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Anti-Apoptotic Defense of *bcl-2* Gene Against Hydroperoxide-Induced Cytotoxicity Together With Suppressed Lipid Peroxidation, Enhanced Ascorbate Uptake, and Upregulated Bcl-2 Protein

Yasukazu Saitoh,[1,2] Rika Ouchida,[1,3] Atsushi Kayasuga,[1] and Nobuhiko Miwa[1]*

[1]Division of Cell Biochemistry, Hiroshima Prefectural University School of BioSciences, Nanatsuka 562, Shobara, Hiroshima 727-0023, Japan
[2]OPHTECS, Inc., Research Center, 156-5, Kamiyoshidai, Toyooka, Hyogo 668-0831, Japan
[3]Division of Clinical Immunology, Advanced Clinical Research Center, Institute of Medical Science, University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo 108-8639, Japan

Abstract Although it is well known that Bcl-2 can prevent apoptosis, the Bcl-2's anti-apoptotic mechanism is not fully understood. Here, we investigate the mechanism of oxidant-induced cell death and to investigate the role of Bcl-2 in the *tert*-butyl hydroperoxide (*t*-BuOOH)-induced oxidant injury in Rat-1 fibroblasts and their *bcl-2* transfected counterparts, b5 cells. Treatment with *t*-BuOOH causes mitochondrial disfunction and induced morphological features consistent with apoptosis more markedly in Rat-1 cells than in b5 cells. The hydroperoxide *t*-BuOOH at concentrations less than 100 nM for as long as 48 h or with higher concentrations (up to 100 μM) for only 3 h induces death in Rat-1 cells, whereas their *bcl-2* transfectants were significantly resistant to cytotoxicity by both time and all concentration other than 100 μM. The similar results were obtained also for DNA strand cleavages as detected by TUNEL stain. The *bcl-2* transfectants significantly suppressed *t*-BuOOH-induced increases in both lipid peroxidation and caspase-3 activation 3 and 1 h after *t*-BuOOH exposure, respectively, but failed to suppress either caspase-1 activation or an enhanced production of the intracellular reactive oxygen species (ROS). Intracellular uptake of [1-^{14}C] ascorbic acid (Asc) into the *bcl-2* transfectants was superior to that into the non-transfectants always under examined conditions regardless of serum addition to culture medium and cell density. Upregulation of Bcl-2 proteins was rapidly induced after *t*-BuOOH exposure in the transfectants, but not in non-transfectants, and restored till 24 h to the normal Bcl-2 level. Thus suppressions of both lipid peroxidation and the subsequent cell death events such as caspase-3 activation and DNA cleavage were concerned with the inhibitory effects of Bcl-2 on the *t*-BuOOH-induced cytotoxicity. And some of these events may correlate with Bcl-2 expression-induced partial enhanced anti-oxidant cellular ability including enrichment of intracellular Asc and oxidative stress-induced upregulation of Bcl-2 protein. On the other hand, ROS production and caspase-1 activation were not related to cytoprotection by Bcl-2. J. Cell. Biochem. 89: 321–334, 2003.

Key words: Bcl-2; cytotoxicity; *tert*-butyl hydroperoxide; reactive oxygen species (ROS); intracellular vitamin C

Apoptosis involves the activation of a genetically determined programmed cell suicide that results in a morphologically distinct from cell death characterized by cell shrinkage, nuclear condensation, DNA fragmentation, membrane reorganization, and blebbing [Kerr et al., 1972]. A number of proteins are important in the apoptotic process. Among them, the *bcl-2* proto-oncogene, a member of the *bcl-2* gene family, is known to be a key regulator of apoptosis, functioning as an anti-apoptotic protein with the ability to protect against a variety of physiologic or pathologic insults and environmental stimuli [Tsujimoto and Croce, 1986; Vaux et al., 1988; Korsmeyer, 1992; Hawkins and Vaux, 1994; Reed, 1994]. Bcl-2 is known to be localized

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*Correspondence to: Nobuhiko Miwa, PhD, Department of Cell Biochemistry, Hiroshima Prefectural University School of BioSciences, Shobara, Hiroshima 727-0023, Japan. E-mail: miwa-nob@bio.hiroshima-pu.ac.jp

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mainly to the outer mitochondrial membrane, as well as to the endoplasmic reticulum and nuclear membranes [Hockenbery et al., 1990; Akao et al., 1994]. The localization of Bcl-2 at the sites of free radical generation such as mitochondria may correlate with its ability to protect the subcellular organization [Tanimoto et al., 1998; Gross et al., 1999] and to function as an apparent anti-oxidant against oxidative stress that may induce apoptosis. In addition, anti-apoptotic effect of Bcl-2 may correlate with repression of reactive oxygen species (ROS) because ROS is generated during the process of apoptosis and mediates at least a part of the overall process [Buttke and Sandstrom, 1995; Jacobson, 1996]. This has been controversial and was, therefore, attempted in the present study to be elucidated for the extent or case of participation of ROS repression in Bcl-2 cytoprotection.

A proposed mechanism to explain Bcl-2's anti-apoptotic function arises from data involving its interaction with members of certain anti-oxidant pathways. In fact, Bcl-2 is suggested to function so as to mimic an anti-oxidant and to interrupt the apoptotic pathway resulting in prevention of cell death [Hockenbery et al., 1993; Kane et al., 1993; Veis et al., 1993; Virgili et al., 1998]. However, the mechanism underlying regulation of apoptosis by Bcl-2 is not fully understood.

The present study was attempted to elucidate the following viewpoints: (1) whether transfection with *bcl-2* genes can protect cells from oxidative injury resulting from the chemical oxidant *tert*-butyl hydroperoxide (*t*-BuOOH), an analogue of short-chain lipid hydroperoxide formed during oxidative stress and a relatively stable hydroxyperoxide that has been used extensively for in vitro studies of oxidative injury [Chance et al., 1979]; (2) whether ROS production, lipid peroxidation, and caspase-1 and caspase-3 activation are participating in *t*-BuOOH-induced cytotoxicity; (3) whether Ascuptake influenced by *bcl-2* transfection; (4) whether the upregulation of Bcl-2 occurs transiently or persistently after *t*-BuOOH exposure. Thus the practical mechanisms involved in Bcl-2 cytoprotection have been elucidated in the present study.

