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Downregulation of *bcl-xL* Is Relevant to UV-induced Apoptosis in Fibroblasts

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Exposure to ultraviolet light (UV) induces apoptosis in mammalian cells. The caspase group of proteases is required for the apoptosis. This pathway is initiated by a release of cytochrome c from the mitochondria into the cytosol. Several Bcl-2 family proteins can regulate the release of cytochrome c by stabilizing the mitochondrial membrane. Here we show that expression of the endogenous *bcl-xL* was strongly downregulated in NIH3T3 cells within 2 h after UV-C irradiation, and that of bax was upregulated from 8 h after irradiation. Apoptosis was induced in more than 50% of the NIH3T3 cells 48 h after irradiation. Constitutive overexpression of *bcl-xL* in NIH3T3 cells protected the UV-induced apoptosis by preventing the loss of mitochondrial membrane potential and the activation of caspase 9. These results suggest that downregulation of Bcl-xL is relevant to UV-induced apoptosis of fibroblasts.

Keywords: Apoptosis, Bcl-xL, Caspase, Mitochondria, Ultraviolet light

Introduction

The development and maintenance of healthy tissues involve apoptosis, a program of physiologically regulated cell death. Dysregulated apoptosis contributes to many pathologies, including tumor promotion, autoimmune and immunodeficiency diseases, and neurodegenerative disorders (Thompson, 1995). Ultraviolet light (UV) irradiation is one of representative apoptotic stimuli (Kulms and Schwarz, 2000) and represents one of the most relevant environmental dangers because of its hazardous effects, including skin aging (Fisher *et al*., 1996), induction of skin cancer (De Gruijil *et al*., 1993, 2001), and exacerbation of infections (Chapman *et al*., 1995). UV irradiation induces a complex cellular response, which includes the post-transcriptional activation of factors including c-Jun N-terminal kinase (JNK) (Dérijard *et al*., 1994), NF-κB and AP-1 (Stein *et al*., 1989), and a release of cytochrome c from mitochondria into the cytosol (Tournier *et al*., 2000). The cytochrome c sequentially activates Apaf-1, caspase 9, and the effector caspase 3 followed by a disruption of inner mitochondrial membrane potential ($\Delta\psi_m$) (Budihardjo *et al*., 1999; Jacotot *et al*., 1999). These events generate reactive oxygen species (ROS), release of apoptosis inducing factors from mitochondria, and induce the nuclear DNA fragmentation of apoptosis (Reed, 1997). Furthermore, UV irradiation induces DNA damage such as cyclobutane pyrimidine dimmers and pyrimidine-pyrimidine photoproducts (Thoma, 1999) and activation of death receptors on the cell surface like as CD95 (Rehemtulla *et al*., 1997; Aragane *et al*., 1998). The activation of death receptors may be related with a disruption of $\Delta\psi_m$ since caspase 8 activated with the stimulation of death receptors cleaves substrate Bid to make it active form that disrupts $\Delta\psi_m$ (Yin *et al*., 1999).

Bcl-2 family proteins play a role in regulating apoptosis (Kroemer, 1997). Bcl-2 and its relatives, Bcl-xL and Bax, are intracellular membrane-bound proteins. Bcl-2 and Bcl-xL reside in the outer mitochondrial and some other intracellular membranes, may form ion channels *in vivo*, and maintain mitochondrial volumes within physiological ranges following apoptotic stimuli, preventing swelling and the subsequent outer membrane rupture that releases cytochrome c (Green *et al*., 1998). Overexpression of Bcl-2 or Bcl-xL enhances the survival of several cell types and prevents apoptosis induced by a wide range of agents including UV irradiation (Shimizu *et al*., 1995; Duckett *et al*., 1998; Kulms and Schwarz, 2000). However, the role of endogenous Bcl-2 family proteins in UV-induced apoptosis is not studied well. Here, we examined expression of the endogenous *bcl-2* family genes in NIH3T3 cells after UV-C irradiation. Expression of *bcl-xL* was strongly downregulated in NIH3T3 cells within 2 h after UV irradiation and apoptosis was induced in many of the cells

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48 h after irradiation. The continuous supplementation of exogenous *bcl-xL* in NIH3T3 cells protected the UV-C induced apoptosis. We discuss the role for Bcl-xL in the apoptotic pathway, especially a disruption of $\Delta\psi_m$ and activation of substrate caspases, in NIH3T3 cells within 48 h after UV-C irradiation.

