Bcl-2 Protein Inhibits Bufalin-induced Apoptosis through Inhibition of Mitogen-activated Protein Kinase Activation in Human Leukemia U937 Cells

Masahiko Watabe, Nobuko Kawazoe, Yutaka Masuda, Shigeo Nakajo, and Kazuyasu Nakaya
Laboratory of Biological Chemistry, School of Pharmaceutical Sciences, Showa University. 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142, Japan

Abstract
In a previous study, we demonstrated that bufalin, which is an active principle of Chinese medicine, chan'su, caused apoptosis in human leukemia U937 cells by abnormal activation of mitogen-activated protein kinase (MAPK) via the signaling pathway of Ras, Raf-1, and MAPK kinase-1. Here, we report the effect of overexpression of bcl-2 in U937 cells on the signaling pathway of apoptosis that is induced by bufalin. The results indicated that the apoptosis induced by bufalin in U937 cells was significantly inhibited by overexpression of the Bcl-2 protein. No significant difference was detected in the activation of MAPK kinase-1 that is induced by bufalin in wild-type or Bcl-2-overexpressed U937 cells; however, the activation of MAPK by bufalin was significantly attenuated in the cells overexpressing Bcl-2. Bufalin treatment activated activator protein-1 transcriptional activity; however, this activation was decreased to 40% in bcl-2-overexpressed U937 cells. These results indicate that Bcl-2 acts downstream of MAPK kinase-1 but upstream of MAPK and suggest that, in the signaling pathway of the apoptotic process induced by bufalin, the transcriptional activity of activator protein-1 may be down-regulated through the inhibition of MAPK activity by Bcl-2.

Introduction
Several genes have been identified as either inducers or repressors of apoptosis. Among these is bcl-2 (B-cell lymphoma-2), which is known as an antiapoptotic gene. It was first discovered by virtue of its involvement in the t(14;18) chromosomal translocations found in the majority of non-Hodgkin's B-cell lymphomas (1, 2). The biochemical mechanism of action of this M. 25,000–26,000 Bcl-2 protein remains enigmatic. The predicted amino acid sequence of Bcl-2 shares no significant homology with other proteins having known functions. Hockenbery et al. (3) proposed that Bcl-2 functions in an antioxidant pathway. On the contrary, Steinman proposed that Bcl-2 acts as a pro-oxidant, generating reactive oxygen intermediates in Escherichia coli (4). Oltvai et al. (5) suggested that some functions of Bcl-2 are mediated through the interaction with Bax, a Bcl-2 homologue. Recently, Bcl-2 protein has been reported to be physically associated with p72 Raf-1, which is a serine/threonine-specific protein kinase (6), and with p23-Ras (7), which is a GTPase member of the Ras family (8). This suggests that this antiapoptotic protein may participate in a signal transduction pathway via Ras/Raf-1. In cotransfection studies using a diploid hematopoietic cell line, the combination of elevations in the Bcl-2 protein level and Raf-1 kinase activity was shown to provide synergistic protection to the cell from death induced by growth factor withdrawal (6). However, the function of Raf-1 and the significance of its interaction with Bcl-2 in the regulation of cell death remains unclear.

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Materials and Methods
Cell Culture. Human promonocytic leukemia U937 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). The cells were grown in RPMI 1640, supplemented with 10% FCS, at 37°C under 5% CO2 in air.

Chemicals and Radioisotopes. Bufalin was purchased from Sigma Chemical Co. (St. Louis, MO); luciferase assay system was from Promega, and [γ-32P]ATP (3000 Ci/mmol) was from Amersham International (Amersham, United Kingdom), respectively. Geneticin (G418 sulfate) and an antibody against Bcl-2 were from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan). The peptides, FLTEYVATRWYRAPEIMLN and KRELVEPLTPAGEAPN, were chemically synthesized with a model 431A peptide synthesizer (Applied Biosystems, Inc.). Cleavage of the 4-hydroxymethylphenoxymethylpolystyrene-resin was performed with 95% (v/v) TFA. The peptides were applied to a C8 reverse-phase high-performance liquid chromatography column (Prep-10 Aquapore, 10 x 250 mm). The column was washed with 0.1% (v/v) TFA and then eluted with a linear gradient of 0–70% (v/v) acetonitrile containing 0.1% (v/v) TFA, at a flow rate of 3 ml/min for 60 min.

Construction of Expression Vector. The 0.9-kb EcorI fragment containing the open reading frame of a human bcl-2 cDNA was inserted into the Hincl site of a pcUC19 cloning vector. The insert was cut with Xhol and HindIII and subcloned into the Xhol and HindIII restriction sites of a pcRS-RSV expression vector.

