**Communication**

**Activation of c-Jun N-terminal Kinase Antagonizes an Anti-apoptotic Action of Bcl-2**

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Bcl-2 is an intracellular membrane-associated protein that prevents cell death induced by a variety of apoptotic stimuli. A mechanism by which Bcl-2 exerts an anti-cell death effect is, however, not fully understood. In the present study, Bcl-2 suppressed cell death of N18TG neuroglioma cells caused by various apoptotic stresses, including etoposide, staurosporine, anisomycin, and ultraviolet irradiation. Concomitantly, Bcl-2 disrupted a signaling cascade to the c-Jun N-terminal kinase activation induced by the apoptotic stresses. Bcl-2 also prevented the etoposide-induced stimulation of MEKK1. Furthermore, overexpression of c-Jun N-terminal kinase antagonized the death-protective function of Bcl-2. These data suggest that suppression of the c-Jun N-terminal kinase signaling pathway may be critical for Bcl-2 action.

Apoptosis is thought to be involved not only in normal physiological processes, but also in pathogenesis of many diseases that result from an imbalance between positive and negative regulators of cell survival (1, 2). Numerous studies have demonstrated that Bcl-2 is a typical positive regulator of cell survival (3). This 26-kDa intracellular membrane-associated protein is capable of protecting various cell types from experimentally induced cell death both in vivo and in vitro (4, 5). For example, Bcl-2 rescues cell death induced by a variety of stresses, including depletion of trophic factors, anti-tumor drugs, oxygen free radicals, viral agents, and heat shock as well as neuronal axotomy (4–7). Furthermore, bcl-2 is thought to be a mammalian counterpart of ced-9, which acts to prevent cell death in Caenorhabditis elegans (8). The molecular mechanism by which Bcl-2 prevents cell death remains unknown, however. Interestingly, it has been demonstrated recently that certain apoptotic stresses can induce activation of the specific signaling systems such as sphingomyelin or c-Jun N-terminal kinase (JNK)³ pathways (9, 10). These findings imply that Bcl-2 might suppress cell death through modulating intracellular signaling cascades associated with apoptosis.

JNK, also termed stress-activated protein kinase (SAPK), is a new member of the family of mammalian mitogen-activated protein (MAP) kinases that mediate intracellular signals originated from diverse extracellular stimuli, including growth factors, cytokines, or various stresses (11). JNK is often activated through upstream protein kinases, including JNKK and MEKK1, in response to a variety of cellular stresses such as ionizing irradiation, alkylating chemicals, ultraviolet (UV) light, or heat shock (12, 13). It is noteworthy that many stresses that induce the stimulation of JNK can eventually cause cell death. In fact, the stimulation of JNK was prerequisite for cell death under various conditions, and a blockade of the JNK activation resulted in the prevention of cell death (9, 10). These findings imply that JNK may mediate an intracellular signaling pathway leading to cell death.

In the present study, we investigated a possible mechanism for anti-apoptotic action of Bcl-2. We observed that Bcl-2 blocked the activation of the JNK signaling pathway by various apoptotic stresses and that the blockade of JNK pathway might be associated with the cell survival effect of Bcl-2.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfections**—N18TG cells were routinely maintained in poly-lysine-coated plates containing Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Cells were transfected with indicated expression vectors by using LipofectAMINE (Life Technologies, Inc.). For bcl-2 transfection, cells were transfected with pcDNA3 (Invitrogen) or pcDNA3 containing a full-length coding sequence of human bcl-2. After 48 h of transfection, cultures were maintained in the complete medium containing G418 (500 µg/ml) to select neomycin-resistant cells. For JNK1 transfection, cells were transfected with pCEP4 (Invitrogen) or pCEP4 containing JNK1 cDNA. Stable cell lines were selected by adding hygromycin (200 units/ml) in culture medium. Heterogeneous populations of the stably transfected cells were used in this study to avoid any possible clonal variations.

**DNA Fragmentation**— Cultured cells were exposed to etoposide (10 µg/ml) or staurosporine (1 µg/ml) overnight or to anisomycin (50 µg/ml) for 4 h. When indicated, cells were exposed to UV light (40 J/m²), then further incubated overnight. After treatment with the indicated agents, cells were harvested, and lysed with a solution containing 20 mM EDTA, 0.8% sodium lauryl sarcosine, 100 mM Tris-HCl, pH 8.0. Cell lysates were treated with RNase A, RNase T1, and proteinase K, as described previously (14). Fragmented DNA samples were separated by electrophoresis on 1.5% agarose gel and visualized with ethidium bromide.