MATERIALS AND METHODS

Cell Culture

Rat fibroblastic cells Rat-1 [Topp, 1981] and their *bcl-2* transfectants b5 [Tsujimoto, 1989] were kindly provided by Dr. Shoji Yamaoka of Tokyo Med. Dent. Univ. Rat-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Nissui Seiyaku, Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco BRL, Grand Island, NY), 4 mM L-glutamine, 50 μ/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The *bcl-2* transfected b5 cells were cultured in DMEM containing 10% heat-inactivated FBS, 4 mM L-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin, and 600 μg/ml Geneticin disulfate (Wako Pure Chemical Industries, Osaka, Japan) at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cell Viability (Mitochondrial Activity) Assay

Cell viability, closely correlated with mitochondrial activity, was evaluated by WST-1 assay. Briefly, the cell layer in a dish was incubated with WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt) (Dojin Laboratories Co., Kumamoto, Japan) solution at 1:10 volume of phenol red-free culture medium for 3 h at 37°C. Viable cells with activity of mitochondrial dehydrogenases such as succinate dehydrogenage are capable of reducing the WST-1 dye to generate the yellowish formazan. At the end of incubation period, the absorbance of each sample was measured at 450 nm with a Bio-Rad absorbance plate reader (Bio-Rad), and the absorbance detected has been demonstrated to be proportional to viable cell number. Since there was no difference in the basal viability (mitochondrial activity) between Rat-1 and b5 cells, the values obtained from control cultures (non-treatment Rat-1 and b5 cells) were represented as 100% viability. Results are expressed as a percentage of those in corresponding control cells.

In Situ Labeling of Nuclear DNA Fragmentation (TUNEL)

Apoptosis was analyzed by terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay using the In Situ Apoptosis Detection Kit (TaKaRa, Kyoto, Japan) according to the manufacture's protocol. Briefly, cells were cultured on coverglasses and incubated for 18–24 h. After *t*-BuOOH treatment, cells were washed three times with phosphate-buffered saline (PBS(−)),

fixed with 3.7% paraformaldehyde for 15 min, and subsequently washed three times with PBS(−). For blocking endogenous peroxide, cells were treated by methanol containing 0.3% hydrogen peroxide for 20 min at room temperature and washed three times with PBS(−). Then, cells were permeabilized with permeabilization buffer for 5 min on ice and washed three times with PBS(−). To label DNA strand cleavages, cells were incubated with TUNEL reaction mixture containing TdT and fluorescein-dUTP in the binding buffer and incubated for 90 min at 37°C in a humidified atmosphere. Thereafter, the preparations were washed three times with PBS(−) and then mounted in a mounting medium (Dako Cytomation, Kyoto, Japan). The coverglasses were examined on a laser scanning confocal fluorescence microscope MRC-600 (Bio-Rad) equipped with argon laser as a light source. All of the TUNEL stains done at the same time and photographed under the same conditions.

Determination of ROS Production

Cells were rinsed twice with PBS(−) and replaced by phenol red-free DMEM containing 20 μM 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CDCFH) (Molecular Probes, Eugene, OR). After 60-min incubation, the fluorescence intensity was measured with a fluorescence microplate reader CytoFluor 2350 (Millipore, Bedford, MA) with excitation and emission wavelengths of 510 and 534 nm, respectively. Fluorescence of the oxidative from of CDCFH increased in a manner dependent on cell numbers and incubation times for viable cells, but not for methanol-killed cells similarly treated as the blank. ROS production is expressed in terms of fluorescence intensity per 10^4 cells. The total number of cells was determined using a Coulter electric particle counter after trypsinization of the cells.

Determination of Lipid Peroxidation

Lipid peroxidative products were measured using the thiobarbituric acid (TBA) assay for malondialdehyde (MDA), an end product of lipid peroxidation in biological tissues. Cells were collected by trypsinization and suspended in a ice-cold buffer containing 10 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, 2 mM phenylmethanesulfonyl fluoride (PMSF), 4 μM leupeptin, and 3 μM pepstatin A. After several times freeze-thawing step, the lysates were subjected to centrifugation at 16,000*g* for 20 min at 4°C and the supernatant was collected. Thereafter, the lysates were mixed with 0.375% TBA, 15% trichloroacetic acid−1 N HCl, and 0.04% 2, 6-di-*tert*-butyl-4-hydroxytoluene in ethanol. The mixture was boiled at 100°C in a water bath for 15 min. Each sample was then centrifuged at 800*g* for 10 min. The supernatant separated was measured at 535 nm with a Bio-Rad absorbance multi-well plate reader and is expressed as nanomoles MDA per 10^4 (10,000) cells. The total number of cells was determined with a Coulter electric particle counter ZB after trypsinization of the cells.

Transport of [1-^{14}C] Ascorbic Acid (Asc) Into the Cell

Cells were seeded on 2 cm^2 wells of a 24-well microplate, preincubated in DMEM containing 1 or 10% FBS for 18 h, and administrated for 1 h with L-[carboxyl-^{14}C] Asc (Amersham-Pharmacia Biotech, England, UK, 80−113 μCi/mg) in DMEM containing 1 or 10% FBS. Then cells underwent aspiration, rinsing twice with PBS, and cytolysis with 0.2% SDS. The cell lysates were measured with an Aloka liquid scintillation counter LC-3600. The control wells containing no cells were similarly operated, and the radioactivity value was subtracted from values of cell-containing wells.

