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4 authors:

Min-Liang Kuo
National Taiwan University

Shine-Gwo Shiah
National Health Research Institutes

Chau-Jong Wang
Chung Shan Medical University

Shuang En Chuang
National Health Research Institutes

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Suppression of Apoptosis by Bcl-2 to Enhance Benzene Metabolites-Induced Oxidative DNA Damage and Mutagenesis: A Possible Mechanism of Carcinogenesis

MIN-LIANG KUO, SHINE-GWO SHIAH, CHAU-JONG WANG, and SHUANG-EN CHUANG

Laboratory of Molecular & Cellular Toxicology, Institute of Toxicology, College of Medicine, National Taiwan University, Taipei, Taiwan (M.-L.K., S.-G.S.); Institute of Biochemistry, Chung-Shan Medical and Dental Collage (C.-J.W.); and Cancer Research Group, National Health Research Institutes (S.-E.C.), Taipei, Taiwan

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ABSTRACT

Apoptosis plays a crucial role in maintaining genomic integrity by selectively removing the most heavily damaged cells from the population. Under that premise, the dysregulation of apoptosis may result in an inappropriate survival of mutated cells. This study demonstrates that ectopic expression of Bcl-2 effectively suppresses benzene-active metabolites, 1,4-hydroquinone- and 1,4-benzoquinone-induced apoptosis in human leukemic HL-60 cells, as evidenced by morphological changes and DNA fragmentation. Although reactive oxygen species production largely contributes to the benzene metabolites-induced apoptotic cell death, Bcl-2 fails to attenuate the benzene metabolites-elicited increase of reactive oxygen species in HL-60 cells, as confirmed by flow cytometry analysis. These data suggest that Bcl-2 prevents benzene metabolites-induced apoptosis at the downstream of oxidative damage events. This study also determines the level of 8-hydroxydeoxyguanosine (8-OH-dGua), an indicator for oxidative DNA damage, in *neo*- and Bcl-2-overexpressing HL-60 cells after treating with 1,4-hydroquinone or 1,4-benzoquinone. Interestingly, our results indicate that a majority of the 8-OH-dGua is efficiently removed in *neo* control cells within 3 to 6 h, whereas only 25 to 35% of 8-OH-dGua is repaired in Bcl-2 transfectants even for 24 h. Similarly, another oxidative DNA base, thymine glycol, failed to repair and was retained in genomic DNA of Bcl-2 transfectants. The above findings suggest that Bcl-2 may retain benzene metabolites-induced oxidative DNA damage in surviving cells. Indeed, the failure of repairing 8-OH-dGua and thymine glycol in benzene metabolites-treated Bcl-2 survivors increases the number of mutation frequencies at the *hprt* locus. Results in this study thus provide a novel benzene-induced carcinogenesis mechanism by which up-regulation of Bcl-2 protein may promote the susceptibility to benzene metabolites-induced mutagenesis by overriding apoptosis and attenuating DNA repair capacity.

Programmed cell death or apoptosis profoundly influences a wide variety of physiological processes. Active physiological cell death selectively removes the most heavily damaged cells from the population. Hence, dysregulation of apoptosis has been implicated in several human diseases, ranging from cancer to autoimmunity, AIDS, and neurological disorders (Reed, 1994; Hanada et al., 1995; Thompson, 1995). According to previous investigations, several chemopreventive agents and tumor promoters exert their activities by inducing or inhibiting apoptosis, respectively (Hall et al., 1994; Wright et al., 1994; Kuo et al., 1996). A related study has indicated that transformation of colorectal epithelium to adenomas and carcinomas is associated with a progressive inhibition of apoptosis (Elder et al., 1996). The above findings reflect the importance of apoptosis as a mechanistic part in the multiple step carcinogenesis. In this regard, the extent to which oncogenes and tumor suppressor genes participate in regulating apoptotic cell death during multistep carcinogenesis has received increasing interest. Studies involving the *bcl*-2-proto-oncogene have provided further insight into the importance of dysregulated apoptotic cell death during the carcinogenic process, which was first identified at the chromosomal breakpoint of t (14;18) found in nonHodgkin's lymphomas (Tsujimoto and Croce, 1986). Overexpression of the Bcl-2 gene in transgenic mice leads to lymphomagenesis, implying that Bcl-2 protein expression could promote oncogenic potency (Korsmeyer, 1992). While corresponding to this observation, histopathological studies have conferred that the Bcl-2 protein is frequently overexpressed in various types of cancer, including lung, breast, and prostate (Reed, 1994; Kaklamanis et al., 1996; Binder et al., 1996). However, exactly how Bcl-2 protein might facilitate oncogenesis is largely unknown.

Chronic exposure to benzene, an ubiquitous pollutant, in-

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ABBREVIATIONS: TG, thymine glycol; 8-OH-dGua, 8-hydroxydeoxyguanosine; ROS, reactive oxygen species; 1,4-HQ, 1,4-hydroquinone; 1,4-BQ, 1,4-benzoquinone; NAC, *N*-acetyl-L-cysteine; DCFH-DA, 2′,7′-dichlorofluorescin diacetate.

duces myelotoxicity, lymphoma, mammary carcinomas, liver cancer, and leukemia in humans (Aksoy, 1989). Sister chromatid exchanges (Tice et al., 1980) and chromosomal loss and breakage (Yardley-Jones et al., 1990) were demonstrated in mice and humans, respectively, upon exposure to benzene. Benzene is metabolized by cytochrome P-450 to various phenolic metabolites, which accumulate in bone marrow. As widely recognized, benzene metabolism plays a prominent role in expressing its toxicity, with many investigators conferring that benzene toxicity is mediated by its metabolites (Dean, 1985). A mechanism by which benzene metabolites induce their genotoxic effects may be by generating one or more reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals (OH·; Yardley-Jones et al., 1991). Supportive of these findings, benzene metabolites 1,2,4-benzenetriol and 1,4-hydroquinone (1,4-HQ) caused oxidative DNA damage, e.g., 8-hydroxydeoxyguanosine (8-OH-dGua), in HL-60 cells in vitro and bone marrow of mice in vivo (Hiraku and Kawaniski, 1996). Thus, these studies indicated the participatory role of ROS in benzene metabolite-induced genotoxicity. Benzene metabolites also induce apoptosis in both bone marrow progenitor HL-60 and $CD34^+$ cells (Moran et al., 1996). The extent of apoptosis closely corresponds to the intensity of oxidative DNA damage. Thus, the fate of cells to apoptosis or mutation is likely dependent on the intensity of DNA damage and the ability to repair DNA.