Materials and Methods

Cell culture The NIH3T3 murine fibroblast cell line was obtained from RIKEN Cell Bank (Tsukuba, Japan). NIH3T3 cells were cultured in RPMI 1640 medium (Sigma Chemical Co., St. Louis, USA) supplemented with 100 μg/ml streptomycin sulfate (Wako Chemical, Osaka, Japan), 100 U/ml penicillin G potassium (Banyu Pharmaceutical, Tokyo, Japan), and 10% (v/v) heat-inactivated fetal calf serum (FCS) (Sigma Product).

UV irradiation NIH3T3 cells, after washing with phosphate-buffered saline (PBS), were exposed to UV-C (254 nm) at a dose rate of 150 mJ/min by a UV crosslinker (Spectrolinker, Spectronics corporation, Westbury, USA) and further cultured in RPMI 1640 with 10% FCS for desired duration.

Northern blot analysis Total RNA was isolated from NIH3T3 cells using the TriZOL RNA isolation reagent (Life Technologies, Gaithersburg, USA). Northern blot analysis was as described (Okada *et al*., 1998). The probes were the full length murine *bcl-2* cDNA (Negrini *et al*., 1987), murine *bax* cDNA (Oltvai *et al*., 1993), and rat *bcl-xL* cDNA (Shiraiwa *et al*., 1996) (a gift from Dr. S. Ohta, Nippon Medical School, Kawasaki, Japan) that were subcloned into pGEM vectors and labeled by digoxigenin using PCR with T7 and SP6 primers (Okada *et al*., 1998).

Western blot analysis Protein extraction and Western blot analysis were performed as described previously (Kojima *et al*., 2001). Blots were incubated with the antibody for Bcl-x (Transduction Laboratories, Lexington, USA) or α-tubulin (Sigma) followed by horseradish peroxidase conjugated donkey anti-rabbit or anti-mouse antibody (Amersham Pharmacia Biotech, Buckinghamshire, UK) respectively, and developed with enhanced chemiluminescence reagents (Amersham).

Transfection of *bcl-xL* cDNA into a NIH3T3 cell line The *Xba*I fragment of *bcl-xL* cDNA (Shiraiwa, 1996) was subcloned into an *Xba*I site of the plasmid pEF-BOS (Shiraiwa *et al*., 1996; Okada *et al*., 1998), which harbors an elongation factor 1α promoter (Mizushima *et al*., 1990). The pEF-BOS/*bcl-xL* (20 μg) was transfected into NIH3T3 cells with 1 μg of the pST-neoB, which carries a neomycin-resistant gene. Transfection was performed by electroporation with a Gene Pulser (Bio-Rad, Richmond, CA) at 0.26 kV in a 0.4-cm cell. After selection with 0.5 mg/ml geneticin G418 (Life Technologies), two clones that stably expressed the exogenous *bcl-xL* gene were established. The clone #26-11 with the higher level and the clone #26-9 with the lower level of *bcl-xL* mRNA among transfectants (data not shown). Parental NIH3T3 cells as well as NIH3T3 clones transfected with pST-neoB were used as controls. Cell morphology, proliferative behavior and expression of *bcl-2* and *bax* mRNA were comparable between *bcl-xL* transfectants and parental NIH3T3 cells (data not shown).

FACS analysis for apoptotic cell death Cell viability was examined by the propidium iodide (PI) exclusion method, as described (Gottschalk *et al*., 1994). Briefly, NIH3T3 cells were harvested by trypsinization and resuspended in staining buffer (0.1% sodium azide, 3% FCS in PBS) with PI (2 μg/ml). PI uptake in each cell was analyzed on FACSCalibur (Becton Dickinson, Mountain View, USA) using Cell Quest software for Macintosh. Data were displayed as percentages of PI-stained cells.