Transfection of U937 Cells. Transfections were performed by electroporation (Bio-Rad Gene Pulser) as described previously (11). The plasmid DNA was delivered to a 0.4-ml U937 cell suspension (4 x 106 cells) containing 10 μg of plasmid DNA. The cells (1 x 106 cells per well) were plated onto 96-well microplates and cultured in RPMI 1640. After 24 h of culture, geneticin was added at a final concentration of 400 μg/ml, and the subclones were isolated 2–3 weeks later.

RNA Isolation and Northern Blot Analysis. Total RNA was prepared by the method of Chomczynski and Sacchi (12) and was subjected to electrophoresis on a 1% (v/v) denatured agarose gel containing formaldehyde and transferred to a Hybond-N (Amersham). Northern blot analysis was performed as described previously (13).

Bufalin Treatment. Unless otherwise indicated, U937 cells (5 x 106 cells) were deprived of serum for 24 h prior to the addition of bufalin. The cells were treated with 10–8 M bufalin in the serum-free RPMI 1640 for the times specified above.

1 The abbreviations used are: MAPK, mitogen-activated protein kinase; AP-1, activator protein-1; TPA, triphosphatase acid; TBS, Tris-buffered saline; DAPI, 4',6-diamidino-2-phenylindole dihydrochloride; MAPKK, MAPK kinase.
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Preparation of Cell Lysate. U937 cells were collected by centrifugation and washed twice with PBS. The washed cells were lysed with 0.4 ml of lysis buffer consisting of 10 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.5 mM EGTA, 0.15 M NaCl, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 5 μg/ml pepstatin A, 5 μg/ml antipain, 0.5 mM phenylmethylsulfonyl fluoride, 50 mM NaF, 2 mM sodium orthovanadate, 10 mM NaPPi, and 1% (w/v) Triton X-100. The cell lysate was centrifuged at 15,000 × g for 20 min. The supernatant thus obtained was used for immunoblotting and for the measurement of protein kinase activities.

Immunoblotting. Cell lysates containing 20 μg of protein were submitted to SDS-PAGE. The proteins were then transferred to a polyvinylidene difluoride membrane using a wet blotting apparatus at 0.5 V/cm² constant voltage for 90 min.

Analysis of DNA Fragmentation by Agarose Gel Electrophoresis. Cells were harvested by centrifugation and washed with PBS. The washed cells were lysed with a solution consisting of 10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% (w/v) SDS, and 0.1% (w/v) RNase A and incubated for 60 min at 50°C. The lysate was incubated for an additional 60 min at 50°C with 1 mg/ml proteinase K and then electrophoresed on a 1% (w/v) agarose gel in 40 mM Tris-acetate (pH 7.5) containing 1 mM EDTA for 60 min at 50 V. After electrophoresis, the DNA was visualized by ethidium bromide staining.

Quantification of DNA Fragmentation. The cells were suspended in a solution consisting of 5 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 0.5% (w/v) Triton X-100 and incubated for 20 min on ice. The suspension was centrifuged at 27,000 × g for 20 min, and the fragmented DNA was recovered from the supernatant. The pellets remaining in the centrifuge tube were sonicated for 1 min at 45 W. The amount of DNA was determined by a fluorometric method using DAPI. The fluorescence intensity was measured at 454 nm, with the excitation at 362 nm. The percent of DNA fragmentation was defined as the ratio of the fragmented DNA to the total DNA.

Luciferase Assay. The promoter region (~517 to +63) of the collagenase gene containing the 12-O-tetradecanoylphorbol-13-acetate response element was generated by PCR. Human genomic DNA was isolated from U937 cells and dissolved in PCR buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1 mM MgCl₂, 50 pmol of the forward primer (ACTCGAGGTACCCAG-GGAGCTTAACA), and 50 pmol of reverse primer (AAGATCTCTTGTCTTCTTCTGTT). The PCRs were performed in the presence of 0.5 units of Taq DNA polymerase (Perkin-Elmer Cetus) for 40 cycles using a step program (94°C, 1 min; 55°C, 1 min; and 72°C, 2 min), followed by a 7-min final extension at 72°C. The 0.5-kb PCR product was cloned into the XhoI and BgIII sites of a Pica gene basic vector (Toyo Inki Co.). U937 cells were transfected with this vector using lipofectin (Life Technologies, Inc.). The transfected U937 cells were treated with 10⁻⁸ M bufalin. The cells were collected, washed with PBS, and lysed in the lysis buffer (Promega). The lysate was centrifuged at 15,000 × g for 5 min, and the supernatant was collected. The supernatant was then subjected to a luciferase assay system.