**Nuclear Staining with Hoechst 33258**—Cells were exposed to 10 µg/ml etoposide for 36 h, fixed with 4% paraformaldehyde, permeabilized with ice-cold ethanol, and stained with 50 ng/ml Hoechst 33258, as described previously (15). Stained nuclei were observed and photographed with a Zeiss Axiovert135 fluorescent microscope with a × 40 objective.

**Enzymatic Assay for JNK1 or MEKK1**—Cells were exposed to the indicated agents, harvested, and lysed with a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1% Nonidet P-40, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate. Solubilized fractions were immunoprecipitated with either a mouse monoclonal anti-JNK1 (PharMingen) or a rabbit polyclonal anti-MEKK1 antibody (Santa Cruz). The immunopellets were assayed for either JNK1 or MEKK1 activity, as described previously (16). Either GST-c-Jun or GST-SEK1 fusion protein was used as a substrate for

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³ The abbreviations used are: JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; SEK, SAPK kinase; JNKK, JNK kinase; MEKK, MEK kinase.
either JNK1 or MEKK1, respectively. The assay reaction mixture was subjected to electrophoresis on a 10% polyacrylamide gel. The phospho-
rylated proteins were quantified by autoradiography and densitometry. Protein concentrations were determined by the BCA method (Pierce), as described in the manufacture’s protocol.

RESULTS AND DISCUSSION

To investigate the mechanism of anti-cell death function of Bcl-2, we transfected a mammalian expression vector containing bcl-2 and named either N18 or N18-Bcl-2 cells, respectively. A, immunoblot of Bcl-2 in cells expressing Bcl-2 (N18-Bcl-2) and control cells (N18). B, viability of either control or Bcl-2-expressing cells in exposure to 10 μg/ml etoposide, as measured by trypan blue exclusion. C, apoptotic DNA fragmentation induced by etoposide. Either control cells (Control) or Bcl-2-expressing cells (Bcl-2) were exposed to 10 μg/ml etoposide (E) for 18 h. DNA was extracted and DNA fragments were resolved on a 1.5% agarose gel.

![Fig. 1. Bcl-2 prevents cell death induced by etoposide. N18TG cells were transfected with either a control vector or a vector containing bcl-2 and named either N18 or N18-Bcl-2 cells, respectively. A, immunoblot of Bcl-2 in cells expressing Bcl-2 (N18-Bcl-2) and control cells (N18). B, viability of either control or Bcl-2-expressing cells in exposure to 10 μg/ml etoposide, as measured by trypan blue exclusion. C, apoptotic DNA fragmentation induced by etoposide. Either control cells (Control) or Bcl-2-expressing cells (Bcl-2) were exposed to 10 μg/ml etoposide (E) for 18 h. DNA was extracted and DNA fragments were resolved on a 1.5% agarose gel.](image)

![Fig. 2. Bcl-2 suppresses the JNK1 stimulation induced by cytotoxic stresses. A, either N18 control cells (control) or Bcl-2-expressing cells (Bcl-2) were exposed to etoposide (10 μg/ml), staurosporine (1 μg/ml), or anisomycin (50 μg/ml) for 1 h or to UV irradiation (40 J/m²). Cells were harvested, lysed, and subjected to immunoprecipitation with mouse anti-JNK1 monoclonal antibody. The immunocomplex JNK1 was assayed by phosphorylating a substrate, GST-c-Jun. Phosphorylated proteins were visualized by SDS-PAGE and autoradiography. B, immunoblot of JNK1 in N18 control cells (N18) and Bcl-2-expressing cells (N18-Bcl-2). C, either control or Bcl-2-expressing cells were exposed to 10 μg/ml etoposide for 40 min. Cells were collected and subjected to immunoprecipitation with rabbit polyclonal anti-MEKK1 antibody. The immunopellets were assayed for MEKK1 activity, as described under “Experimental Procedures.”](image)

![Fig. 3. Overexpression of JNK1 in Bcl-2-expressing cells overrides the suppressive effect of Bcl-2 on JNK1 activity. JNK1 activity was stimulated by exposing either Bcl-2-transfected (N18-Bcl-2) or Bcl-2/JNK1-transfected (N18-Bcl-2/JNK1) cells to etoposide (10 μg/ml), staurosporine (1 μg/ml), anisomycin (50 μg/ml) for 1 h or to UV irradiation (40 J/m²) and was assayed for phosphorylating GST-c-Jun, as for Fig. 2.](image)

