived from CH11-treated cells induced almost complete cytochrome *c* release from the mitochondria when was added to permeabilized cells. However, like lysate derived from untreated cells, the lysate derived from CH11/D609-treated cells did not induce cytochrome *c* release (Figure 5a). The addition of D609 to the lysates *in vitro* did not have any influence on cytochrome *c* release (Figure 5a, lower panel), suggesting that D609 did not have direct protective effect to the mitochondria. These results suggested that Fas stimulation not only activates the caspase-8/Bid pathway but also another signaling pathway to the mitochondria with a D609-sensitive step.

To confirm the existence of a Fas-activated caspase-8-independent signaling pathway to the mitochondria and to investigate whether this pathway could be inhibited by D609, cells were incubated with or without CH11 plus z-VAD-fmk in the presence or absence of D609, or D609 alone. After 30 min, the cells were permeabilized, incubated with different concentrations of rBid, and then cytochrome *c* release was detected by immunostaining. As shown in Figure 5b, particularly when rBid was added at a low concentration (0.01 μg/ml), it induced very little release of cytochrome *c* in untreated cells, but it released cytochrome *c* from the mitochondria in CH11/z-VAD-fmk-treated cells, suggesting that CH11/z-VAD-fmk treatment sensitized the mitochon-

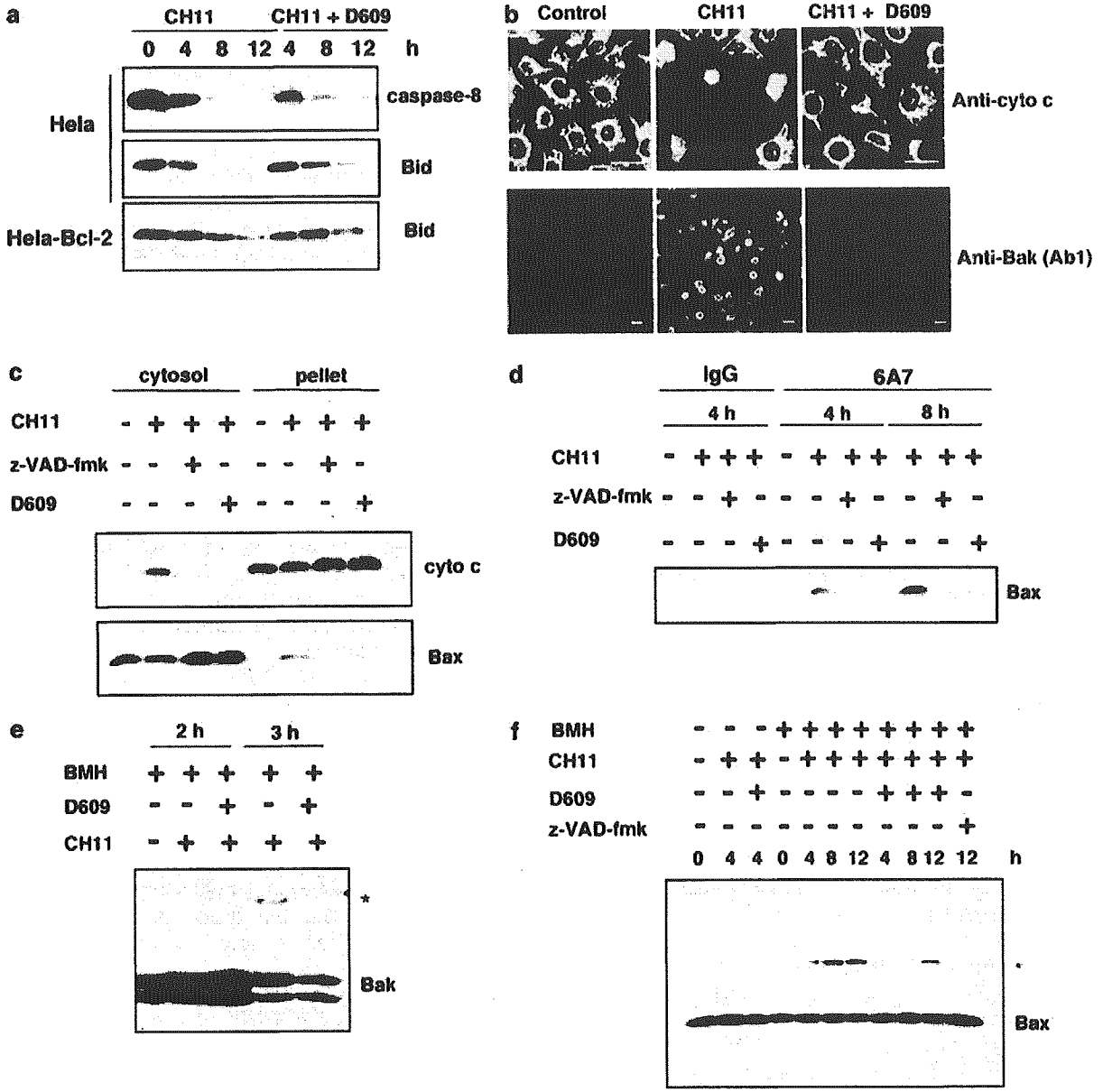

Figure 3 The protective effect of D609 occurs upstream of or at the mitochondria. (**a**) Hela cells and Hela cells overexpressing Bcl-2 (Hela-Bcl-2) were treated with or without 0.25 μg/ml CH11 in the presence or absence of D609 (200 μM) for the indicated times, lysed, and subjected to Western blot analysis with antibodies for caspase-8 and Bid. (**b**) D609 inhibits CH11-induced release of cytochrome *c* and exposure of the N-terminus of Bak. Hela cells were incubated with or without 0.25 μg/ml CH11 in the presence or absence of D609 (200 μM) for 5 h, and then immunostained with antibodies to cytochrome *c* (upper panel) or Bak (Ab1) (lower panel). Scale bar = 20 μm. (**c**) D609 inhibits CH11-induced release of cytochrome *c* and mitochondrial translocation of Bax. Hela cells were treated with or without CH11 (0.25 μg/ml) in the presence or absence of D609 (200 μM) or z-VAD-fmk (100 μM) for 5 h, and then fractionated into cytosolic (cytosol) and organellar (pellet) fractions, followed by Western blot analysis with antibodies to cytochrome *c* and Bax. (**d**) D609 inhibits CH11-induced exposure of the N-terminus of Bax. Hela cells were treated as in (**c**) for 4 or 8 h. Cell extracts were immunoprecipitated with anti-Bax (6A7) monoclonal antibody that only recognized activated