In light of the above developments, this study is designed to explore whether Bcl-2 overexpression alters the susceptibility of cells to apoptosis induced by benzene metabolites 1,4-benzoquinone (1,4-BQ) and 1,4-HQ. ROS generation, oxidative DNA damage, and *hprt* gene mutation are determined in Bcl-2-overexpressing and *neo* control cells exposed to benzene metabolites. Results presented herein demonstrate that overexpression of Bcl-2 prevents benzene metabolites-induced apoptosis and attenuates the repair of oxidative DNA damage, ultimately leading to an enhancement in *hprt* gene mutation in survivors.

Materials and Methods

Chemicals. 1,4-HQ, 1,4-BQ, propidium iodide, *N*-acetyl-L-cysteine, proteinase K, ribonuclease A, nuclease P1, and alkaline phosphatase were purchased from Sigma Chemical Co. (St. Louis, MO). 2′,7′-dichlorofluorescin diacetate (DCFH-DA) was obtained from Molecular Probes, Inc. (Eugene, OR).

Cell Culture. HL-60 cells obtained from the American Type Culture Collection (Rockville, MD) were cultured in RPMI 1640 supplemented with fetal bovine serum (10%) and gentamicin sulfate (50 μg/ml). Cells were grown in a humidified atmosphere in 5% CO_2 at 37°C. Cell viability was determined using trypan blue exclusion in which 200 cells/culture were analyzed. All initial viabilities were greater than 95%.

Establishment of *bcl*-2 Overexpressing Clones. HL-60 cells constitutively expressing human *bcl*-2 were created by electroporation of HL-60 cells with *bcl-2* expression vector, pCΔj-*bcl*-2 (kindly donated by Dr. S.-F. Yang of the Institute of Molecular Biology, Academic Sinica, Taiwan) as described elsewhere (Kuo et al., 1996). Briefly, cells were suspended in 1 ml HEPES-buffered saline containing plasmid DNA and then received electric treatment with optimal conditions as follows: electric amplitude, 350 V; pulse width, 99 μs; subsequently, the population was cultured in G418 (100 μg/ml)-selective medium for 2 weeks. The survivors were administered a series dilution for single cells in 96-well plates in G418 medium for an additional 4 weeks. Finally, several independent resistant clones were obtained and subjected to determine Bcl-2 protein levels by immunoblotting.

DNA Fragmentation Assay. Cells were harvested and washed with PBS; DNA fragmentation was analyzed as described elsewhere (Kuo et al., 1996, 1997).

Quantification of Apoptosis by Flow Cytometry. Cells used for cytometry were prepared as described elsewhere (Kuo et al., 1996, 1997). Briefly, 10^6 cells were washed with PBS and resuspended in 500 μl of a buffer (0.5% Triton X-100/PBS/0.05% RNase A) and incubated for 30 min. Finally, 0.5 ml of propidium iodide solution (50 μg/ml) was added; cells were left on ice for 15 to 30 min. Fluorescence emitted from the propidium iodide-DNA complex was quantified after laser excitation of the fluorescent dye by FACSsor flow cytometry (Becton Dickinson, Mountain View, CA). Finally, the extent of apoptosis was determined by counting cells of DNA content below the Go/G1 peak.

Detection of Peroxides by Flow Cytometry. HL-60 cells (1×10^6 cells/ml) were incubated with either 1,4-HQ or 1,4-BQ in RPMI medium for 2 h at 37°C. DCFH-DA, a sensitive fluorometric probe of peroxides (Gupta, 1984; Ubezio and Civoli, 1994), was dissolved in ethanol, 10 uM DCFH-DA was added to the medium, and the cells were incubated for 30 min at 37°C. After incubation, the medium was removed and the cells were washed once with, then suspended in, PBS. Finally, the cells were analyzed with a FACScan (Becton Dickinson).

Determination of 8-OH-dGua in DNA. DNA was isolated from HL-60 cells and *bcl*-2 transfectants by the phenol extraction procedure of Gupta (1984). To avert any additional oxidative damage to the DNA due to peroxide or quinone contaminants in phenol, high-purity double distilled phenol was used for extractions. About 200 to 400 μg DNA were resuspended in 200 μl 20 mM sodium acetate (pH 4.8) and digested to nucleotides with 20 μg nuclease P1 at 70°C for 15 min. To adjust the pH, 20 μl of 1 M Tris-HCl (pH 7.4) were added to the nucleoside mixture, which was then treated with 1.5 U alkaline phosphatase and incubated at 37°C for 60 min. These hydrolyzed DNA solutions were then filtered using an Ultrafree Millipore filtration system (10,000-Da cutoff). Kalachana et al. (1993) have described the HPLC conditions used in this study. Briefly, the amount of 8-OH-dGua in the DNA was analyzed by flow-through electrochemical detection using an ESA model 5100 Coulochem detector (ESA, Inc., Bedford, MA) equipped with a 5011 high-sensitivity analytical cell with the oxidation potentials of electrodes 1 and 2 adjusted to 0.1 and 0.35 V, respectively. A C_{18} HPLC column (15×4.6 mm) was utilized to separate 8-OH-dGua. The mobile phase consisted of 10% methanol and 50 mM KH_2PO_4 buffer, pH 5.5, run isocratically at a flow rate of 1 ml/min.