JNK Antagonizes Action of Bcl-2

The signaling mechanism for the anti-apoptotic action of Bcl-2 remains unclear. The mammalian MAP kinases, which include JNK, extracellular signal-regulated kinase (ERK), and p38 kinase, are parts of signal transduction cascades leading to a variety of cellular events (11). In particular, JNK has been shown to mediate intracellular signals leading to apoptosis (9, 10, 18). All of the apoptotic stresses used in this study, which included etoposide, staurosporine, UV light, and anisomycin, could induce the stimulation of the JNK signaling pathway in N18 control cells (Fig. 2A). Using these apoptotic stresses, we investigated a possibility that Bcl-2 might modulate the JNK signaling cascade. In Bcl-2-expressing cells, etoposide had little effect, if at all, on JNK1 activity (Fig. 2A). Bcl-2 also suppressed the stimulation of JNK1 activities induced by staurosporine.

2 J. Park and E.-J. Choi, unpublished observations.
Overexpression of JNK1 counteracts the anti-apoptotic function of Bcl-2. Cultured cells were exposed to etoposide (10 μg/ml), staurosporine (1 μg/ml), UV light (40 Jm–2), or anisomycin (50 μg/ml). N18 control N18TG cells; N18-JNK1, cells transfected with JNK1 gene; N18-Bcl-2; cells transfected with bcl-2 and JNK1 gene. A, viability of cells after treatment with apoptotic agents. Cultured cells were exposed to etoposide or staurosporine overnight or anisomycin for 4 h. For UV experiments, cells were exposed to 40 Jm–2 UV light, then further incubated overnight. After treatment of cells with the indicated agents, percentage of viability was determined by trypan blue exclusion. B, DNA fragmentation induced by apoptotic agents. Either N18-Bcl-2 cells (Bcl-2) or N18-Bcl-2/JNK1 cells (Bcl-2/JNK1) were exposed to etoposide (E), staurosporine (S), anisomycin (A), or UV light (U), the same as for A.

DNA fragmentation was visualized, the same as for Fig. 1, C–F, staining of apoptotic cells with Hoechst 33258. Cultured cells were incubated without (C) or with 10 μg/ml etoposide (D–F) for 36 h, and morphological changes in cells stained with Hoechst 33258 were examined and photographed with a Zeiss Axiovert135 fluorescence microscope with a × 40 objective. C and D, N18 control cells; E, N18-Bcl-2 cells; F, N18-Bcl-2/JNK1 cells.
through the endoplasmic reticulum in some experimental models (21–23). We report here that various stresses which induce the stimulation of JNK activity can cause cell death and that Bcl-2 prevents both cell death and JNK stimulation induced by those stresses. Moreover, overexpression of JNK1 counteracted the anti-apoptotic function of Bcl-2 in response to the apoptotic stresses. Interestingly, a recent study with PC12 cells also reported that JNK activation induced by nerve growth factor withdrawal was blocked by Bcl-2 (24). Although a detailed mechanism by which Bcl-2 blocks JNK activation is not clear yet, we found that Bcl-2 disrupted the stimulation of MEKK1, an upstream protein kinase in JNK signaling pathway (19). It suggests that Bcl-2 might act on its cellular target(s) that might be involved in the stimulation of MEKK, which can activate the JNK pathway. A precise mechanism by which overexpression of JNK1 overcomes the antagonistic effect of Bcl-2 on the stress-induced stimulation of endogenous JNK1 is not clear yet. One possibility could be that it might result from a dose effect of the overexpressed JNK1 protein. That is, JNK1 activity could be enhanced to a significant level in JNK1-overexpressing cells in response to apoptotic stresses, if Bcl-2 could not completely block the MEKK/JNK1 signaling pathway. Another possibility could be that there might exist alternative pathways for stress-induced stimulation of JNK1. In this regard, it is noteworthy that JNK1 signaling pathway can be also regulated through other MAPKKKs such as ASK1 (25), independent of MEKK1. In any event, our findings in this study strongly suggest that the JNK signaling pathway may be downstream from the target(s) of Bcl-2 action. Notably, overexpression of Bcl-XL, a functional analog of Bcl-2 (26), in N18TG cells also resulted in the suppression of the JNK stimulation after treatment of cells with etoposide or the other stresses used in this study.7 Therefore, it appears that suppression of the JNK pathway may be crucial for the anti-cell death function of both Bcl-2 and Bcl-XL. Our findings presented in this study provide new insights into the mechanism by which Bcl-2 and its related proteins regulate cell death and survival.

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