In Vitro Kinase Assay Using a Synthetic Peptide. MAPKK and MAPK activities were measured in cell lysates (40 μg of protein) prepared from the expression vector-transfected U937 cells treated with 10⁻⁸ M bufalin. The MAPKK assay was performed in a final volume of 100 μl of assay mixture consisting of 50 μM [γ-³²P]ATP (0.1 mCi), 40 μg of cell lysate, 10 μg of synthetic peptide (FLTEYVRWYRAPEIMLN), 12.5 mM MOPS (pH 7.2), 12.5 mM MgCl₂, 0.5 mM EDTA, 0.05 mM NaF, 2 mM DTT, and 0.5 mM sodium orthovanadate. After a 10-min incubation at 30°C, the reaction was terminated by the addition of 1 ml of 1 N HCl. After boiling for 10 min, 1 ml of 0.1% (w/v) TFA was added to the sample, and it was then applied to a Sep-pak C18. The column was washed with 0.1% (w/v) TFA, and the substrate peptide was eluted with 10% acetonitrile. The radioactivity incorporated into the peptide was measured with a liquid scintillation counter. The MAPK assay was performed in a final volume of 30 μl of assay mixture containing 50 μM [γ-³²P]ATP (0.1 mCi), 40 μg of cell lysate, 2 μM synthetic peptide (KRELVEPLTAGEAPNQLAR), 25 mM HEPES (pH 7.4), 1.5 mM MgCl₂, and 0.1 mM sodium orthovanadate. After a 10-min incubation at 30°C, the reaction was terminated by the addition of 300 mM orthophosphoric acid, and the reaction mixture was spotted onto a 1.5-cm² Whatman P81 disc of phosphocellulose paper. The papers were washed in 1% phosphoric acid more than seven times by shaking for at least 5 min. The radioactivity incorporated into substrate peptide was measured with a liquid scintillation counter.

Results

Expression of bcl-2 mRNA in Transfected U937 Cells. To examine the involvement of the Bcl-2 protein in the bufalin-induced apoptotic process, the bcl-2 gene was introduced into human leukemia U937 cells. The expression of bcl-2 mRNA in eight clones of G418-resistant cells was evaluated by Northern blot analysis (Fig. 1A). Although the expression of endogenous bcl-2 mRNA could scarcely be detected in both parental cells (U937wt) and cells transfected with...
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pRc-RSV vector alone (U937v), a significant expression of bcl-2 mRNA was detected in two clones transfected with a pRc-RSV vector carrying the cDNA of human bcl-2. Relatively low and high bcl-2-expressing cells were designated U937bcl-2L and U937bcl-2H, respectively. Cell lysates prepared from these cells were analyzed with an antibody against Bcl-2 by immunoblotting (Fig. 1B). High and low levels of Bcl-2 protein expression were detected in U937bcl-2H and U937bcl-2L cells, respectively. These two cell lines were used for additional experiments.

Inhibition of Bufalin-induced Apoptosis by Bcl-2. To avoid the effects of growth factors in the serum, cells were maintained in the medium without FCS for 24 h and then treated with $10^{-8}$ M bufalin in this medium. Cell viability was measured at the times indicated in Fig. 2A. Cell viability of the U937v clone was decreased by the bufalin treatment after 24 h to 40%, which is similar to the viability obtained for U937WT cells (10). As compared to U937v and U937WT cells, U937bcl-2L and U937bcl-2H clones were resistant to bufalin treatment. To evaluate whether the suppression of cell death was due to the inhibition of apoptosis, we analyzed DNA fragmentation induced by bufalin in these clones. Following agarose gel electrophoresis of U937v cells treated with $10^{-8}$ M bufalin for 6 h, a typical ladder pattern of internucleosomal fragmentation was observed, which is considered to be an early event of apoptosis (Fig. 2B). In contrast, DNA fragmentation in U937bcl-2L and U937bcl-2H was significantly inhibited. Fragmented DNA was quantified by the fluorometric method using DAPI, and the results are shown in Fig. 2C. DNA fragmentation induced in U937v cells was 49%, which is almost identical to that reported for U937WT cells (10). This revealed that DNA fragmentation in U937bcl-2L and U937bcl-2H induced by bufalin decreased to 70 and 36%, respectively, as compared with that in U937v. This result was consistent with that of the agarose gel electrophoresis. These results indicate that apoptosis induced by bufalin in U937 cells was inhibited by the overexpression of Bcl-2 protein.