Assay of Caspase-1 and Caspase-3 Activities

Caspase-1 and caspase-3 activities were assessed by a fluorometric assay quantifying the extent of cleavages of the fluorometric peptide substrate using a CaspACE™ Assay System kit (Promega, Madison, WI). Cells were collected by trypsinization and suspended in an ice-cold buffer containing 10 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 5 mM EDTA, 2 mM DTT, 2 mM PMSF, 4 μM leupeptin, and 3 μM pepstatin A. After several times freeze-thawing steps, the lysates were obtained by centrifugation at 16,000*g* for 20 min at 4°C and the supernatant was collected, and the amount of protein was measured using DC Protein Assay kit (Bio-Rad). The lysates were mixed with ICE-Like Assay Buffer containing 312.5 mM HEPES (pH 7.5), 31.25% sucrose, 0.3125% CHAPS, 2% DMSO, 10 mM DTT, and 50 μM inhibitor of caspase-1 and caspase-3, respectively. After 30-min incubation at 30°C,

50 μM of tetrapeptide substrate, acetyl-tyrosyl-valyl-alanyl-aspartic acid 7-amino-4-methyl coumarin; (Ac-YVAD-AMC) for caspase-1 or acetyl-aspartyl-glutamyl-valyl-aspartic acid 7-amino-4-methyl coumarin; (Ac-DEVD-AMC) for caspase-3 were added to the lysates and incubated for 60 min at 30°C. The intensity of fluorescence which was derived from substrate cleavage to release free aminomethylcoumarin (AMC) was measured with a fluorescence plate reader CytoFluor 2350 (Millipore) with excitation and emission wavelengths of 360 and 460 nm, respectively. Fluorescent units were converted to picomoles of AMC using a standard calibration curve generated with free AMC per microgram protein.

Western Blot Analysis

Cells were washed twice with PBS and lysed with an ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM dithiothreitol (DTT), 1 mM PMSF, 1% IGEPALCA-630, 1% sodium dodecyl sulfate (SDS), 4 μM leupeptin, and 3 μM pepstatin A. After being several times freeze-thawed, the lysate was centrifuged at 20,000*g* for 5 min at 4°C and the supernatant was collected. The amount of protein was measured using DC Protein Assay kit (Bio-Rad). The cell lysates were resuspended in buffer containing 62.5 mM Tris-HCl (pH 6.8), 15% glycerol, 10% β-mercaptoethanol, 0.005% bromophenol blue, and 4% SDS. Then the cell lysates were boiled for 3 min and applied to a 12% SDS–polyacrylamide gel, and the separated proteins were blotted to 0.45-μm polyvinylidene difluoride (PVDF) membranes (Millipore). Non-specific binding was blocked by incubating the membranes for 2 h at room temperature in a blocking buffer containing 50 mM Tris-HCl (pH 7.5), 3% bovine serum albumin, and 150 mM NaCl. The membranes were then stained with 1:2,500 diluted mouse monoclonal antibody against human Bcl-2 (product number sc-509; Santa Cruz Biotechnology, Santa Cruz, CA) in blocking buffer overnight at 4°C with agitation. After they were washed three times with washing buffer containing 50 mM Tris (pH 7.9), 100 mM NaCl, and 0.05% Tween-20, the membranes were incubated with 1:3,000 diluted horseradish peroxidase-coupled anti-mouse antibody in a blocking buffer for 30 min at room temperature. After they were washed twice with the washing buffer, the membranes were washed with the blocking buffer. The specific bands were detected using an enhanced chemiluminescence (ECL) detection system (Amersham-Pharmacia Biotech), and blots were exposed to Hyperfilm MP (Amersham-Pharmacia Biotech) for 0.5–2 min. Laser scanning densitometry was conducted for semiquantitative analysis of the data. Loading of approximately equivalent amount of protein content was confirmed by the densitometric values of a randomly selected band on the Coomassie blue stained gel.

Statistical Analysis

Unpaired student's *t*-test was used to evaluate the significance of differences between groups, and the criterion of statistical significance was taken as $P < 0.05$.

RESULTS

Effects of *t*-BuOOH on Cell Viability of Rat Fibroblasts Rat-1 and Their *bcl-2* Transfectants b5

To examine the role of *bcl-2* genes in the cytotoxic response to *t*-BuOOH, Rat-1, and b5 cells were exposed to the indicated concentrations of *t*-BuOOH (0–100 μM) for 3 h (Fig. 1A). After the indicated exposure time, cells were incubated for 24 h in fresh medium, and then were assessed for the cell viability by WST-1 assay. Treatment with *t*-BuOOH for 3 h decreased the cell viability of both the parent and *bcl-2* transfected cells in a dose-dependent manner. Cell viability of b5 cells was more markedly retained than that of the parent rat-1 cells against *t*-BuOOH-induced injuries, especially at 10, 20, and 50 μM of the hydroperoxide. However, at 100 μM of *t*-BuOOH, the highest concentration examined, there was no difference in cell viability between both the cell lines. The results showed that transfection with *bcl-2* genes exerts a cytoprotective effect against *t*-BuOOH-induced injuries as moderate as induced at concentrations of 5–50 μM but not against injuries as drastic as induced at 100 μM of *t*-BuOOH.