Apoptotic cells are detected by the annexin V staining method (Vermes *et al*., 1995). Cells were assessed for staining with annexin V using the Annexin V-FITC kit (Bender MesSystem, Vienna, Austria) following the manufacture's instructions. Briefly, UV-irradiated cells were trypsinized and resuspended in staining buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$). Then, the cells were stained with FITC-labeled annexin V and PI (1 μg/ml) for 10 min and analyzed on FACSCalibur.

Since the nuclei in apoptotic cells show a uniform reduction in DNA stability with PI, which is indicated by the appearance of a subdiploid fraction of cells on the DNA histogram (Nicoletti *et al*., 1991), FACS analysis of PI-stained nuclei was performed to detect apoptotic cells. UV-irradiated cells were incubated in Krishan's reagent (0.05 mg/ml PI, 0.1% Na citrate, 0.02 mg/ml Ribonuclease A, 0.3% NP-40, pH 8.3) on ice for 30 min. Fluorescence from PI-nuclear DNA complexes was analyzed using FACSCalibur. Data were displayed as percentages of apoptotic (hypodiploid) nuclei.

Measurement of mitochondrial membrane potential ($\Delta\psi_m$) by flow cytometry UV-irradiated cells were incubated with 10 μg/ml of Rhodamine-123 (Rh-123) (Molecular Probe, Eugene, USA) for 30 min at 37°C. The cells were washed twice with PBS, resuspended in RPMI 1640 with 10% FCS, and incubated for another 30 min to remove unbound fluorochrome. The cells were trypsinized, washed twice with PBS, resuspended in staining buffer with PI (2 μg/ml), and analyzed on FACSCalibur. Loss of mitochondrial membrane potential was visualized as a reduction in the signal in FL1.

Detection of caspase 3, caspase 8 and caspase 9 activity Activity of caspase 3, caspase 8, and caspase 9 was detected using assay kits (caspase 3; CaspACETM Assay system, Colorimetric, Promega, Madison, WI, caspase 8; FLICE/Caspase-8 Colorimetric Protease Assay Kit, MBL, Nagoya, Japan, caspase 9; Caspase 9/Mch6 Colorimetric Protease Assay Kit, MBL). The assay procedures were followed according to the manufacture's instructions. Briefly, UV-irradiated NIH3T3 cells were washed with ice-cold PBS, and lysed in 50 μl of chilled cell lysis buffer (accompanied in assay kit). The cell lysate was frozen at −80°C and thawed quickly. The homogenates were centrifuged, and supernatants (cytosolic extract) were collected. Protein concentration was determined using a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA). Then, 100 μl aliquots of protein extracts (100 μg protein) were incubated with DEVD-pNA (200 μM, IETD-pNA (200 μM or LETD-pNA (200 μM) substrate in reaction buffer containing 10 mM DTT for 2 to 4 h at 37°C. The absorbance of samples was measured on an ELISA plate reader (Bio-Rad) at 405 nm.

Results

Expression of *bcl-xL* is downregulated in NIH3T3 cells after UV irradiation NIH3T3 cells were irradiated with various doses (30-100 mJ/cm^2) of UV-C, and viability of the cells was examined 24 and 48 h after irradiation by the PI exclusion method with FACS. Viability of NIH3T3 cells was 90% and 15% 24 h after irradiation with 60 and 100 mJ/cm^2 of UV-C, respectively. The viability became 50% and 10% 48 h after irradiation, respectively. In order to examine the relation between UV-induced cell death and expression of the endogenous *bcl-2* family genes (*bcl-2*, *bcl-xL*, and *bax*), NIH3T3 cells were irradiated with 60 mJ/cm^2 of UV-C and the expression in those irradiated cells was analyzed up to 24 h after irradiation by Northern blot. Figure 1A shows that expression of *bcl-xL* and *bax* mRNA but not that of *bcl-2* mRNA was detected in NIH3T3 cells before irradiation. Expression of *bcl-xL* mRNA decreased within 2 h, and sustained at least 12 h after irradiation. Expression of *bax* mRNA also decreased until 4 h and then upregulated from 8 h after irradiation. However, *bcl-2* mRNA was not detected at all even after irradiation. Expression of Bcl-xL protein in those irradiated NIH3T3 cells was further analyzed by Western blot (Fig. 1B). Although the amount of *bcl-xL* mRNA decreased 2 h after irradiation, Bcl-xL protein was still detectable in NIH3T3 cells until 2 h after irradiation and then decreased such as the reduction of *bcl-xL* mRNA.