Bax, and then were subjected to Western blotting with a polyclonal anti-Bax antibody (N20). (**e** and **f**) D609 inhibits the oligomerization of Bak/Bax. Jurkat cells (**e**) and Hela cells (**f**) were treated as indicated, harvested, and incubated with or without BMH for 30 min at room temperature. Oligomerization of Bak (**e**) and Bax (**f**) was detected by Western blotting. Asterisks denote dimers of respective proteins

dria to apoptotic signaling. When cells treated with CH11/z-VAD-fmk in the presence of D609 were permeabilized and incubated with the same concentration of rBid, the release of cytochrome *c* was markedly inhibited (Figure 5b). Incubation of cells with D609 alone for 30 min did not have any influence on rBid-induced cytochrome *c* release (Figure 5b), indicating that incubation with D609 for a short time could not confer the mitochondria resistance to apoptotic signal. Since Fas-activated caspase-8/Bid pathway is completely inhibited by z-VAD-fmk but not by D609, all these results suggested that D609 could inhibit CH11-induced

caspase-8-independent apoptotic signaling to the mitochondria. Similar to the results revealed in Figure 5a, addition of D609 *in vitro* did not inhibit rBid-induced cytochrome *c* release (Figure 5b).

To further confirm the inhibition by D609 of CH11-induced caspase-8-independent apoptotic signaling to the mitochondria, caspase-8-deficient murine embryonic fibroblasts (caspase-$8^{-/-}$ mouse embryonic fibroblasts (MEFs)) were employed. The cells were incubated with or without agonistic anti-mouse Fas antibody (Jo2), Jo2 plus D609, or D609 alone for 4 h, and then cytosols were prepared. Permeabilized Hela cells were incubated with isotonic buffer or various cytosols for 10 min, and then stimulated with or without 0.01 μg/ml rBid. As shown in Figure 5c, any cytosol alone did not induce release of cytochrome *c*. Treatment with rBid induced the release of cytochrome *c* from the mitochondria in a small fraction of cells, and preincubation of permeabilized cells with the cytosol derived from control caspase-$8^{-/-}$ MEFs only slightly enhanced rBid-induced release of

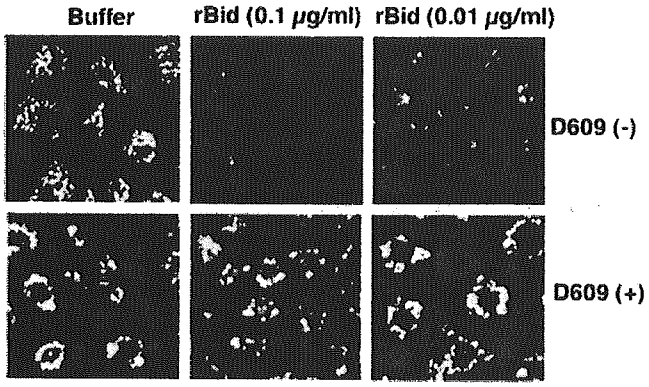

Figure 4 D609 promotes mitochondria resistance to Bid. Hela cells were incubated with or without 200 μM D609 for 2 h, permeabilized by incubation with 20 μg/ml digitonin for 3 min at room temperature, and incubated with rBid for 10 min at 37°C. As a negative control, isotonic buffer was added instead of rBid. After washing three times, the cells were immunostained with anti-cytochrome *c* antibody. Note that cytochrome *c* release from the mitochondria caused loss of immunoreactivity to cytochrome *c* antibody due to leakage through the permeabilized plasmamembrane