On the other hand, effects of treatment with more dilute *t*-BuOOH for longer periods were examined (Fig. 1B). Rat-1 or b5 cells were exposed to *t*-BuOOH at graded concentrations as low as 0–100 nM (that is 0.1 μM) for 24 or 48 h, and were determined for cell viability. The hydroperoxide reduced the cell viability of

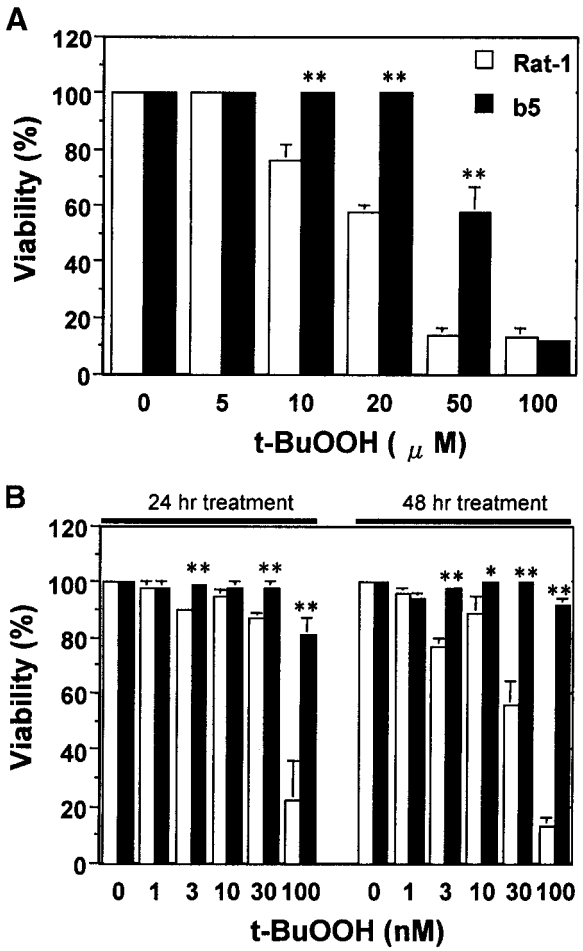

Fig. 1. Effects of the higher concentrations—shorter period (**A**) or the lower concentrations—longer period (**B**) treatment of fibroblastic cells Rat-1 and their *bcl-2* transfectants b5 with *tert*-butylhydroperoxide (*t*-BuOOH) on cell viability. Rat-1 and b5 cells were plated at a density of $1.0–5.0 \times 10^4$ cells/cm^2 in 24-well plates. After preincubation for 18–24 h, the medium was replaced by FBS-free medium containing different concentrations of *t*-BuOOH (0–100 μM) for 3 h (A) or different concentrations of *t*-BuOOH (0–100 nM) being lower than those in (A) for a period as long as 24 or 48 h (B). After removal of *t*-BuOOH-containing medium, cells were rinsed and further cultured in FBS-containing medium for 24 h. Then cell viability was assessed by WST-1 assay. The data shown are typical of three independent experiments. The bar represents the SD of wells in triplicate. Significantly different from Rat-1 cells: $^*P < 0.05$; $^{**}P < 0.01$.

non-transfectants markedly at 100 nM alone after 24-h treatment, whereas 48-h treatment decreased cell viability in a dose-dependent manner at 3–100 nM. In contrast, cell viability of the *bcl-2* transfectants was not decreased at any examined concentrations of *t*-BuOOH after 24 or 48 h. As compared to Rat-1 cells, b5 cells were markedly prevented from cell damage that was induced with *t*-BuOOH at 100 nM for 24-h treatment, or at 3, 10, 30, and 100 nM for 48 h, respectively.

Cell Morphological Aspects of *t*-BuOOH Treated Cells and *bcl-2* Transfectants

Typical microphotographs of Rat-1 (A, B, E, F) and b5 (C, D, G, H) cells immediately before (A, C, E, G) and after (B, D, F, H) 20 μM *t*-BuOOH exposure were taken under the phase-contrast microscope (Fig. 2). For Rat-1 cells, the density of adherent cells was markedly diminished as compared with the cells immediately before *t*-BuOOH exposure. A considerable number of dead cells were detached from the culture dish into the supernatant, and in part cytolysed into cell debris. For b5 cells, only a few round, detached cells were visible, but the majority of cells were rigidly attached and spread on the culture dish. Thus, b5 cells suffered from less marked cellular degenerations than Rat-1 cells. The results showed that transfection with *bcl-2* genes exerts a cytoprotective effect against *t*-BuOOH-induced injuries.

Inhibitory Effects of Bcl-2 on *t*-BuOOH-Induced DNA Strand Cleavages

We investigated whether *t*-BuOOH-exposed cells undergo apoptosis, and whether *bcl-2* gene transfection affects *t*-BuOOH-induced DNA cleavages (Fig. 3). The DNA 3′-cleaved terminals that are typically observed upon apoptotic cell death were detected for *t*-BuOOH-exposed Rat-1 cells by TUNEL assay. Rat-1 and b5 cells that were treated with 50 μM *t*-BuOOH showed an intense green fluorescent staining in the nuclei, indicating the incorporation of fluorescein dUTP onto nicked DNA strand ends. Temporal analysis shows an increase in the population of DNA-cleaved cells. The maximum degree of DNA cleavages occurred in Rat-1 cells that were incubated for 1 h after 3-h exposure with *t*-BuOOH. In contrast most of b5 cells were shown to be negative in TUNEL staining for up to 3 h after the exposure with *t*-BuOOH. These results show that transfection with *bcl-2* genes inhibited *t*-BuOOH-induced apoptosis-like cell death.

Increases in Intracellular ROS Upon Addition of *t*-BuOOH

We attempted to examine whether the addition of *t*-BuOOH can cause accumulation of intracellular ROS and whether *bcl-2* gene