***hprt* Gene Mutation Assay.** Bcl-2-overexpressing and *neo* HL-60 cells were diluted daily to a density of 4×10^5 cells/ml to maintain them in exponential growth. Four to five days before chemical treatment, cells were pretreated with hypoxanthine, aminopterin, and thymidine to remove any pre-existing *hprt*-deficient mutants from the population. Two days after hypoxanthine, aminopterin, and thymidine treatment, cells were resuspended in standard growth medium. Replicate cultures (up to 1.5×10^8 cells/group) were exposed to 1,4-HQ or 1,4-BQ to ensure a sufficient number of surviving mutants for good statistics. To determine the surviving fraction, an aliquot of cells was immediately seeded after benzene metabolite exposure in 96-well microtiter dishes at densities of 20 cells/well. Macroscopic colonies scored after 11 days of growth and relative surviving fractions were calculated according to standard methods (Yandell et al., 1990). After waiting 3 or 6 days for expression of *hprt* or mutant phenotypes, respectively, cells were seeded in the presence of 6-thioguanine selective agent in 96-well flat-bottomed microtiter plates. Each culture was also plated at 1 cell/well without selective medium to determine the plating efficient. Mutation frequencies were calculated according to standard methods (Yandell et al., 1990).

Results

Bcl-2 Protects HL-60 Cells from Benzene Metabolites-Induced Apoptosis. To verify whether Bcl-2 can affect benzene metabolites-induced apoptosis, this work initially established Bcl-2 overexpressing clones via transfecting HL-60 cells with *bcl*-2 expression vector pCΔj-*bcl*-2 and the native *neo* vector alone. Each expression vector contains the *neo* gene, which confers resistance to the antibiotic G418. After selection in G418, stable transfectants were analyzed by Western blotting for production of Bcl-2 protein. According to Fig. 1A, four independent clones of HL-60 cells were identified as overexpressed 3- to 5-fold Bcl-2 protein. Next, the growth properties of *bcl*-2 transfectants and vector-transfected control were determined. Under standard culturing conditions, the growth rates among *bcl*-2 transfectants and its respective vector control cell line did not significantly differ (data not shown). Two representative *bcl*-2 transfectants, HL-60/B*cl*-2–1 (5-fold increase in Bcl-2 protein) and HL-60/B*cl*-2–3 (3-fold increase in Bcl-2 level), were selected to examine their susceptibility to cytotoxicity induced by benzene metabolites, e.g., 1,4-HQ and 1,4-BQ. Trypan blue exclusion assay indicated that both *bcl*-2 transfectants remarkably resisted 1,4-HQ (Fig. 1B) or 1,4-BQ (Fig. 1C) treatment. In contrast, the *neo* control cells were sensitive to benzene metabolites. Generally, the higher Bcl-2 expression level implies a more resistant phenotype of these transfectants. The survivors of benzene metabolites-treated Bcl-2 transfectants still maintained membrane integrity and proliferating activity for several days (Fig. 1D). Our data suggest that Bcl-2 overexpression effectively protects cells from benzene metabolites-induced cytotoxicity in bone marrow HL-60 cells.

Agarose gel electrophoresis revealed that upon 1,4-HQ or 1,4-BQ treatment, the DNA from *neo* control cells displayed a dose-dependent increase in DNA fragmentation characteristic of apoptotic cell death (Fig. 2, A and B). In contrast, Bcl-2-overexpressing cells did not give rise to any type of DNA fragmentation when exposed to equal concentrations of both benzene metabolites. To quantitate the apoptosis, the number of hypodiploid cells (apoptotic cells), which are stained less intensely with propidium iodide, can be unequivocally quantitated from the peak in the flow cytometry subG1