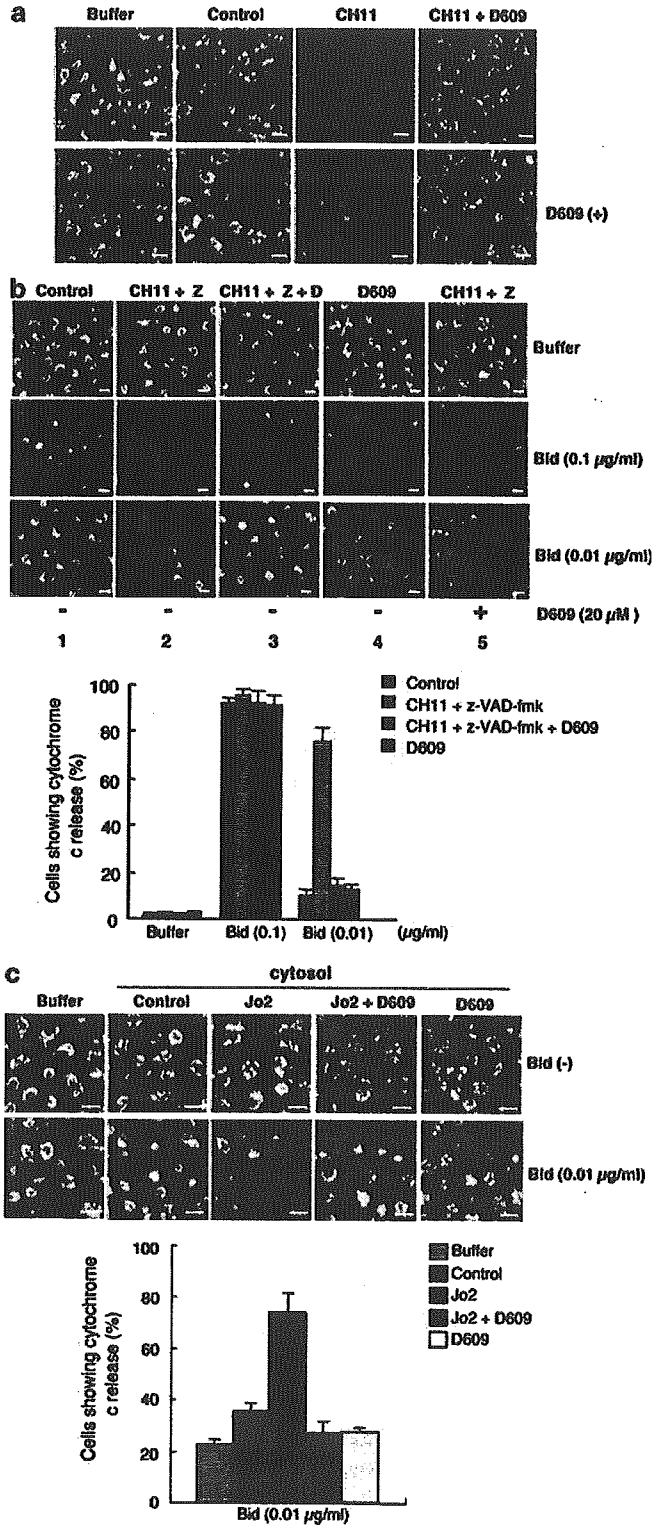

Figure 5 D609 inhibits CH11-induced caspase-8-independent apoptosis signaling. (**a**) Cytosol was prepared from Hela cells incubated with or without 0.25 μg/ml CH11 in the presence or absence of 200 μM D609 for 30 min. Cytosols were added to permeabilized Hela cells in the presence (lower panel) or absence (upper panel) of 20 μM D609 for 10 min at 37°C. As a negative control, isotonic buffer was added instead of cytosol. After washing three times, immunostaining was carried out with anti-cytochrome *c* antibody. Scale bar = 20 μm. (**b**) Hela cells were incubated with (panels 2 and 5) or without (panel 1) 0.25 μg/ml CH11 plus 100 μM z-VAD-fmk, or with a combination of CH11, z-VAD-fmk, and 200 μM D609 (panel 3), or with D609 (200 μM) alone (panel 4) for 30 min, permeabilized, and then incubated with rBid (panels 1–4) or rBid plus 20 μM D609 (panel 5) for 5 min at 37°C. Isotonic buffer was added instead of rBid as a negative control. After washing three times, immunostaining was carried out with anti-cytochrome *c* antibody. Scale bar = 20 μm. Cells in which cytochrome *c* had been released from the mitochondria were counted. (**c**) Caspase-$8^{-/-}$ MEFs were incubated with or without 0.5 μg/ml Jo2, Jo2 plus D609 (200 μM), or D609 (200 μM) alone for 4 h, and then the cytosols were prepared. Cytosols were added to permeabilized Hela cells for 10 min at 37°C, followed by incubation with or without rBid (0.01 μg/ml) for 5 min. Isotonic buffer was added instead of rBid as a negative control. After washing three times, immunostaining was done with anti-cytochrome *c* antibody. Scale bar = 20 μm. Cells in which cytochrome *c* had been released from the mitochondria were counted

cytochrome *c*, whereas preincubation with the cytosol derived from Jo2-treated cells markedly promoted rBid-induced cytochrome *c* release (Figure 5c). This result is consistent with our previous finding (Zhang *et al.*, 2004). However, the cytosol derived from Jo2/D609-treated cells did not enhance rBid-induced cytochrome *c* release (Figure 5c). The cytosol derived from D609-treated cells did not affect rBid-induced cytochrome *c* release (Figure 5c), and, when was added to the cytosol derived from Jo2-treated cells, it could not inhibit Jo2 lysate-induced enhancement of rBid-induced cytochrome *c* release (data not shown). These results further indicated that Fas stimulated a caspase-8-independent apoptotic signaling to sensitize the mitochondria to apoptotic stimuli and this pathway could be prevented by D609.

D609 inhibits caspase-8-independent exposure of the N-terminus of Bak activated by Fas stimulation

Our previous study has revealed that Fas stimulation activates a caspase-8-independent signaling pathway that induces exposure of the N-terminus of Bak, and this change primes Bak for full activation and thus sensitizes the mitochondria to apoptotic stimuli (Zhang *et al.*, 2004). We examined whether D609 could inhibit this caspase-8-independent signaling pathway. As shown in Figure 6a, D609 completely inhibited CH11-induced caspase-8-independent exposure of the N-terminus of Bak. Since D609 might inhibit CH11-induced caspase-8-independent exposure of the N-terminus of Bak at the mitochondria level, we examined whether it inhibited caspase-8-independent signaling upstream of the mitochondria by employing a permeabilized cell system. As reported previously, cytosol derived from cells incubated with CH11 in the presence of z-VAD-fmk induced exposure of the N-terminus of Bak (Figure 6b) (Zhang *et al.*, 2004), while cytosol from cells incubated with CH11 in the presence of z-VAD-fmk plus D609 failed to induce this exposure (Figure 6b). The addition of D609 to the lysate did not inhibit exposure of the N-terminus of Bak (data not shown). These results further confirmed that other signaling pathway(s) originated from the death receptor to participate in the regulation of apoptosis in addition to caspase-8/Bid pathway.