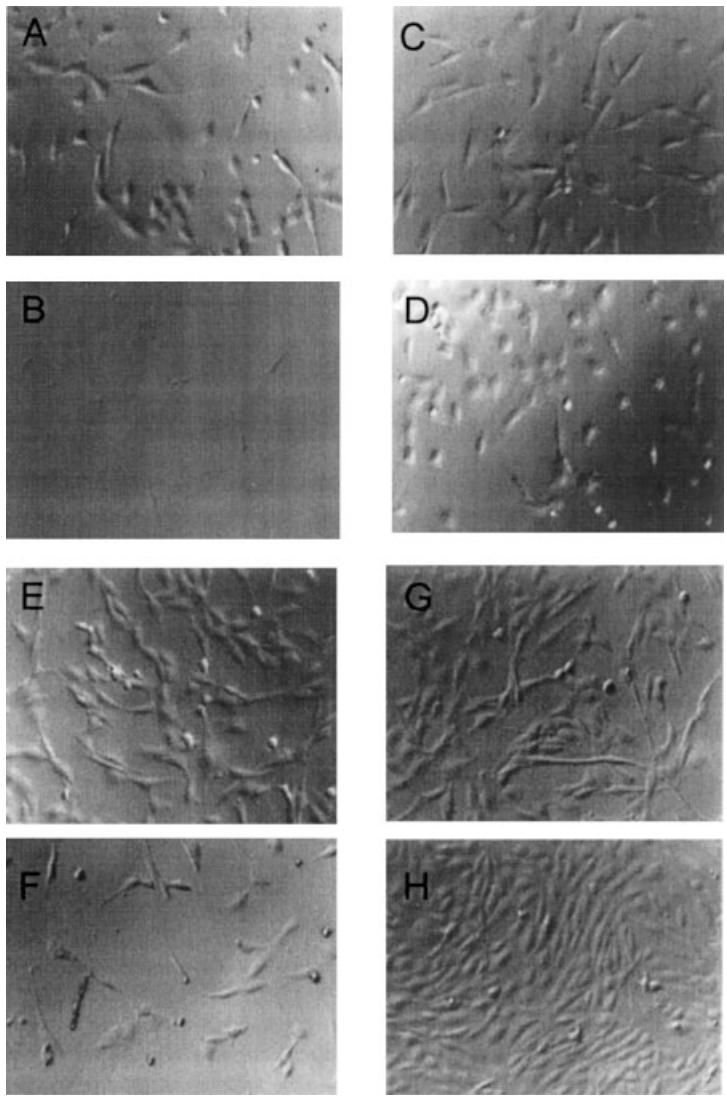

Fig. 2. Morphological aspects of Rat-1 cells and their bcl-2 transfectants b5 that underwent treatment with *t*-BuOOH. Cells were plated at densities of $1.0–2.0 \times 10^4$ (subconfluence; **A**–**D**) or $3.0–4.0 \times 10^4$ (confluence; **E**–**H**) cell/cm^2 in 24-well plates. After preincubation for 18–24 h, the medium was replaced by FBS-free medium containing 20 μM *t*-BuOOH for 3 h. After removal of *t*-BuOOH-containing medium, cells were rinsed and cultured in medium containing 10% FBS. After 24 h, cells were observed under the phase-contrast microscope. Rat-1 cells: (A, E) immediately before *t*-BuOOH exposure; (B, F) after *t*-BuOOH exposure and the subsequent treatment. Their bcl-2 transfectants b5: (C, G) immediately before *t*-BuOOH exposure; (D, H) after *t*-BuOOH exposure and the subsequent treatment, respectively. Magnification: 100-fold.

transfection in the transfected cells could influence ROS levels. This is because the role of oxidative stress in induction of apoptosis has generated considerable debate [Clement and Stamenkovic, 1996; Kazzaz et al., 1996; McGowan et al., 1996; Quillet-Mary et al., 1997; Tan et al., 1998]. The intracellular ROS levels were quantified using the fluorescein derivative CDCFH as a redox indicator by fluorometry. CDCFH that is taken up into cells is esterolyzed to be membrane-impermeable, and oxidized to highly fluorescent CDCF primarily by H_2O_2, hydroxyl radicals, and diverse peroxides [Sejda et al., 1984].

Figure 4A demonstrates the accumulation of ROS after treatment with 50 μM *t*-BuOOH. A 2.5-fold increase in the ROS level was observed 3 h after treatment with *t*-BuOOH, and was restored to the baseline level 24 h after treatment. These peaks of maximal ROS production were formed at similar times for both cell lines at 3 h after *t*-BuOOH treatment. No significant difference in ROS production was observed both cell lines. For both cell lines, the increase in ROS

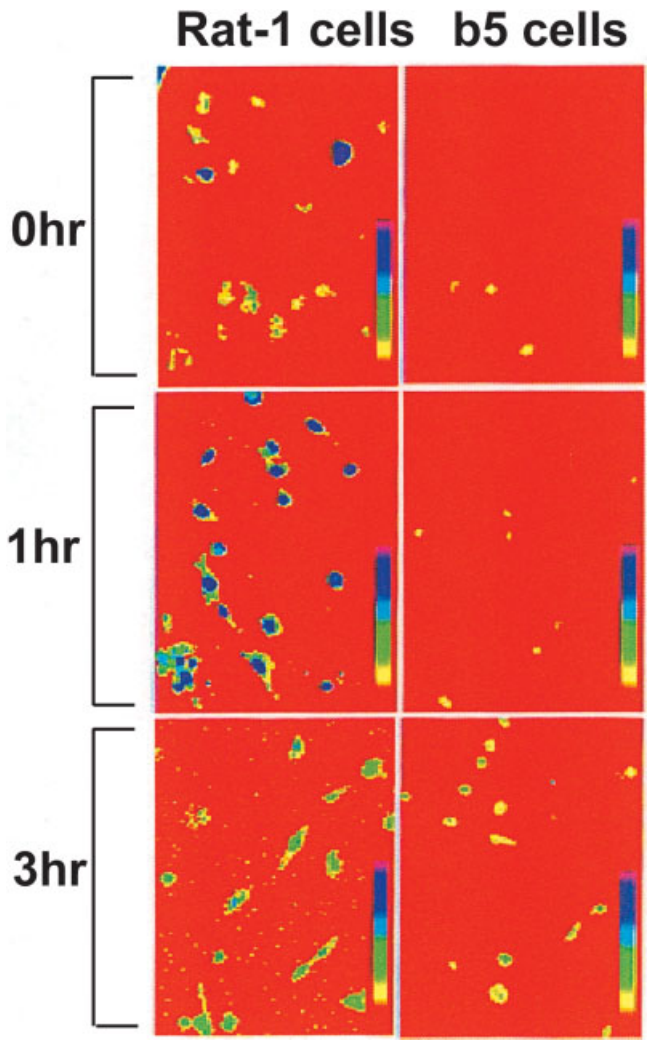

Fig. 3. Time course of DNA strand cleavage after exposure to 50 μM *t*-BuOOH for 3 h. Photomicrographs of TUNEL-stained cells in response to *t*-BuOOH exposure of Rat-1 and b5 cells. Cells were preincubated for 18–24 h, and treated with 50 μM *t*-BuOOH for 3 h. Then, cells were further incubated in fresh culture medium for the indicated times and stained with fluorescein-based TUNEL assay for DNA cleavage sites. The fluorescence was measured by laser scanning confocal microscopy. Magnification: 100-fold. The rainbow colored bar means graded degrees of TUNEL-attributed fluorescence intensities.