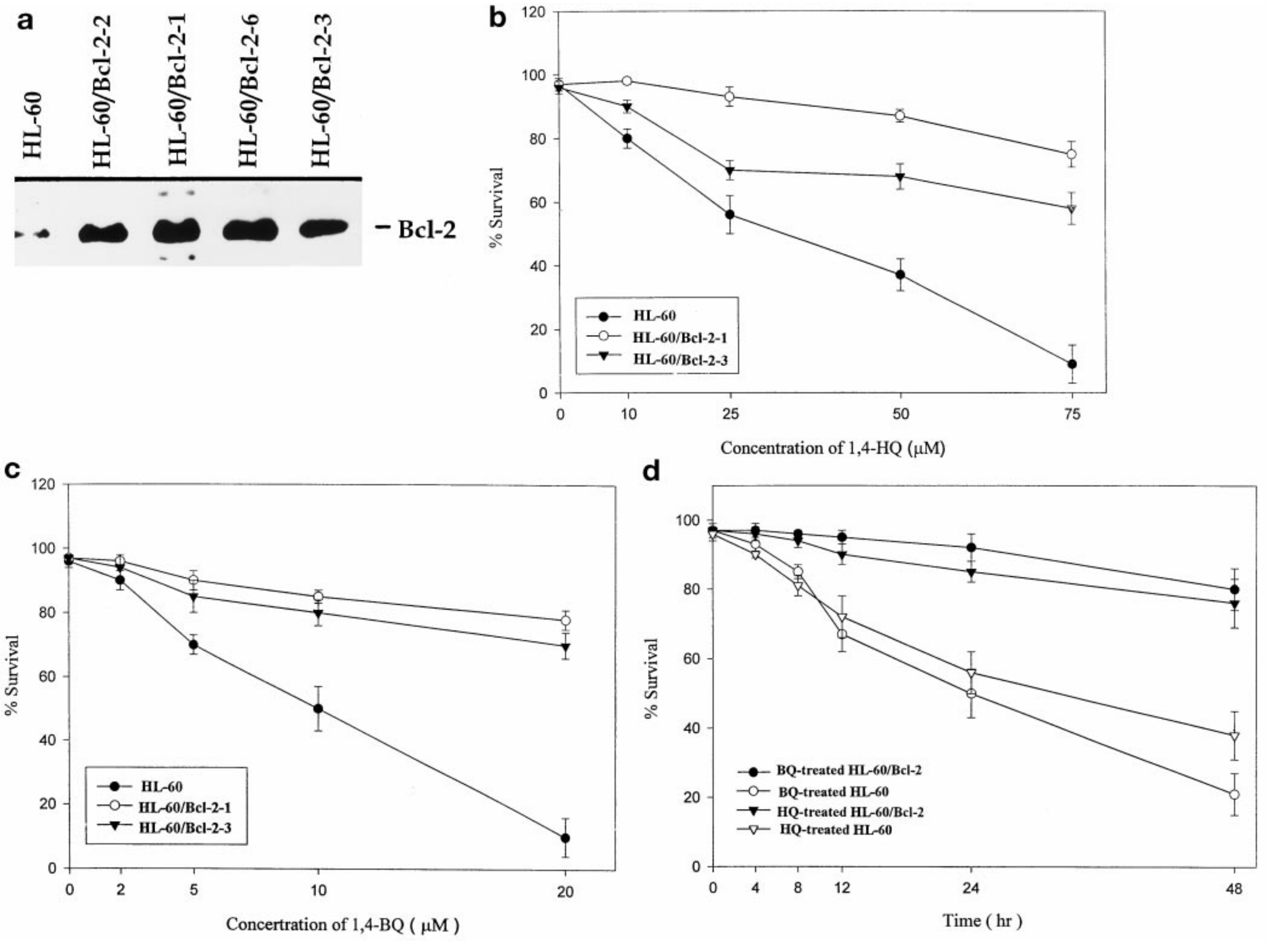

Fig. 1. Effect of Bcl-2 overexpression on benzene metabolite-induced cell death. A, expression of Bcl-2 protein in several clonal cell lines by Western blotting. Sensitivity of Bcl-2 overexpressed cells and vector control cells to 1,4-HQ (B) and 1,4-BQ (C). Briefly, the *neo* vector control cells and two Bcl-2 overexpressed clones were plated in a density 5×10^5 cells/60-mm dish in the presence of various concentrations of 1,4-HQ, 1,4-BQ, or 0.1% dimethyl sulfoxide for 24 h. D, cytotoxicity of HL-60 and Bcl-2 overexpressed cells treated with benzene metabolites for different periods of time. Both cells were treated with 10 μM 1,4-BQ or 25 μM 1,4-HQ for indicated time points. The percentage of viable cells was measured by a trypan blue exclusion assay. Data points are the mean of two highly reproducible experiments. Bar, S.D.

region. Figure 3 indicates that Bcl-2-overexpressing cells did not show significant levels of apoptosis (less than 25%) when exposed to either 25 μM 1,4-HQ or 10 μM 1,4-BQ. Under the same conditions, both benzene metabolites induced more than 70% of the *neo* control cells to become apoptotic. Notably, treating *neo* or Bcl-2 overexpressing HL-60 cells with the antioxidant *N*-acetyl-L-cysteine (NAC) nearly completely inhibited benzene metabolites-induced hypodiploid cells. This finding corresponds to other reports (Hiraku and Kawaniski, 1996) that suggested that ROS significantly contribute to the apoptosis elicited by benzene metabolites. The above results suggest that Bcl-2 overexpression could inhibit benzene metabolites-induced apoptotic cell death.

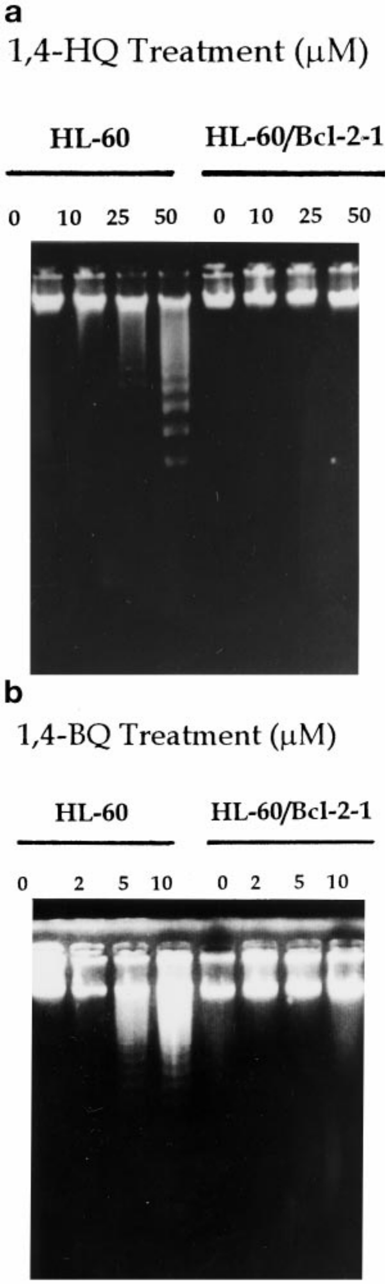

Fig. 2. Internucleosomal DNA fragmentation in Bcl-2 overexpressed clone and control cells treated with 1,4-HQ (A) and 1,4-BQ (B). Both cells were exposed to various concentrations of 1,4-HQ or 1,4-BQ or 1% dimethyl sulfoxide for 12 h. DNA from cells was extracted, electrophoresed through 1.2% agarose gels, and visualized by staining with ethidium bromide.

Bcl-2 Overexpression Fails to Inhibit Benzene Metabolites-Induced ROS. A previous study has contended that Bcl-2 may act as an antioxidant to protect cells from oxidative damage (Vaux, 1993). We speculate that if Bcl-2 against benzene metabolites-induced apoptosis is mediated by disruption of ROS production. To address this issue, we determined the intracellular peroxide level in benzene metabolites-treated Bcl-2 transfectants and *neo* control cells by using a dye DCFH-DA. Flow cytometric analysis shows that Bcl-2-overexpressing cells and *neo* control cells produced similar peroxide levels when exposed to 1,4-HQ or 1,4-BQ, implying that Bcl-2 overexpression did not attenuate benzene metabolites-elicited ROS generation (Fig. 4, A and B). However, NAC treatment effectively abolished 1,4-HQ- or 1,4-BQ-elicited peroxide production in both Bcl-2 transfectants and parental HL-60 cells (Fig. 4, A and B). The above results suggest that Bcl-2 effectively suppresses benzene metabolites-induced apoptotic cell death is mediated by other mechanism(s) rather than by interfering with the production of ROS.