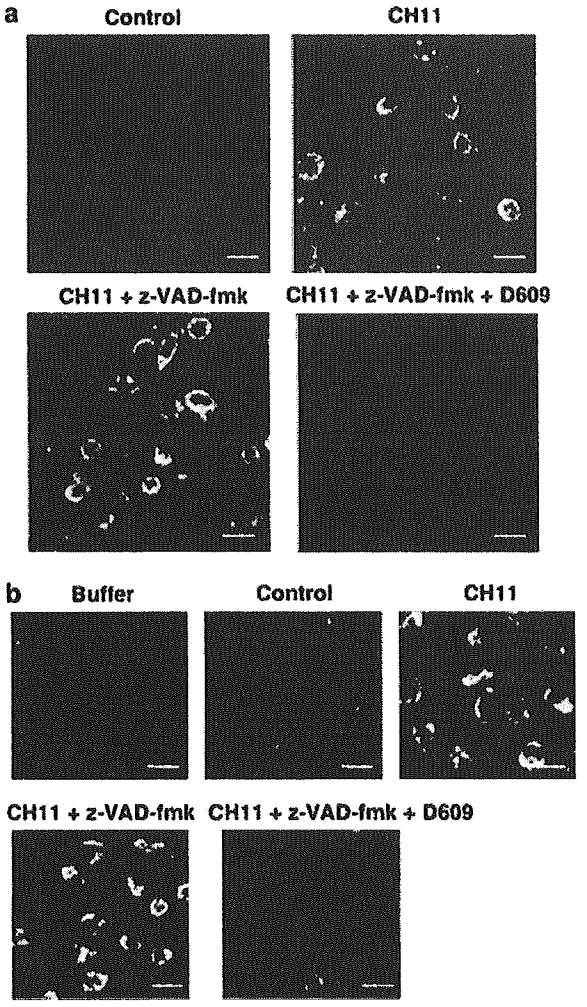

Figure 6 D609 inhibits CH11-induced caspase-8-independent exposure of the N-terminus of Bak. (**a**) Hela cells were incubated for 2 h without or with 0.25 μg/ml CH11 in the presence or absence of 100 μM z-VAD-fmk, or with a combination of CH11, z-VAD-fmk, and 200 μM D609, and then were immunostained with anti-Bak (Ab1) antibody. (**b**) Digitonin-permeabilized Hela cells were incubated for 10 min with cytosol derived from Hela cells treated as in (**a**), followed by immunostaining with an anti-Bak monoclonal antibody (Ab1). Isotonic buffer was added instead of cytosol as the negative control. Scale bar = 20 μm

Discussion

In type II cells, mitochondria-mediated apoptotic signaling plays an essential role in Fas-induced apoptosis, since such apoptosis is completely blocked when mitochondrial permeabilization is prevented either by overexpression of Bcl-2/Bcl-x_L (Scaffidi *et al.*, 1998) or deficiency of Bax/Bak (Wei *et al.*, 2001), while apoptosis is severely delayed when Bid, which transmits apoptotic signals to the mitochondria, is eliminated by genetic alteration (Yin *et al.*, 1999). Fas stimulation does not only activate the caspase-8/Bid pathway but also other signaling pathway(s) such as ERK (Shinohara *et al.*, 2000; Desbarats *et al.*, 2003), PC-PLC (Cifone *et al.*, 1995), JNK (Yang *et al.*, 1997), and aSMase (Cifone *et al.*, 1995). To date, whether these pathways play any roles in the regulation of apoptosis is still unclear. Here, we showed that D609 prevents Fas-induced apoptosis not by interfering caspase-8/Bid activation but, at least partly, by inhibiting Fas-mediated caspase-8-independent apoptotic signaling to the mitochondria (Figure 7). This result suggested that in addition to caspase-8/Bid pathway, Fas stimulation also activates other signaling to regulate apoptosis. It is currently unclear how D609 inhibited this signaling pathway, since D609 has been reported to inhibit PC-PLC and SMase activity (Schutze *et al.*, 1992; Wiegmann *et al.*, 1994; Amtmann, 1996; Machleidt *et al.*, 1996), and is also known as an antioxidant (Zhou *et al.*, 2001). Although the target of D609 has not been identified, D609 has enabled us to identify a novel apoptotic signaling pathway that is

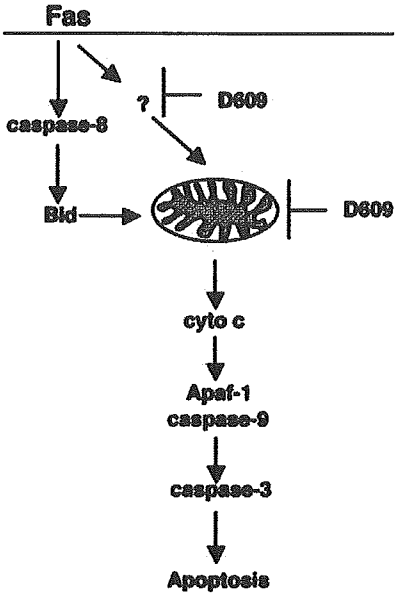

Figure 7 Two independent signaling pathways activated by Fas stimulation in type II cells. Fas activates two distinct signaling pathways to the mitochondria to regulate apoptosis: the caspase8/Bid-dependent and -independent pathways. Although this caspase-8-independent pathway might not induce apoptosis directly, it sensitizes the mitochondria to apoptosis stimulation. D609 prevents Fas-induced apoptosis by inhibiting the caspase-8-independent signaling pathway to the mitochondria as well as protecting the mitochondria

activated by Fas and is independent of caspase-8/Bid. Our previous study also revealed that Fas stimulates a caspase-8-independent signaling pathway, which is staurosporine-inhibitable, and, although this pathway may not be able to induce directly apoptosis, it sensitizes the mitochondria to apoptotic stimuli by causing exposure of the N-terminus of Bak (Zhang *et al.*, 2004). We found that D609 completely inhibited the CH11-induced caspase-8-independent signaling pathway leading to the exposure of Bak N-terminus. These findings also suggested that in addition to the caspase-8/Bid pathway, Fas stimulates other signaling pathway(s) to regulate apoptosis.