production (levels) reached maximal values (approximately 2.5-fold increase) at the same time. The results showed that transfection with *bcl-2* genes does not exert an inhibitory effect against *t*-BuOOH-induced ROS production.

Formation of Lipid Peroxides in *t*-BuOOH-Treated Cells

We next examined whether lipid peroxidation is induced after *t*-BuOOH treatment of Rat-1 cells and their *bcl-2* transfectants b5 (Fig. 4B). The amount of lipid peroxides in the cells was determined by TBA assay. There is no significant difference in lipid peroxidation levels of both cell lines before *t*-BuOOH exposure. However, upon the treatment of Rat-1 cells with 50 μM *t*-BuOOH for 3 h, lipid preoxidation levels reached the maximum value (approximately 2.1-fold increases vs. the untreated level), whereas the peroxylipid level in b5 cells reached the maximal value (approximately 1.6-fold increases) at the same time. The rise of lipid peroxide levels was significantly suppressed in b5 cells when compared to Rat-1 cells. The increase in lipid peroxide levels was reversed down to the control level at 24 h after 3-h exposure with *t*-BuOOH. The results showed that transfection with *bcl-2* genes exerts inhibitory effects against *t*-BuOOH-induced lipid peroxidation.

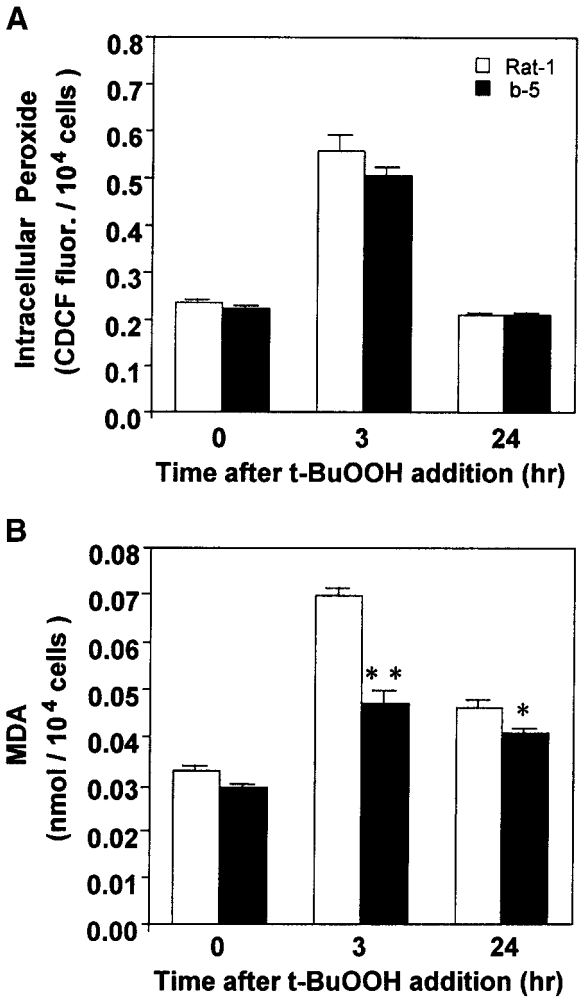

Fig. 4. Intracellular reactive oxygen species (ROS) production (**A**) or lipid peroxidation (**B**) in Rat-1 cells and their *bcl-2* transfectants b5 exposed to *t*-BuOOH. Cells were preincubated for 18–24 h, and treated with 50 μM *t*-BuOOH for 3 h. After removal of *t*-BuOOH-containing medium, cells were rinsed and further cultured in fresh medium containing 10% FBS for the indicated periods. ROS production was evaluated using the redox indicator dye 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (CDCFH) (A). The lipid peroxide production was determined by TBA assay. Lipid peroxide levels are expressed as nanomoles malondialdehyde (MDA) per 10,000 cells (B). The data shown are typical of three independent experiments. The bar represents the SD of wells in triplicate. Significantly different from Rat-1 cells: $*P < 0.05$; $**P < 0.01$.

Effects of *bcl-2* Overexpression on Asc Uptake Into the Cell

Our previous study [Saitoh et al., 1997] demonstrates that uptake of Asc into aortic endothelial cells is affected by concentrations of serum contained in the culture medium. So, we investigated whether Asc uptake into Rat-1 cells and the *bcl-2* transfected cells b5 was affected by serum concentrations (Fig. 5). Cells were forced to be cultured under the serum-sufficient (10% FBS) or serum-poor (1% FBS) condition, and then were administrated for 1 h with [^{14}C] Asc of 100 μM which is included an Asc concentration range in the normal human plasma. Two conditions such as serum-sufficient or poor medium were examined also upon the administration with Asc, followed by scintillascopic quantification of intracellular radioactivity. It was consistently shown, unexpectedly, that Asc uptake is more markedly efficient in b5 cells than in Rat-1 cells in common with all the four conditions. This superiority of b5 cells to Rat-1 cells in terms of Asc uptake seems not to be influenced by concentrations of serum in the culture medium upon either preculture or uptake-culture, but to be indirectly related with overexpression of exogenous *bcl-2* gene that was transferred into b5 cells.