Effect of Bcl-2 Overexpression on Benzene Metabolites-Induced Oxidative DNA Damage. If apoptosis selectively removes the most heavily damaged cells from the population, it may play a crucial role in the prevention of carcinogenesis by preserving genomic integrity. To test this hypothesis, we examined the extent of oxidative DNA damage, i.e., the formation of 8-OH-dGua, in Bcl-2 transfectants and *neo* control HL-60 cells after treatment with 1,4-HQ or 1,4-BQ. Figure 5A indicates that treatment of *neo* control cells with 25 μM 1,4-HQ and 10 μM 1,4-BQ for 30 min resulted in a 2.7- and 3.5-fold increase of 8-OH-dGua levels, respectively (Fig. 5B). However, this increase obviously declined toward background levels after 1 h and remained constant through 24 h. A slight amount or no cytotoxicity was observed from exposure to both compounds for at least 6 h (Fig. 1D), indicating that 8-OH-dGua formation in cells does not occur after cell death. Again, NAC treatment effectively inhibited 1,4-HQ- or 1,4-BQ-induced 8-OH-dGua formation in *neo* HL-60 cells (data not shown). Notably, a similar maximum 8-OH-dGua level was detected in Bcl-2-overexpressing cells as compared to that in *neo* control cells after a 30-min exposure to 1,4-HQ or 1,4-BQ (Fig. 5, A and B). However, over 70% of 8-OH-dGua was retained in Bcl-2 transfectants after 3 h of treatment. After a 24-h benzene metabolites treatment, approximately 50 to 60% of 8-OH-dGua was retained in genomic DNA of Bcl-2-overexpressing cells.

Furthermore, we used gas chromatography-mass spectroscopy to determine the amount of another oxidized DNA base, the thymine glycol (TG), in benzene metabolites-treated Bcl-2 transfectants and HL-60 cells. It is of interest to note that over 50% of TG remained in Bcl-2 transfectants treated with 1,4-HQ or 1,4-BQ for 24 h, but all of TG was repaired in HL-60 cells for the same time period (Fig. 6, A and B). The above results indicate that Bcl-2 overexpression may interfere with the cellular functions that possibly regulate and maintain genomic integrity.

Bcl-2 Overexpression Enhances Benzene Metabolites-Induced *hprt* Locus Mutation. Failing to remove benzene metabolites-induced oxidative DNA bases in Bcl-2-overexpressing cells may make the cells more susceptible to gene mutation. To test this hypothesis, we examined the *hprt* gene mutation in *neo* control and Bcl-2-overexpressing cells treated with 1,4-HQ or 1,4-BQ. Figure 7A reveals that the 1,4-HQ-induced *hprt* gene mutation frequencies in the Bcl-2 transfectants showed a 2- to 3-fold increase over that in the *neo* control cells. Figure 7B reveals that overexpression of Bcl-2 protein resulted in a 6-fold increase in 1,4-BQ-induced *hprt* gene mutation in HL-60 cells. Each experimental point was corrected for the background *hprt* mutation frequencies in parallel untreated cultures. Experimental results also demonstrated that overexpression of Bcl-2 protein enhances the total number of benzene metabolites-induced *hprt* mutants by affecting the overall number of surviving cells and increasing the number of mutants per surviving cell.

Discussion

Bcl-2 protein, which plays a central role in regulating apoptosis, is expressed in a variety of hematopoietic lineages (Reed, 1994). Bcl-2 has been localized to the mitochondria membrane, the nuclear membrane, and the endoplasmic reticulum (Korsmeyer, 1992). Many in vitro studies have conferred that *bcl*-2 overexpression promotes cell survival by inhibiting apoptosis induced by a variety of stimuli including radiation, hyperthermia, glucocorticoids, and DNA-damaging agents (Liu et al., 1997). For the first time, this study demonstrates that overexpression of Bcl-2 can effectively suppress apoptotic cell death induced by the benzene metabolites 1,4-HQ and 1,4-BQ in human promyeloid leukemic