We also showed that D609 decreased susceptibility of the mitochondria to apoptotic signal (Figure 7). Consistently, D609 inhibited not only Fas-induced apoptosis but also etoposide-induced apoptosis (unpublished data). D609 may decrease mitochondrial susceptibility by inhibiting the exposure of N-terminus of Bak, although another possibility exists because D609 also inhibited the activation of Bax. It has been reported that dynamic changes of mitochondrial membrane lipids occur during Fas- and radiation-induced apoptosis (Matsko *et al.*, 2001). In the present study, we found that Fas stimulation increased PC-PLC activity, and D609, but not caspase inhibitor z-VAD-fmk, inhibited this increased activity (data not shown). PC-PLC is a phospholipase; the hydrolysis of lipid and/or the reaction products could affect lipid homeostasis in the mitochondria. Since Bak is localized on the outer mitochondrial membrane, it is conceivable that changes of the membrane lipids could affect the conformation of Bak. To exert proapoptotic activity, Bax undergoes translocation and is inserted into the mitochondrial membrane, where it oligomerizes to contribute to the formation of a protein-conducting pore (Wolter *et al.*, 1997; Antonsson *et al.*, 2000, 2001; Eskes *et al.*, 2000). Recent studies have revealed that Bax-induced permeabilization of liposomes is dependent on cardiolipin, which is a specific mitochondrial membrane lipid (Epand *et al.*, 2002; Kuwana *et al.*, 2002). In the absence of liposomes, tBid failed to induce the conformational change and oligomerization of Bax (Yethon *et al.*, 2003), suggesting that an appropriate lipid environment is required for Bax to undergo translocation and insertion into the mitochondrial membrane. It is possible that mitochondrial membrane lipids are altered by PC-PLC or other molecule to facilitate the conformational change of Bax, as well as its mitochondrial translocation and membrane insertion. It is currently unclear whether the protective effect of D609 is exerted via inhibition of PC-PLC, and also whether PC-PLC really plays an important role in the regulation of Fas-induced apoptosis, since PC-PLC itself has not been identified in eukaryote. This will be unveiled when eukaryotic PC-PLC is cloned or when other target(s) of D609 are found. Alternatively, death stimulation induces generation of reactive oxygen species (ROS), and ROS influences lipid homeostasis in the mitochondria, therefore promoting activation of Bak or Bax. D609 has been reported as a potent antioxidant, and this effect may interpret that D609 inhibited etoposide-induced apoptosis at the mitochondrial level (unpublished data).

It has been reported that aSMase/ceramide plays an important role in Fas-induced apoptosis and that D609 inhibits aSMase activation (Schutze *et al.*, 1992; Boesende Cock *et al.*, 1999; Lin *et al.*, 2000; Paris *et al.*, 2001). Therefore, it is possible that the inhibition of Fas-induced apoptosis by D609 is due to inhibition of aSMase/ceramide. However, this is unlikely, because blocking the production of ceramide with an aSMase inhibitor (imipramine) or an inhibitor of ceramide synthase (fumonisin B1) did not have any protective effect against Fas-induced apoptosis (data not shown), suggesting that aSMase may not participate in this form of apoptosis and that protective effect of D609 is not mediated via inhibition of aSMase in these cells. Identification of true targets of D609 would develop a better understanding about apoptosis regulation.

Materials and methods

Antibodies and Reagents

Anti-Bak (Ab1) monoclonal and polyclonal antibodies were purchased from Oncogene Research Products (Boston, MA, USA) and Upstate Biotechnology (Lake Placid, NY, USA), respectively. Anti-Bax (N20) and anti-Bid polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and R&D System (Minneapolis, MN, USA), respectively. Anti-cytochrome *c* (6H2 and 7H8), anti-Bax (6A7) and anti-mouse Fas (Jo2) monoclonal antibodies were

from Pharmingen (San Diego, CA, USA). Anti-human Fas (CH11), anti-caspase-3, and anti-caspase-8 monoclonal antibodies were obtained from MBL (Nagoya, Japan), while anti-lamin B1 and anti-active caspase-3 antibodies were purchased from Zymed Laboratories (San Francisco, CA, USA) and Promega (Madison, WI, USA), respectively. All of the secondary antibodies were purchased from Molecular Probes (Eugene, OR, USA).

The crosslinker bismaleimidohexane (BMH) came from Pierce Biotechnology (Rockford, IL, USA). D609 was purchased from Calbiochem (San Diego, CA, USA), while the other chemicals were obtained from Sigma (Saint Louis, MS, USA). rBid was prepared as described previously (Shimizu and Tsujimoto, 2000).

Cell culture

The human Hela cell line, human Jurkat T-leukemic cell line, and human SKW6.4. B lymphoblastoid cell line were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS). MEFs derived from caspase-8-deficient mice were grown in Dulbecco's modified Eagle's medium supplemented with 10% FBS.

Immunofluorescence staining

Cells were fixed in 4% parafomaldehyde for 30 min, and then permeabilized with 0.1% Triton X-100 for 15 min at room temperature. After incubation with 2% FBS in phosphate-buffered saline (PBS) for 1 h, the cells were incubated with anti-Bak (Ab1) (1 : 500) or anti-cytochrome *c* (1 : 500) for 1 h. After washing three times with PBS, the cells were incubated with the secondary antibody (Alexa 488-conjugated anti-mouse IgG) for 1 h. Then fluorescence was detected under a confocal microscope (Zeiss, LSM510). In all immunostaining experiments, normal mouse IgG was added instead of the primary antibody (IgG) as a negative control.