Exogenous *bcl-xL* protects NIH3T3 cells from UV-induced apoptosis. The exogenous *bcl-xL* gene was constitutively overexpressed in NIH3T3 cells and its effect on UV-induced cell death was analyzed. We used two lines ($^{\#}$26-9 and $^{\#}$26-11) of the *bcl-xL* stable transfectants of NIH3T3 cells. Those transfectants constitutively expressed the exogenous *bcl-xL* gene after irradiation with 60 mJ/cm^2 of UV-C (data not shown). Dead cells in those *bcl-xL* transfectants 24 and 48 h after irradiation were detected by the PI exclusion method with FACS. Figure 2A shows that approximately 50% of NIH3T3 cells and neo transfectants but not *bcl-xL* transfectants were dead 48 h after irradiation. Moreover, the clone $^{\#}$26-11 with the higher level of *bcl-xL* mRNA is more viable than the clone $^{\#}$26-9 with the lower level of *bcl-xL* mRNA. When those cells were irradiated with 100 mJ/cm^2, more than 80% of control and neo transfectants were dead 48 h after irradiation. However, both *bcl-xL* transfectants were resistant for the dose (100 mJ/cm^2) of UV-C irradiation (data not shown). These data suggest that Bcl-xL prevents cell death of NIH3T3 cells induced by irradiation with 60-100 mJ/cm^2 of UV-C at least until 48 h after irradiation.

To investigate whether type of cell death induced in NIH3T3 cells by irradiation with 60 mJ/cm^2 of UV-C was apoptosis or not, the annexin V labeling method was used. When NIH3T3 cells were irradiated with 60 mJ/cm^2 of UV-C, more than 70% of cells were annexin V positive 48 h after irradiation (Fig. 2B). Since annexin V$^+$/PI$^-$ cells are apoptotic cells (Vermes *et al*., 1995) and 22% of the cells were in the apoptotic subset 48 h after irradiation, cell death of NIH3T3 cells induced by UV irradiation is apoptosis. However, percentages of annexin V positive cells did not increase in *bcl-xL* transfectants after irradiation. Furthermore, apoptotic cells can be identified by cell cycle analysis with their lower DNA contents than those of cells in G_1 phase (Nicoletti *et al*., 1991), and apoptotic cell death of NIH3T3 cells irradiated with 60 mJ/cm^2 of UV-C was confirmed by the DNA contents with a flow cytometry. The apoptotic cells were detectable in NIH3T3 cells (46%) 48 h after irradiation (Fig. 2C). However, apoptotic cells in $^{\#}$26-11 cells (11%) were lower than that of NIH3T3 cells after irradiation. These results suggest that the downregulation of endogenous *bcl-xL* is relevant to induction of apoptosis in NIH3T3 cells after UV-C irradiation.

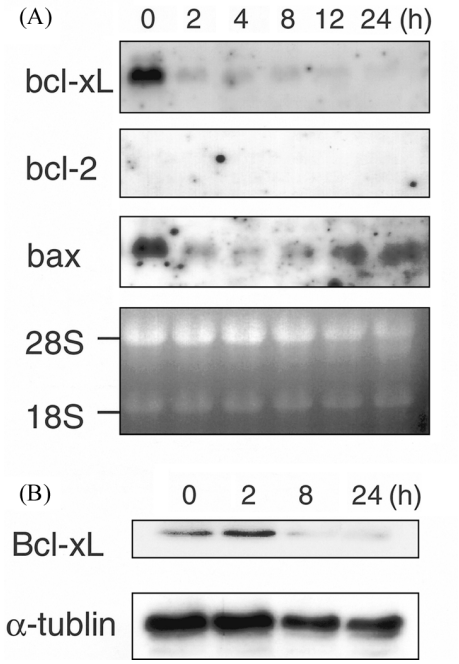

Fig. 1. Kinetics of *bcl-2* family gene expression in UV-irradiated NIH3T3 cells. NIH3T3 cells were UV-irradiated (60 mJ/cm^2), and total RNA and protein were isolated from NIH3T3 cells at the indicated times after UV irradiation. (A) Levels of *bcl-xL*, *bcl-2* and *bax* mRNA in total RNA were analyzed by Northern blot. (B) Levels of Bcl-xL and α-tubulin in total protein were analyzed by Western blot.