Caspase-1 and Caspase-3 Activation by *t*-BuOOH

Caspase-1 and caspase-3, families of cysteine aspartic acid-specific proteases play key roles in inflammation and apoptosis in mammalian cells [Thornberry et al., 1992; Nicholson et al., 1995; Tewari et al., 1995; Fernandes-Alnemri et al., 1996]. To investigate the role of *bcl-2* genes in *t*-BuOOH-induced cell death in Rat-1 and

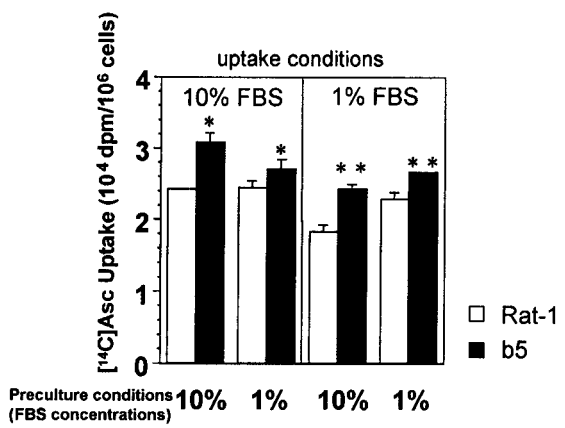

Fig. 5. Intracellular uptake of [^{14}C]-labeled ascorbic acid (Asc) into Rat-1 and their *bcl-2* transfectant, b5 cells undergoing previous and/or simultaneous starvation with serum or not. Cells were previously cultivated in Dulbecco's modified Eagle's medium (DMEM) containing 10 or 1% FBS for 18 h, and then exposed to [1-carboxy-^{14}C] Asc of 100 μM for 1 h in DMEM–10 or 1% FBS. The intracellular contents were subjected to determination for Asc contents by liquid scintillascopy. The data shown are typical of three independent experiments. The bar represents the SD of wells in triplicate. Significantly different from Rat-1 cells: $*P < 0.05$; $**P < 0.01$.

b5 cells, the activation of caspase-1 and caspase-3 were monitored as a hallmark of apoptosis (Fig. 6). The activation was measured spectrofluorometrically by assaying the hydrolysis of a fluorophore-conjugated tetrapeptide substrate that can be selectively cleaved only by the caspases-1 or caspase-3. Caspase-1 activity in both cells, after 1 h of exposure to *t*-BuOOH, was significantly increased about threefold increase as compared to before the exposure. The activation and repression of caspase-1 may be involved in the *t*-BuOOH-induced cell death mechanisms, but were not prevented by *bcl-2* gene transfection. Thus, the cytoprotective effect of transfection with *bcl-2* genes does not be mediated through their effects on caspase-1 activation.

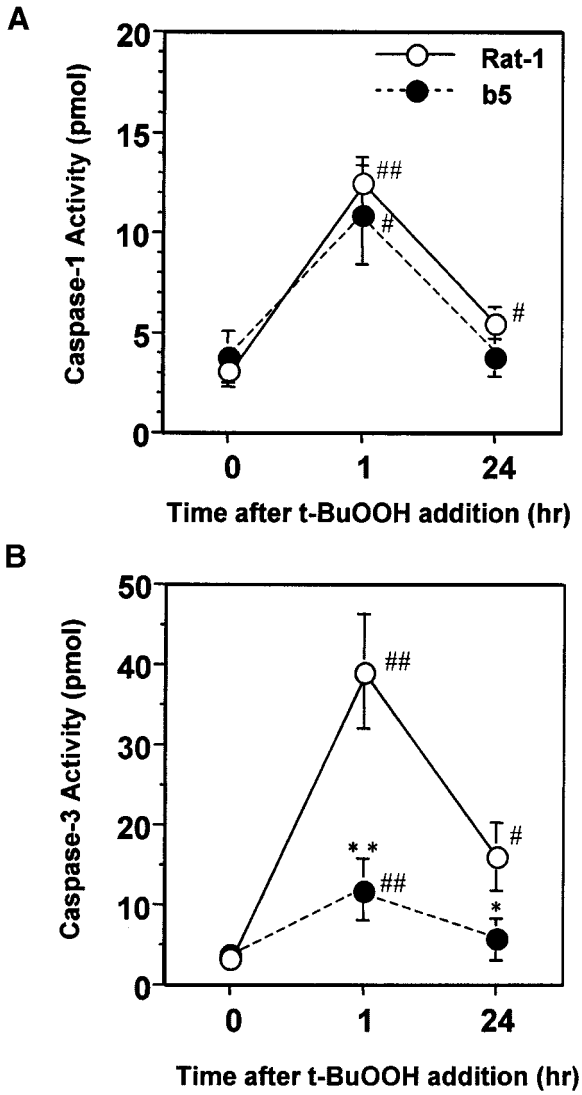

Fig. 6. Activation of caspase-1 (**A**) and caspase-3 (**B**) in Rat-1 cells and their *bcl-2* transfectants b5 exposed to *t*-BuOOH. Cells were preincubated for 18–24 h, and treated with 50 μM *t*-BuOOH for 3 h. After removal of *t*-BuOOH-containing medium, cells were rinsed, and further cultured in medium containing 10% FBS for 24 h. Cytosolic protein were extracted from cells immediately before *t*-BuOOH exposure or at 1 or 3 h after 3-h exposure to *t*-BuOOH, and measured for caspase-1 and caspase-3 activities. The data shown are typical of three independent experiments. The bar represents the SD of dishes in triplicate. Significantly different from Rat-1 cells: $*P < 0.05$; $**P < 0.01$, and from time zero: $\#P < 0.05$; $\#\#P < 0.01$.

On the other hand, caspase-3 activity in Rat-1 cells after 1 h of exposure to *t*-BuOOH was increased about eightfold as compared to the control cells. In contrast, a threefold increase in caspase-3 activity was detected for b5 cells. The degree of caspase-3 activation was significantly suppressed in b5 cells when compared to Rat-1 cells at both 1 and 24 h. These results indicate that activation and repression of caspase-3 may be involved in the mechanisms whereby cell death was induced by *t*-BuOOH and prevented by *bcl-2* gene transfection.