Immunoprecipitation

Hela cells were treated, harvested, and lysed with HNC buffer (25 mM HEPES (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), 2% CHAPS). The extracts were precleared, incubated with a monoclonal anti-Bax antibody (6A7) (1 μg) or normal mouse IgG (1 μg) for 3 h at 4°C, and then incubated with protein A beads for further 3 h. After washing five times with HNC buffer, the beads were suspended in sample buffer and then were subjected to Western blotting with a polyclonal anti-Bax antibody (N20).

Analysis of cell death

Cell viability was assessed by staining with Annexin-V or Hoechst 33342. Briefly, cells were stained with Cy3-conjugated Annexin-V or 1 μM Hoechst 33342 for 5 min at room temperature, and then analysed using a flow cytometer or examined under a fluorescence microscope (Olympus, BX50). Cells with condensed and fragmented nuclei were classified as apoptotic cells and were counted in four randomly chosen fields.

Crosslinking

Cells were harvested, incubated with 1 mM BMH for 30 min at room temperature, and then incubated with 1 mM DTT for 15 min to quench the cross-linker. Next, the cells were solubilized in sample buffer and analysed by Western blotting. As a control, the same concentration of Me_2SO (DMSO) was added instead of BMH.

Permeabilization of cells

Hela cells were cultured in 10-well glass slides. After washing twice with isotonic buffer (20 mM HEPES-KOH (pH 7.4), 1.5 mM $MgCl_2$, 10 mM KCl, 250 mM sucrose), the cells were incubated with isotonic buffer containing 20 μg/ml digitonin for 3 min at room temperature. After washing three times with isotonic buffer, the cells were incubated with cytosol, or rBid at 37°C. After washing another three times with isotonic buffer, the cells were subjected to immunostaining.

Preparation of cytosol

After washing three times with isotonic buffer, cells were suspended in the same volume of isotonic buffer supplemented with protease inhibitors, and then homogenized using a Dounce homogenizer. After centrifugation at 100 000 *g* for 1 h, the supernatant was obtained and used as the cytosol.

Subcellular fractionation

Cells were fractionated as described previously (Nomura *et al*., 1999). Briefly, the cells were harvested, washed three times with isotonic buffer, and then incubated with 30 μg/ml digitonin in isotonic buffer for 5 min at 37°C. After centrifugation at 3000 r.p.m. for 3 min, aliquots of the supernatant (cytosolic fraction) and the pellet (containing the mitochondria) were analysed by Western blotting with anti-cytochrome *c* and anti-Bax antibodies.

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An investigation into the role of Bcl-2 in neuroendocrine differentiation

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Abstract

Introduction: In addition to its role in apoptosis suppression, Bcl-2 has been reported to be co-expressed with neuroendocrine markers in several tissues, leading to speculation that this oncoprotein may promote neuroendocrine differentiation.

Aim: This study investigated whether Bcl-2 modulated neuroendocrine biopeptide expression.

Methods: Levels of chromogranin A, neurone specific enolase, protein gene peptide 9.5, pancreatic polypeptide, and the chromogranin-derived peptides, intervening peptide and vasostatin-1 were examined by immunocytochemistry in rat phaeochromocytoma (PC12) cell lines genetically engineered to over-express Bcl-2 and their mock-transfected controls. Intensity of fluorescence was graded using a semi-quantitative scale from (−) indicating negative expression to (+++) indicating intense positivity.

Results: Mann–Whitney *U* analysis indicated that no significant differences in expression existed between control and Bcl2 over-expressing cell lines for any of the six peptides examined.

Conclusions: The results of this study do not support the hypothesis that Bcl-2 promotes the acquisition of a neuroendocrine phenotype.

Keywords: Differentiation; Phaeochromocytoma; Chromogranin A; PC12; Biopeptide; Immunocytochemistry

Bcl-2 is an anti-apoptotic cellular protein [1]. Studies have shown that elevated expression of this protein is associated with several malignancies [1–4]. Models of over-expression suggest that Bcl-2-dependent inhibition of apoptosis is important in tumour formation [1–4]. In addition to its well-characterised role in the suppression of programmed cell death, evidence exists that Bcl-2 is expressed both in true neuroendocrine cells and in cells that have acquired a neuroendocrine phenotype [5–7], leading to speculation that this protein may not be involved solely in cytoprotection. For example, the anterior pituitary neuroendocrine cells of the adult rhesus monkey represent the only brain cell population consistently exhibiting a non-microglial pattern of Bcl-2 expression [5]. Bcl-2 and neuroendocrine marker co-expression has also been demonstrated in a range of histologically distinct lung tumours [6]. In this study Bcl-2 expression was found to be closely associated with the neuroendocrine differentiation of tumour cells, leading the authors to suggest that this protein may be involved in cell development and differentiation [6]. Similarly, in colon carcinoma cells and neighbouring crypt cells, elevated Bcl-2 expression has been found to correlate with the expression of a number of bioactive neuroendocrine markers. Double immunostaining techniques were used to retrospectively analyse whether advanced colorectal carcinomas and tumour neighbouring mucosa produced both Bcl-2 and a range of gastrointestinal neuropeptides.

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These workers provided evidence that Bcl-2 immunoreactivity correlated with the expression of key neurohormonal polypeptides and amines including vasointestinal polypeptide (VIP), pancreatic polypeptide (PP), and somatostatin in both carcinoma tissue and in the neighbouring mucosa, and suggested that in this setting Bcl-2 may promote endodermal stem cell neuroendocrine differentiation [7]. Similarly, Azzoni et al. [8] reported that in the normal oxyntic mucosa Bcl-2 was consistently expressed by a distinct population of neuroendocrine cells located predominantly in the central mucosal layer, suggesting a role for the protein in the migration of maturing endocrine cells. These authors also found that increased probability of gastric carcinoid development was paralleled by greater Bcl-2 expression in hyperplastic oxynic endocrine cells. Indeed, they found that the ratio of Bcl-2 to chromogranin A (CGA)-positive cells was highest in atrophic fundal gastritis, a condition conferring the highest risk of carcinoid development, and lowest in sporadic Zollinger–Ellison syndrome, a condition characterised by virtually no risk [8].