Exogenous *bcl-xL* blocks a loss of mitochondrial membrane potential and caspase 9 activation induced by UV irradiation One of the protective action of Bcl-xL against apoptosis is the prevention of mitochondrial depolarization (Zamzami, 1995). Mitochondria lose their membrane potential as a result of the opening of permeability transition pores. To examine mitochondrial depolarization following UV-C irradiation with 60 mJ/cm^2, $\Delta\psi_m$ was monitored by Rh-123 fluorescence in NIH3T3 cells and $^{\#}$26-11 cells. Rh-123 accumulation reflected combined changes in

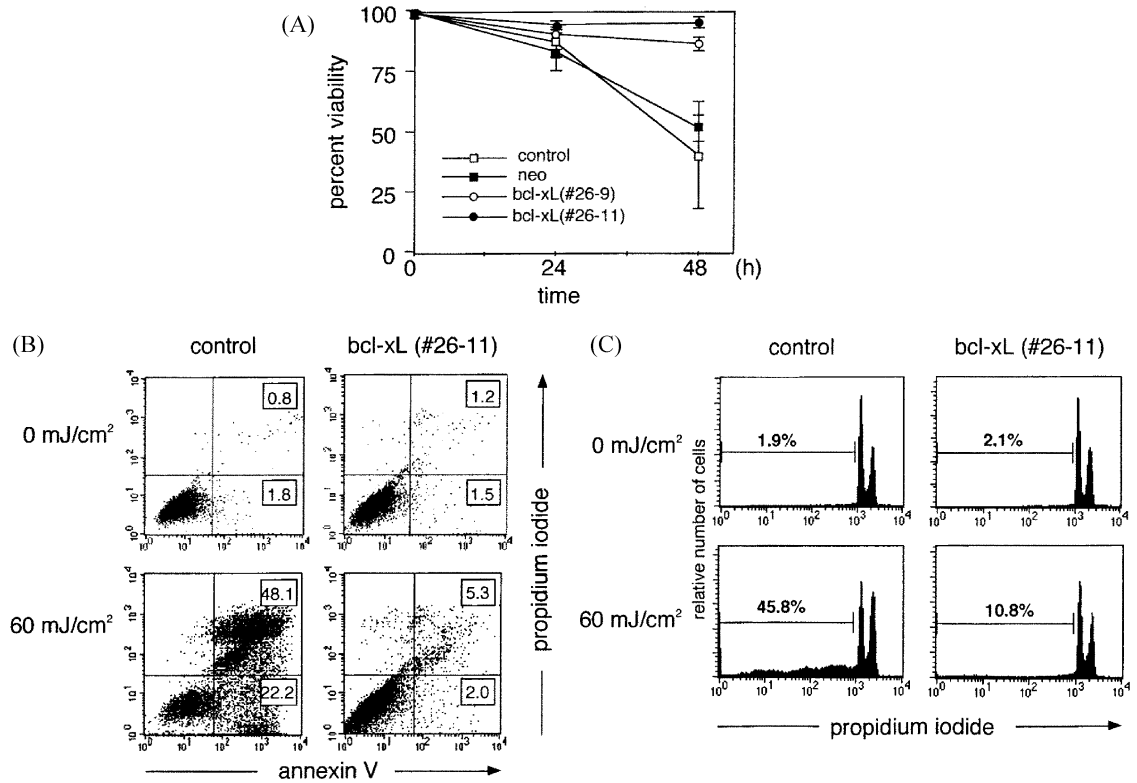

Fig. 2. Exogenous *bcl-xL* protects NIH3T3 cells from UV-induced apoptosis. (A) NIH3T3 cells transfected with a *bcl-xL* (#26-9 and #26-11) expression vector, the vector control (neo), or parental NIH3T3 (control) cells were exposed with UV irradiation (60 mJ/cm^2), and viability was determined at the indicated time points by PI exclusion. The data presented are the mean and standard deviation of three independent determinations and are representative of three independent experiments. (B, C) Apoptosis of NIH3T3 cells (control) and *bcl-xL* transfectants (#26-11) was detected 48 h after UV irradiation by FACS with the annexin-V staining method (B) and PI staining method of nuclei (C). The data represented are representative of three independent experiments.