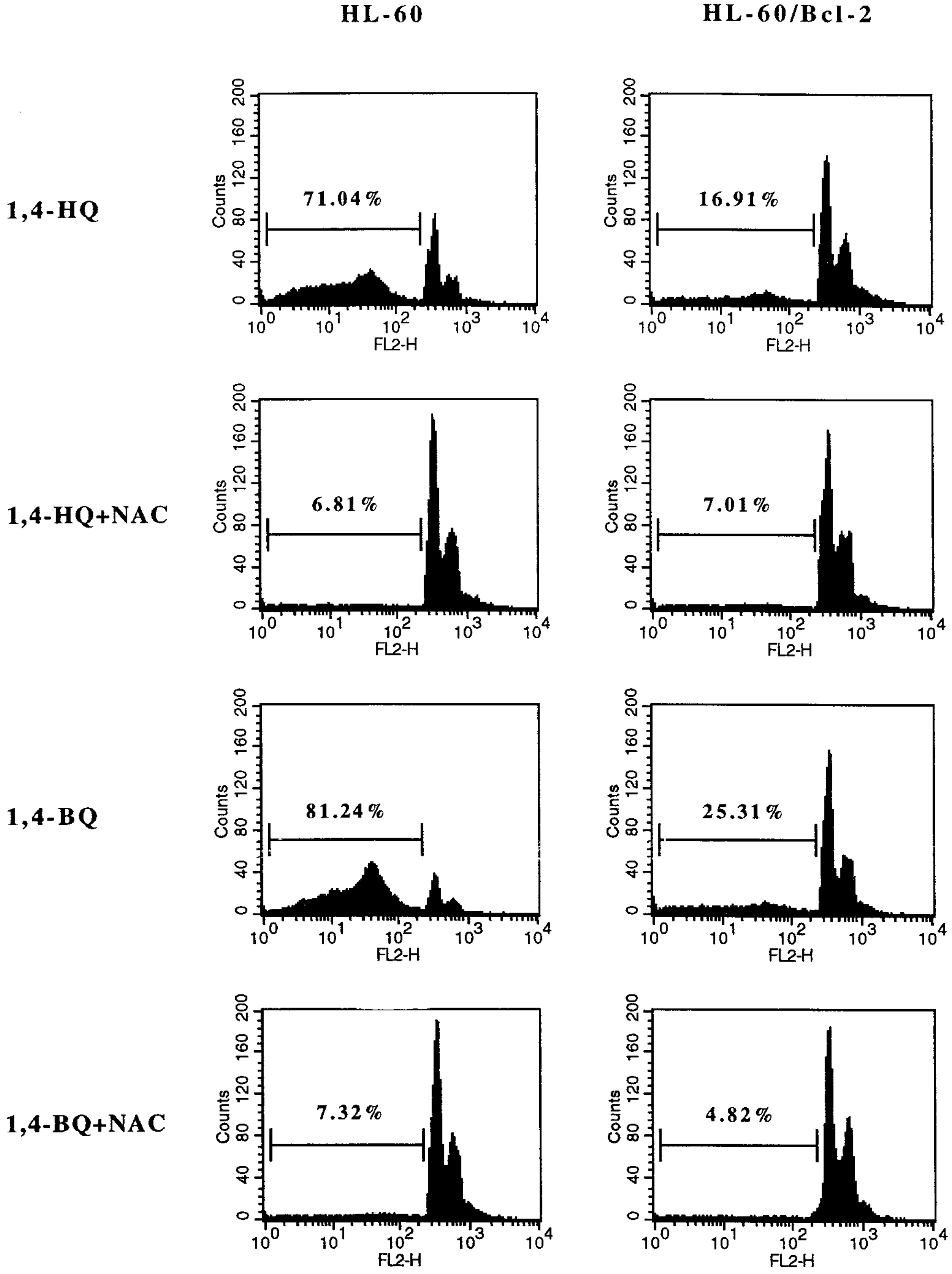

Fig. 3. Effect of Bcl-2 on benzene metabolites-induced hypodiploid cells. Bcl-2 transfectants (Bcl-2–1) and HL-60 cells (*neo*) were exposed to 25 μM 1,4-HQ, 10 μM 1,4-BQ, 1,4-HQ plus 30 mM NAC, or 1,4-BQ plus NAC for 16 h as indicated in the figure. Hypodiploid cells (apoptotic cells) were quantified by the flow cytometry analysis of propidium iodide-stained samples, as described in *Materials and Methods*. Data are representative of three independent experiments. The values indicated represent the percentage of apoptosis.

HL-60 cells. Trypan blue exclusion assay confirmed again that Bcl-2 also retained cell membrane integrity and long-term survival (Fig. 1D) for HL-60 cells after 1,4-HQ and 1,4-BQ treatment. The fact that antioxidant NAC treatment nearly inhibited both benzene metabolites-induced apoptosis implies that ROS generation contributes to benzene metabolites-mediated cell death. However, our results demonstrate that *bcl*-2 overexpression did not attenuate the increase of intracellular peroxides induced by 1,4-HQ or 1,4 BQ. This finding contradicts that of another report (Vaux, 1993), which suggested that Bcl-2 countered apoptotic death via an antioxidant pathway operated at sites of free radical generation induced by dexamethasone. Possibly, this discrepancy is at least partially due to a different cellular context. Our findings, however, suggest that Bcl-2 prevents benzene metabolites-induced apoptosis that may occur downstream of the oxidative damage event. More recent studies have clearly indicated that Bcl-2 inhibits mitochondrial cytochrome c release, thereby blocking caspase activation and subsequent apoptotic death (Yang et al., 1997). Therefore, whether Bcl-2 counter benzene-induced apoptosis occurs at the site of

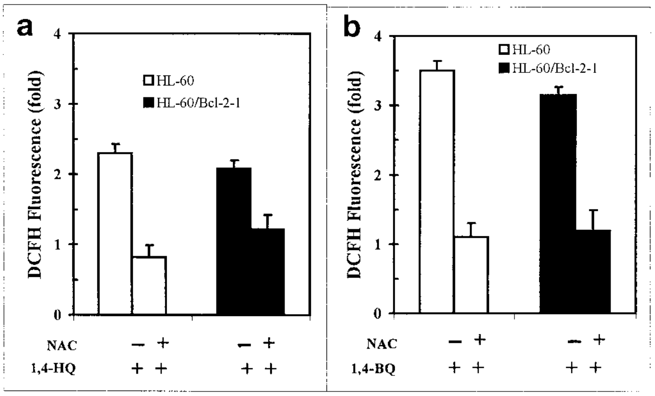

Fig. 4. Effect of Bcl-2 on benzene metabolites-induced intracellular peroxides level. HL-60/Bcl-2 to 1 and parental HL-60 cells were exposed to 25 μM 1,4-HQ or 1,4-HQ plus 30 mM NAC (A) and 10 μM 1,4-BQ or 1,4-BQ plus NAC (B) for 2 h. Intracellular peroxides level was quantified by DCFH fluorescence using flow cytometer as described in *Materials and Methods*. Each value represents the mean ± S.D. of three different experiments.