In addition to studies reporting a correlation between the levels of expression of Bcl-2 and those of neuroendocrine biopeptides, this oncoprotein has also been reported to be physically associated with the neuroendocrine-associated reticulon (RTN) family member RTN-xs [9]. Tagami et al. [9] found that the interaction between Bcl-2 and RTN-xs sequestered the former in the endoplasmic reticulum, thereby reducing its anti-apoptotic activity. While this study employed cell culture models of Bcl-2 over-expression, the putative modulation of levels of RTN-xs, its family members, or indeed any other neuroendocrine markers, by this oncoprotein was not reported. The aim of the current study was, therefore, to examine the relative levels of a panel of neuroendocrine markers in an established cell culture model of Bcl-2 over-expression in order to determine whether Bcl-2 positively modulates levels of these biopeptides.

Materials and methods

The cell lines employed in the current study were rat adrenal phaeochromocytoma (PC12) cells genetically engineered to constitutively over-express Bcl-2 (PC12 HB2-2 and PC12 HB2-3) and their mock-transfected controls (PC12 V1 and PC12 V4) [10]. In addition, a well-characterised PC12 model of Cox-2 over-expression (PCXII) and their mock-transfected controls (PC-MT) were employed in optimisation experiments [11]. In PCXII cells exogenous Cox-2 expression is induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 2.5 mM) to the cell culture media [11].

All cell lines were cultured on sterile plasticware obtained from Iwaki, Japan. Cell culture media including Dulbecco's modified Eagle's media (DMEM), heat-inactivated horse serum (HS), and foetal calf serum (FCS) were obtained from Invitrogen Life Technologies, CA, USA. Trypsin, versene, and all supplements used during culture were obtained from Sigma Aldrich, Missouri, USA. Rat-tail collagen type I was from Roche Diagnostics, Germany.

For immunocytochemistry, cells were cultured in collagen-coated BD Falcon slides which were obtained from BD Biosciences, UK.

The primary antibodies for immunocytochemistry included anti-Cox-2 (SC-1745, Santa Cruz Biotechnologies, USA), anti-CGA (20085, Diasorin, USA), and anti-Protein Gene Peptide 9.5 (PGP 9.5) from UltraClone, UK. The remaining primary antibodies used in immunocytochemistry [namely anti-vasostatin (VST-1), anti-intervening peptide (IP), anti-neuron specific enolase (NSE), and anti-pancreatic polypeptide (PP)] were produced in-house [12–14]. Swine anti-rabbit fluorescein isothiocyanate (SWAR-FITC) was obtained from DakoCytomation, Denmark (F0205), the biotinylated donkey anti-rabbit (BAR)-streptavidin-FITC system (RPN 1004 and 1232, respectively) came from Amersham Biosciences, UK, and rabbit anti-goat fluorescein isothiocyanate (RAG-FITC) was also supplied by DakoCytomation (F0233).

For experiments all cells were routinely subcultured in growth media onto collagen-coated slides. The following day growth media were replaced with restriction media (DMEM, 0.5% horse serum/calf serum). In the case of PC-MT and PCXII cells restriction media also routinely included IPTG (2.5 mM). Media were removed from cells 18–24 h later and these were washed three times in PBS (0.15 M sodium chloride, pH 6.0, containing 0.03 M sodium dihydrogen orthophosphate-1-hydrate and 0.09 M anhydrous disodium hydrogen orthophosphate). The cells were then fixed in 4% paraformaldehyde (30 min, 4 °C) after which they were washed three times in PBS. Primary antibodies, namely anti-Cox-2 (1:100) anti-CGA (1:500), anti-NSE (1:50), anti-PGP 9.5 (1:400), anti-IP (1:400), anti-PP (1:200), and anti-VST-1 (1:200), were then applied as appropriate. These primary antibodies were diluted in PBS containing 0.5% bovine serum albumin Fraction V, 0.5% Trition X-100, and 0.015 M sodium azide (antibody diluent). Brain cortex, brain cerebellum, pancreas head, and ileal carcinoid tissue sections were employed as positive controls for the expression of NSE, PGP 9.5, PP, and VST-1, respectively, with adrenal medulla tissue sections serving as positive control for CGA and IP. For immunocytochemistry, negative controls included were a secondary antibody only (antibody diluent) control and the appropriate serum control (1:500). The cells were then placed in an immunostaining chamber and left at 4 °C overnight.

Following this incubation the cells were washed with PBS (3 × 5 min). The appropriate secondary antibodies were then applied to the cells, namely SWAR-FITC for anti-IP, anti-PP, and anti-VST-1 (1:100, for 1 h at room temperature in darkness), RAG-FITC for anti-Cox-2, and BAR-streptavidin for anti-CGA, anti-NSE, and anti-PGP 9.5. The latter was applied in a two-step process. First cells were incubated with BAR (1:100 for 30 min at room temperature). Following a PBS wash, the cells were then incubated in streptavidin-FITC (1:200 for 30 min in darkness at room temperature).

Following incubation, the cells were washed in PBS in the dark for 20 min. Coverslips were then applied to the slides using 0.1 M PBS/glycerol (1:9; v/v) containing 2.5% 1,4 diazabicyclo(2,2,2)octane (anti-fade). The slides were viewed under the fluorescence microscope and images were taken. The fluorescent microscope used was fitted with exciter filters that produced blue light (495 nm) for the excitation of fluorescein.

Quantification of the fluorescence intensity emitted by the FITC tag was undertaken by two independent observers. Fluorescence intensity was scored on 4 mark scale ranging—negative (−), weakly positive (+), moderately positive (++) or intensely positive (+++). For each of the six neuroendocrine markers, five independent experiments were carried out on all four cell lines and images were recorded.