the $\Delta\psi_m$ and mitochondrial volume. The number of NIH3T3 cells that were loosing $\Delta\psi_m$ (Rh-123^{low}, PI$^-$) increased 24 and 48 h after irradiation (Fig. 3). In contrast, even 48 h after irradiation, the majority of #26-11 cells did not undergo a loss of $\Delta\psi_m$.

More than 10 different mammalian caspases have been characterized (Los *et al.*, 1999), and changes in the $\Delta\psi_m$ release cytochrome c that activates Apaf-1 and caspase 9 followed by the activation of caspase 3 (Reed, 1997). UV irradiation also activates death receptors followed by activation of caspase 8 (Rehemtulla *et al.*, 1997; Aragane *et al.*, 1998). To determine which caspases might be responsible for apoptosis in NIH3T3 cells induced by irradiation with 60 mJ/cm^2 of UV-C, we performed *in vitro* cleavage assays for caspase 8, caspase 9, and caspase 3. Activity of caspase 8, caspase 9, and caspase 3 was detected in NIH3T3 cells 6 h after irradiation and increased 24 h after irradiation (Fig. 4). Activation of caspase 9 was completely blocked in #26-11 cells until 24 h after irradiation although activation of caspase 8 and caspase 3 could not be blocked in the #26-11 cells. These results indicate that the pathway of apoptosis induced by UV irradiation involves the loss of $\Delta\psi_m$ and activation of caspase 9, and that Bcl-xL can inhibit the UV-induced apoptosis by preventing the loss of $\Delta\psi_m$ and the activation of caspase 9.

Discussion

Bcl-2 family proteins play an important role in a programmed cell death pathway. The mechanism of Bcl-2 family proteins to protect apoptosis is the prevention of mitochondrial depolarization (Zamzami *et al.*, 1995). Mitochondria lose their membrane potential as a result of the opening of permeability transition pores in the Fas-induced apoptosis (Yin *et al.*, 1999). Isolated mitochondria that undergo permeability transition in response to any of a variety of treatments have been shown to release substances that are capable of inducing apoptotic changes in isolated nuclei (Liu *et al.*, 1996). Mitochondria isolated from cells overexpressing Bcl-xL are resistant to membrane depolarization in response to oxidizing agents or drugs that uncouple oxidative phosphorylation (Boise *et al.*, 1997), and overexpression of Bcl-2 in a human T cell line prevented UV-induced cytochrome c release (Keogh *et al.*, 2000). Exposure of fibroblasts to UV-C irradiation caused a large increase in cytoplasmic cytochrome c and decreased mitochondrial membrane potential (Tournier *et al.*,