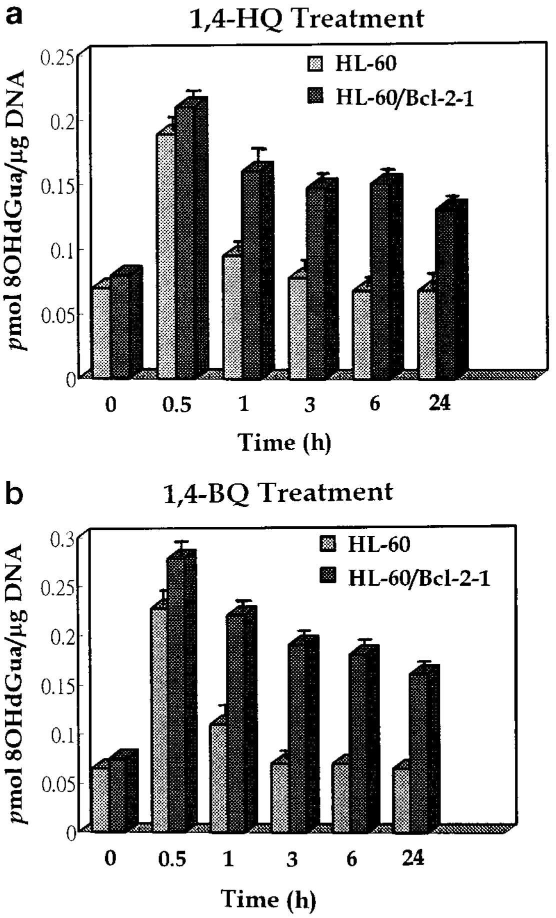

Fig. 5. Time course of benzene metabolites-induced DNA 8-OH-dGua formation in Bcl-2 overexpressing and HL-60 cells. Both cells (2×10^6) were treated with 25 μM 1,4-HQ (A) or 10 μM 1,4-BQ (B) for different periods of time as indicated. After treatment, DNA in each sample was extracted and 8-OH-dGua was determined by using HPLC as described in *Materials and Methods*.

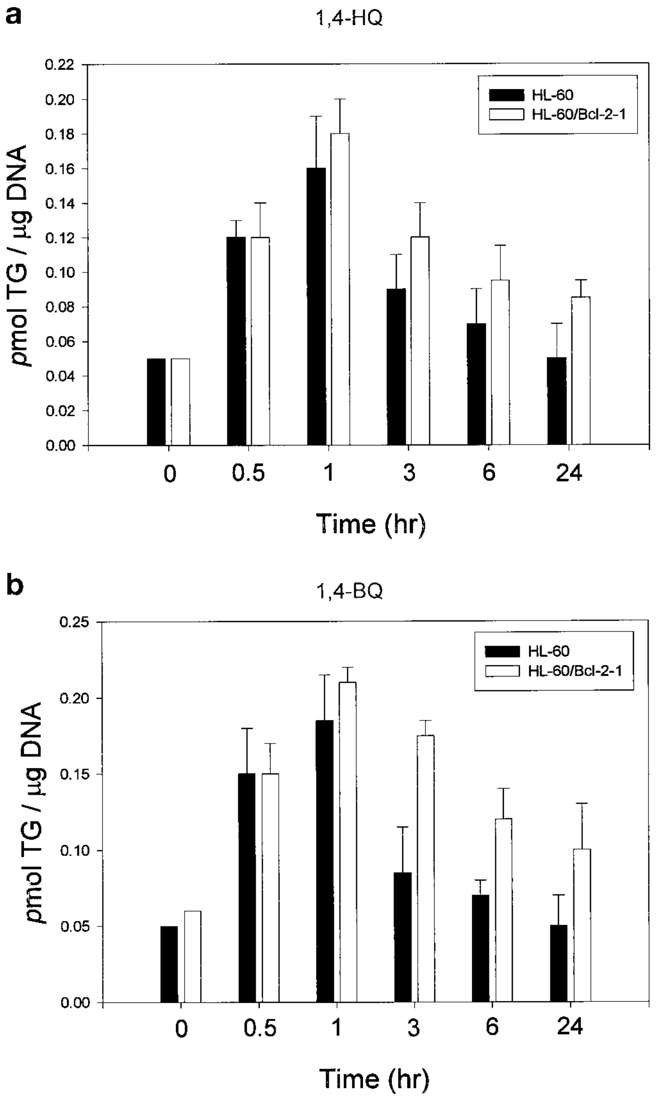

Fig. 6. Time course of benzene metabolites-induced TG formation in Bcl-2 overexpressing and HL-60 cells. Briefly, both cell lines (2×10^6) were treated with 25 μM 1,4-HQ (A) or 10 μM 1,4-BQ (B) for various periods of time as indicated. Measurement of TG was performed using gas chromatography-mass spectroscopy-SIM (see *Materials and Methods*) and data are mean ± S.D., $n = 4$.

caspase activation is of worthwhile interest and needs further investigation.

As we know, 8-OH-dGua is the most abundant product of oxidative damage to DNA by ROS and induces G-T and A-C base substitutions (Kolachana et al., 1993). This fact suggests that formation of this hydroxylated base may contribute to mutagenic and carcinogenic properties of chemicals that generate active oxygen. Herein, we report that 1,4-HQ and 1,4-BQ increase the steady-state level of 8-OH-dGua and peak at 30 and 60 min, respectively, in the DNA of HL-60 cells. Both oxidized bases were effectively removed when HL-60 cells were exposed to benzene metabolites for 6 h. This finding correlates with the in vivo study by Kolachana et al. (1993), which demonstrated that the maximum level of 8-OH-dGua in mouse bone marrow induced by benzene was observed at 1 h, ultimately decreasing to 20 to 30% by 3 h. The maximal level of 8-OH-dGua and TG induced by benzene metabolites in *bcl*-2 transfectants is similar to that in parental HL-60 cells; however, the removal of 8-OH-dGua and TG is not obvious in *bcl*-2 transfectants. This finding suggests that Bcl-2 protein may attenuate certain repair enzyme activity, subsequently delaying oxidative DNA base removal. The base excision repair enzyme has been found to be responsible for the removal of oxidative DNA lesions (Matsuba et al., 1997). Supportive of our findings, Liu et al. (1997) recently observed that the cyclobutane pyrimidine dimers induced by UV irradiation were efficiently removed in HL-60 cells, but failed to be repaired in Bcl-2-overexpressing HL-60 cells. Their results suggested that Bcl-2 overexpression may affect nucleotide excision repair in UV-irradiated cells.