For immunoblotting, cell lines were routinely subcultured from confluent T25 flasks at a split ratio of 1:10 in order to achieve 70–80% confluency at time of cell lysis. Growth media were replaced with restriction media, −/+ 2.5 mM IPTG, 18–24 h prior to cell lysis. Routinely, cells were placed on ice and restriction media were removed. Cells were then washed three times with ice-cold PBS. Following the complete removal of PBS, cells were lysed in hypotonic lysis

buffer, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (Hepes), pH 7.5, containing 150 mM NaCl, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride, and 200 μM orthovanadate (100 μl) as described previously [11]. Samples standardised for protein were separated by SDS–polyacrylamide gel electrophoresis using 5% and 8% acrylamide stacking and resolving gels, respectively, and immunoblotted for Bcl-2, Cox-2 or CGA expression according to the manufacturer's instructions (anti-Bcl-2, Catalogue No. 610538, BD transduction Labs, Oxford, UK; 1:500, anti-Cox-2 Catalogue No. SC-1745, Santa Cruz, Biotechnology, USA, 1:200, and anti-CGA, Catalogue No. SC-1488, Santa Cruz, Biotechnology, USA, 1:200, in Tris buffered saline, pH 7.8, containing 5% [v/v] BSA). Blots were visualised using an ECL Western blotting detection kit, also exactly according to the manufacturer's instructions.

Statistical analysis. For each of the six neuroendocrine markers, five independent experiments were carried using the two control (V1/V4) and two Bcl-2 over-expressing (HB2-2/-3) cell lines. On the advice of a statistician, expression of the neuroendocrine markers by the control (V1/V4) cell lines was compared by means of the Mann–Whitney *U* test. Similarly, neuroendocrine marker expression in the Bcl-2 over-expressing (HB2-2/-3) cell lines was compared by means of this statistical test. Mann–Whitney *U* analysis was then carried out using grouped data from the control and Bcl-2 over-expressing cell lines.

Results and discussion

In view of the reports implicating the anti-apoptotic oncoprotein Bcl-2 in the acquisition/promotion of a neuroendocrine phenotype, the current study sought to determine whether forced expression of this protein was associated with modulated levels of the neuroendocrine markers, CGA, NSE, PGP 9.5, PP, and the CGA-derived peptides VST-1 and IP. A PC12 cell culture model of constitutive Bcl-2 over-expression ([10]; Fig. 1) was used in conjunction with immunocytochemical methods. Since PC12 cells are a neuroendocrine line—derived originally from a rat adrenal phaeochromocytoma—which do not basally express Bcl-2 [10,15,16], they represent an ideal model in which to examine the putative effects of exogenously expressed Bcl-2 on neuroendocrine peptide expression. Moreover, the use of immunohistochemical methods in the present study facilitated the semi-quantitative determination of marker expression, some of which would have proven difficult to detect by immunoblotting due to their low molecular weight.

Immunoblot analysis of the four cell lines confirmed that under basal conditions the PC12 HB2-2 and PC12 HB2-3 cell lines expressed Bcl-2, whilst the control cell lines, PC12 V1 and V4, did not (Fig. 1; [10,15,16]). It was next necessary to validate the methodologies chosen in order to confirm that immunohistochemical protocols would be sufficiently sensitive to detect differential neuroendocrine marker expression. In parallel with the current study expression array analysis in our laboratory involving a second, and equally well-characterised, PC12 model of Cox-2 over-expression [11] had indicated that levels of CGA were 2-fold higher in PCXII than their control (PC-MT) counterparts (Connolly et al., unpublished observations). Immunocytochemical and immunoblotting analyses were subsequently carried out to verify this observation. These experiments confirmed that, following the induction of Cox-2 expression in response to IPTG in PCXII cells (Fig. 2A), levels of CGA were elevated compared to those found in their control cell line (Figs. 2B and C). Interestingly, levels of NSE were similar in the two cell lines indicating that the effect of Cox-2 on CGA expression was specific and not due to a global enhancement of neuroendocrine marker expression/differentiation. Critically for the purposes of the current study, the finding that Cox-2-dependent modulation of CGA expression was clearly detected by immunocytochemistry (Fig. 2B) validated the use of this technique for the detection of putative Bcl-2-dependent modulation of neuroendocrine marker expression in PC12 HB2-2/HB2-3 cells and their mock-transfected controls.

Having confirmed that increased expression of neuroendocrine marker expression could be detected immunocytochemically using a PC12 cell model of over-expression, levels of the neuroendocrine markers CGA, NSE, PGP 9.5, PP, IP, and VST-1 were next examined by this method in the HB2-2, HB2-3, V1, and V4 lines. This particular panel of markers was chosen since CGA, NSE, PGP 9.5, and PP represent established neuroendocrine-specific proteins/peptides whose co-expression with Bcl-2 has been demonstrated in a range of tissues [6–8] and whose expression in PC12 cells has also been reported [17]. The expression of the CGA-derived peptides IP and VST-1 was also examined since, as well as representing additional, somewhat less ubiquitous, neuroendocrine biopeptides, it was reasoned that

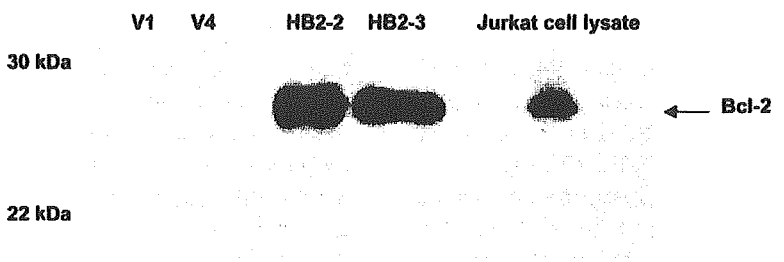

Fig. 1. Immunoblot analysis of Bcl-2 expression in control (PC12 V1 and V4) and Bcl-2 over-expressing (PC12 HB2-2 and HB2-3) cell lines.