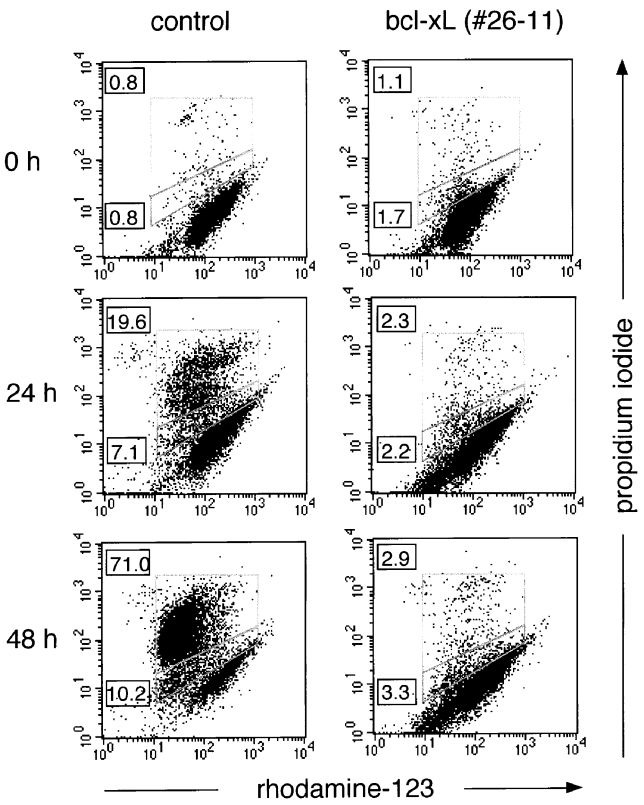

Fig. 3. Bcl-xL can block loss of mitochondrial membrane potential induced by UV irradiation. NIH3T3 cells (control) and *bcl-xL* transfectants (#26-11) were UV-irradiated (60 mJ/cm^2) and analyzed 24 and 48 h after UV irradiation. Viability and changes in mitochondrial membrane potential of those cells were analyzed by PI staining and Rhodamine-123 staining, respectively. The data represented are representative of three independent experiments.

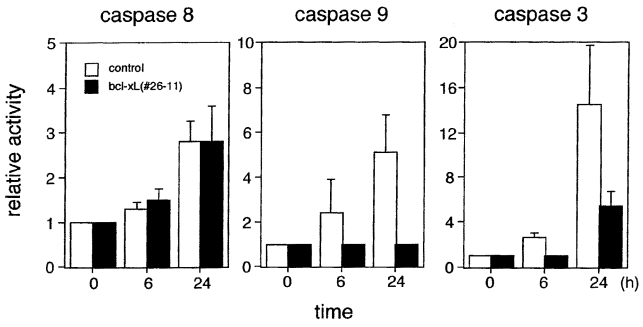

Fig. 4. Bcl-xL inhibits UV-induced activation of caspases. Activity of caspase 8, caspase 9, and caspase 3 was detected in NIH3T3 cells (control; open bar) and compared with that in *bcl-xL* transfectants (#26-11; closed bar). Cells were treated with 60 mJ/cm^2 of UV-C and analyzed 6 and 24 h after UV irradiation. The results represent the average valves from three independent experiments.

2000). Here we clearly showed that murine fibroblasts (NIH3T3 cells) lost mitochondrial membrane potential within 24 h after UV-C irradiation and that apoptosis was induced in approximately 50% of them 48 h after irradiation. Bcl-xL but not Bcl-2 was detected in NIH3T3 cells, and the Bcl-xL was downregulated in NIH3T3 cells within 8 h after irradiation. Overexpression of Bcl-xL protected NIH3T3 cells from the UV-induced apoptosis. Thus, the endogenous Bcl-xL in NIH3T3 cells may play a physiologic role in the regulation of UV-induced apoptosis.

Expression of the endogenous *bcl-xL* mRNA is downregulated in NIH3T3 cells after UV-C irradiation (Fig. 1A). Since ROS are responsible for the decrease in Bcl-xL mRNA level during apoptosis mediated by TGF-beta in fetal hepatocytes (Herrera, *et al.*, 2001), ROS induced by UV irradiation (Kroemer, 1997) may downregulate the expression of Bcl-xL in NIH3T3 cells. Bcl-2 was also downregulated in human epidermis and fibroblasts by UV-B irradiation (Isoherranen *et al.*, 1999). Bcl-2 has been reported to be phosphorylated and inactivated by JNK (Maundrell *et al.*, 1997). Although UV irradiation induces JNK activation (Dérijard *et al.*, 1994), Bcl-2 was not phosphorylated in embryonic fibroblasts exposed to UV-C irradiation (Tournier *et al.*, 2000). Thus, downregulation of Bcl-2 family gene expression may be a major regulatory mechanism of function of Bcl-2 family proteins in cells after UV irradiation. Protection of human endothelial cells from UV-induced apoptosis was correlated with upregulaton of the endogenous Bcl-2 (Suschek *et al.*, 1999). Furthermore, overexpression of Bcl-2 (Keogh *et al.*, 2000) or Bcl-xL (Fig. 1A) protected UV-induced apoptosis, indicating that the downregulation of Bcl-2 and Bcl-xL may be required for UV-induced apoptosis.