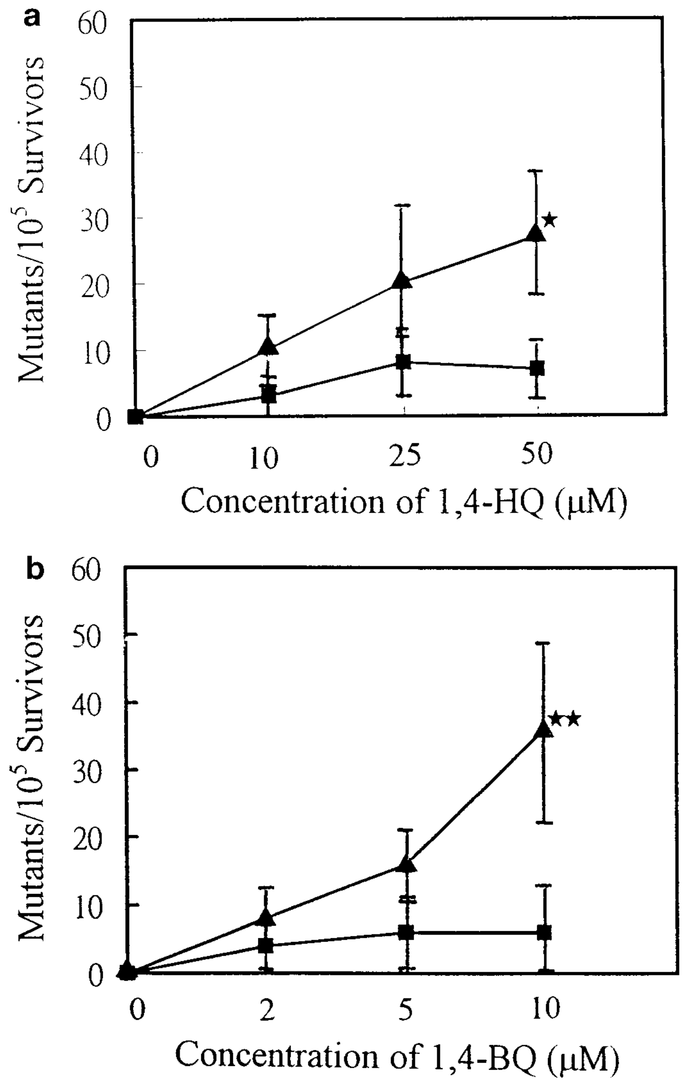

Fig. 7. Effect of Bcl-2 on 1,4-HQ- (A) or 1,4-BQ- (B) induced *hprt* locus mutation in HL-60 cells. Briefly, Bcl-2-overexpressing (▲) and *neo* HL-60 cells (■) were seeded at a cell density of 1.5 × 10^8 cells/100-mm dish. Replicate cultures were exposed to varying concentrations of **1,4-HQ** or **1,4-BQ** as indicated for 16 h. After waiting 6 days for expression of *hprt*-mutant phenotype, cells were seeded in the presence of 6-thioguanine selective agent. Mutation frequencies were calculated according to previous studies (Yandell et al., 1990). *$P < .05$ versus *neo* cells at the same concentration. **$P < .01$ versus *neo* cells. Bar, S.D.

As expected, the failure of repairing 1,4-HQ or 1,4-BQ-induced oxidized base, 8-OH-dGua and TG, in Bcl-2-overexpressing survivors enhanced mutation frequencies at the *hprt* locus. Consistent with oxidative DNA damage, no significant *hprt* locus mutation was observed in benzene metabolites-treated *neo* survivors. As reported elsewhere, benzene metabolites exhibited a low mutagenicity to *hprt* or other gene loci (Ward et al., 1992). A closely related observation made by Cherbonnel-Lasserre et al. (1996) reveals that Bcl-2 and Bcl-xL overproduction prevents apoptosis and enhances mutagenesis by hydrogen peroxide in cells with wild-type p53 or with mutant p53 protein. Thus, our data and others suggest that Bcl-2 overexpression perturbs the normally physiologic surveillance in genomic stability that causes cells to become more susceptible to genotoxic agents-induced genetic mutation.

A previous investigation indicated that Bcl-2 overexpression contributes to oncogenesis in *Eu-bcl*-2 transgenic mice in that they develop clonal B-cell lymphomas by extending the viability of B-cell precursors (McDonnel and Korsmeyer, 1991). It has also been demonstrated that overexpression of Bcl-2, through the delayed commitment to apoptosis, increased DHFR gene amplification frequency in BH2 cells (Yin and Schimke, 1996). More recent evidence has indicated that overexpression of Bcl-2 definitely promotes radiation-induced mutagenesis in human cells (Thompson, 1995). Furthermore, the Bcl-2 protein is produced at high levels in many types of tumors, including 90% of colorectal, 30 to 60% of prostate, 70% of breast, 20% of nonsmall cell lung cancer, and 65% of lymphomas (Hanada et al., 1995).

Conclusively, our studies demonstrate that up-regulation of Bcl-2 protein may actively enhance mutagenesis and carcinogenesis by both attenuating DNA repair processes and overriding apoptosis. Under that premise, we believe that modulation of apoptosis threshold by *bcl*-2 family members in bone marrow progenitors may promote benzene-induced carcinogenesis.

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Send reprint requests to: Dr. Min-Liang Kuo, Ph.D., Laboratory of Molecular & Cellular Toxicology, Institute of Toxicology, College of Medicine, National Taiwan University, No. 1, Section 1, Jen-Ai Road, Taipei, Taiwan. E-mail: toxkml@ha.mc.ntu.edu.tw