Effect of Bcl-2 Overexpression on Activation of MAPK Cascade. Recently, we have demonstrated that bufalin induces activation of the MAPK signaling pathway in U937 cells (10). As shown in Fig. 3A, MAPK in U937v cells was activated by treatment with $10^{-8}$ M bufalin, as reported previously (10). The degree of activation of MAPK in U937v cells caused by bufalin was quite similar to that reported for U937WT cells (10). The activation was significantly suppressed in the Bcl-2-overexpressing cells. In accordance with our previous report (10), MAPKK-1 in U937v cells was also markedly
activated by bufalin treatment (Fig. 3B). In the present study, no significant inhibition of the MAPKK-1 activation induced by bufalin was observed in U937<sub>bc1,2L</sub> or U937<sub>bc1,2H</sub> cells. This is in contrast to the inhibitory effect of Bcl-2 on the MAPK activation induced by bufalin. These results suggest that Bcl-2 protein acts downstream of MAPKK-1 and inhibits the activity of MAPK.

**Effect of Bcl-2 Overexpression on AP-1 Transcriptional Activity.** Because AP-1 is reported to be downstream of the MAPK cascade, we examined the effect of the overexpressed Bcl-2 protein on AP-1 transcriptional activity. When U937<sub>wt</sub> and U937<sub>v</sub> cells were treated with 10<sup>-8</sup> M bufalin for 6 h, AP-1 transcriptional activity was markedly increased (Fig. 4). However, the activation of AP-1 activity induced by bufalin was slightly reduced in bcl-2-transfected U937<sub>bc1,2L</sub> cells and significantly in Bcl-2-transfected U937<sub>bc1,2H</sub> cells. This result indicates that Bcl-2 protein inhibits the activation of MAPK induced by bufalin, and this leads to the decreased transcriptional activity of AP-1.

**Discussion**

Here, we investigated the effect of Bcl-2 overexpression on the induction of apoptosis by bufalin treatment in U937 cells. When wild-type U937 cells were treated with 10<sup>-8</sup> M bufalin, several lines of evidence, such as cell viability, chromosomal DNA laddering, and morphological change of the cells and nuclei, indicated that bufalin induced typical apoptosis as described previously (14). In contrast, the apoptosis induced by bufalin was attenuated in bcl-2-transfected U937 cells.

When wild-type U937 cells were treated with bufalin, the anamalous activation of MAPK was observed (10). However, little is known about the relationship between the expression of Bcl-2 and the signal pathway of MAPK. To examine the effect of Bcl-2 on the MAPK pathway, we measured the activities of MAPKK-1 and MAPK in Bcl-2-overexpressing U937 cells that had been treated with bufalin. There was a marked activation of MAPKK-1 induced by bufalin in U937<sub>bc1,2H</sub>, as well as in U937<sub>bc1,2L</sub> and U937<sub>v</sub> cells, suggesting that Bcl-2 does not act upstream of MAPKK-1 in the signal transduction pathway in U937 cells. Consistent with our result, Wang et al. (6) demonstrated that Bcl-2 protein did not affect the activity of Ras or Raf-1 in the apoptosis induced by growth factor deprivation of a diploid hematopoietic cell line.

In contrast to the effect of Bcl-2 overexpression on MAPKK-1 activity, a significant inhibition of the MAPK activity was observed. Although the MAPK activity in the vector-transfected cells increased approximately 1.9-fold upon treatment with 10<sup>-8</sup> M bufalin, the activation was significantly inhibited in Bcl-2-overexpressing cells, especially in U937<sub>bc1,2H</sub> cells. The results indicate that Bcl-2 plays an important role downstream of MAPKK-1 and upstream of MAPK in the MAPK signaling pathway in cell death induced by bufalin.

AP-1 is thought to be located downstream of MAPK, and the transcriptional activity of AP-1 is affected by MAPK (15). As demonstrated in the present study, bufalin increased the activity of AP-1 in both wild-type and vector-transfected cells, but approximately 63% of the increased activity was attenuated in bcl-2-overexpressed U937<sub>bc1,2H</sub> cells. It is possible that this inhibition is due to the attenuation of the MAPK activity caused by the overexpression of Bcl-2. This may be one of the mechanisms by which Bcl-2 suppresses cell death. Further work is needed to clarify the physiological role of Bcl-2.

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**References**

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