UV irradiation induces DNA damage by multiple causes, such as protein-DNA crosslinks, oxidative base damage (forming 8-oxo-7,8-dihydroxyguanine), and single strand DNA breaks (de Gruijl *et al.*, 2001). These effects are not mutually exclusive and contribute independently to a complete response to UV-B irradiation (Kulms *et al.*, 1999). UV-C irradiation induces DNA damage followed by the cell cycle arrest predominantly at G1 phase and DNA repair or by apoptosis (Geyer *et al.*, 2000). Cell death induced by DNA damaging agents is a late event occurring later than 48 h after treatment and it was preceded by decrease in Bcl-2 protein level (Ochs and Kaina, 2000). Since Bcl-2 overexpression in marsupial cells was found to delay but not to prevent cell death 14 days after UV-C irradiation (Miyaji and Menck, 1998), Bcl-2 family proteins may not be able to protect cell death induced by DNA damage after UV irradiation. When NIH3T3 cells were irradiated with 60 mJ/cm^2 of UV-C, cell cycle was not arrested in G1 phase 24 h (data not shown) and 48 h (Fig. 2C) after irradiation and overexpression of Bcl-xL protected NIH3T3 cells from the UV-induced apoptosis until 48 h after irradiation. These results suggest that DNA damage is not the major cause of death of NIH3T3 cells until 48 h after irradiation with this dose of UV-C.

UV-C irradiation directly activates death receptors such as

CD95 (Fas/APO-1) (Kulms and Schwarz, 2000), which activate substrate caspases (caspase 8 and caspase 3) to induce apoptosis (Boise *et al*., 1997; Yin *et al*., 1999). We demonstrated that UV-C irradiation activates caspase 8, caspase 9 and caspase 3 in NIH3T3 cells within 6 h after irradiation (Fig. 4) and the caspase inhibitor zVAD inhibited the apoptosis (data not shown). These results indicate that activation of caspases is a major cause of the UV-induced apoptosis in NIH3T3 cells until 48 h after irradiation. Overexpression of Bcl-xL can inhibit the apoptosis with the complete inhibition of caspase 9 activation but not caspase 8 and caspase 3 in NIH3T3 cells after irradiation. Thus, a major cause of the apoptosis is the activation of caspase 9 but not that of caspase 8. This has been supported using Jurkat T lymphocytes (Vu *et al*., 2001). Gene knockout studies also demonstrate that caspase 8 is not required for UV-induced apoptosis (Los *et al*., 1999). Caspase 9 is activated with Apaf-1 and cytochrome c in cytosole, suggesting that the activation of caspase 9 is considered to be a substrate of depolarization of mitochondrial membrane potential. Therefore, direct activation of death receptors on the cell surface are not a major cause of apoptosis in NIH3T3 cells until 48 h after UV-C irradiation at a dose we used.

Caspase 3 was slightly activated in *bcl-xL* transfectants 24 h after UV-C irradiation (Fig. 4) probably because caspase 8 was activated in the transfectants after UV-C irradiation and it can directly activate caspase 3 (Kulms *et al.*, 1999). Caspase 8 also activates Bid, a proapoptotic BH3-only member of the Bcl-2 group. Bid is proteolytically activated to generate a fragment that translocates to the mitochondria and induces cytochrome c release (Yin *et al*., 1999). Bid cleavage is induced in embryonic fibroblasts by UV-C irradiation (Tournier *et al*., 2000). However, the caspase inhibitor zVAD did not inhibit Bid cleavage and cytochrome c release caused by UV irradiation, indicating that Bid cleavage and cytochrome c release in response to UV irradiation may be caspase-independent (Tournier *et al.*, 2000). Thus, UV-induced apoptosis is mainly to through mitochondrial pathway at first, whether it occurs after activation of death receptors or direct stimulation of mitochondria, independent of the existence of genomic DNA damage.

In conclusion, Bcl-xL is downregulated in NIH3T3 cells after UV-C irradiation, and that downregulation is related with a loss of mitochondrial membrane potential and substrate caspase activation. Since overexpression of Bcl-xL prevents the loss of mitochondrial membrane potential and activation of caspase 9, an early event in the irreversible phase of programmed cell death, the downregulation of Bcl-xL may be required for apoptosis in NIH3T3 cells induced by UV-C irradiation.

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