Expression of Bcl-2 Protein After *t*-BuOOH Treatment

To obtain further insight into the mechanism of *t*-BuOOH induced apoptosis, we also analyzed Bcl-2 expression after *t*-BuOOH treatment by Western blots and densitometry (Fig. 7). Before *t*-BuOOH exposure, Bcl-2 expression in

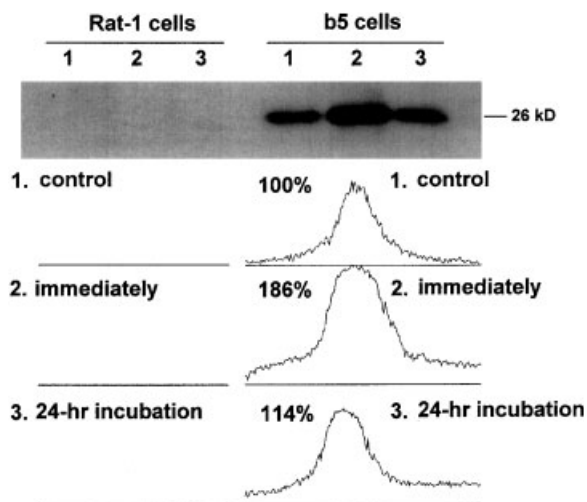

Fig. 7. Expression of Bcl-2 proteins in Rat-1 cells and their bcl-2 transfectants b5 that were treated with *t*-BuOOH. Cells were preincubated for 18–24 h, and treated with 50 μM *t*-BuOOH for 3 h. Removal of *t*-BuOOH-containing, cells were rinsed, and further cultured in medium containing 10% FBS for 24 h. Cellular proteins were extracted immediately before *t*-BuOOH exposure (control), immediately after 3 h exposure to *t*-BuOOH, or 24 h after *t*-BuOOH, and subjected to Western blots using anti-Bcl-2 antibody and densitometry.

b5 cells were markedly overexpressed when compared to their *bcl-2* transfected counterparts, Rat-1 cells. And Bcl-2 expression in b5 cells were increased to approximately 186% of the unexposed control immediately after 3-h exposure to *t*-BuOOH, whereas the expression was restored to the control level 24 h after termination of *t*-BuOOH exposure. Thus, the Bcl-2 upregulation was transiently inductive and reversible. In contrast, Bcl-2 expression in Rat-1 cells, since the expression level of Bcl-2 protein was low, not only before *t*-BuOOH exposure but also after *t*-BuOOH exposure was undetectable.

DISCUSSION

In the present study, cytoprotective effects of *bcl-2* genes against oxidative injuries were demonstrated by the finding that *t*-BuOOH causes three cell death-related symptoms such as mitochondrial disfunction, morphological cell degeneration, and DNA strand cleavage more markedly in rat fibroblasts Rat-1 than their *bcl-2* transfectants b5. We demonstrated that *t*-BuOOH-induced oxidative stress causes cell death and morphological changes markedly in rat fibroblasts Rat-1 whereas their *bcl-2* transfectants b5 have more appreciable tolerances to oxidative stress (Figs. 1 and 3). Rat-1 cells underwent cell death not only at higher concentrations of *t*-BuOOH for a shorter period (higher concentrations—shorter period—induced cytotoxicity) but also at lower concentrations for a longer period (lower concentrations—longer period—induced cytotoxicity). The *bcl-2* gene seems not to be an almighty anti-apoptotic regulator because of failure to protect against the cytotoxicity with *t*-BuOOH higher than 50 μM (Fig. 1A), although Bcl-2 expression was enhanced to a 1.9-fold level (Fig. 7) being consequently a wasteful gene expression in terms of cytoprotection. In contrast, a complete cytoprotective effect of Bcl-2 was achieved upon lower concentrations—longer period cytotoxicity with *t*-BuOOH as dilute as 10–100 nM (Fig. 1B). It seems that Bcl-2 can confer cellular protection only against oxidative stimulus below definite strength, whereas a stronger oxidative stimulus induces cell death despite Bcl-2 expression.

Enforced overexpression of Bcl-2 in cultured cells confers the resistant ability to overcome apoptosis that is induced by diverse cytotoxic triggers [Vaux et al., 1992; Hockenbery et al., 1993; Mah et al., 1993; Zhong et al., 1993; Raffo et al., 1995; Lock and Stribinskiene, 1996; Kirshenbaum and de Moissac, 1997; Mirkovic et al., 1997], but the preventive mechanisms underlying in common with diverse ROS-generators have not been resolved clearly yet. The model prooxidant *t*-BuOOH was shown to induce a cell death in Rat-1 cells by apoptosis-like mechanism as analyzed by TUNEL stain directed for the DNA 3′-OH cleavage terminals as previously described [Adams et al., 1996; Cai et al., 1999; Cregan et al., 1999; Barak et al., 2001; Haidara et al., 2002]. Apoptosis-like cell death was prevented by Bcl-2 because of negativity in TUNEL staining for the *bcl-2* transfectants but not for the parent cells after the exposure to *t*-BuOOH. Although multiple processes can be involved in apoptosis, extensive investigations indicate that activation of two cysteine proteases, caspase-1 and caspase-3, are participated in the execution of the cell death process [Martins et al., 1997; Yuan, 1997; Thornberry and Lazebnik, 1998]. Especially, caspase-3, when it is once activated, is responsible for many of the downstream events associated with apoptosis. Our results showed that caspase-3 activation was prevented in the *bcl-2* transfectants more appreciably than the parent cells after *t*-BuOOH exposure. On the other hand, caspase-1 activation was not suppressed in the *bcl-2* transfectants after *t*-BuOOH exposure. Previous report showed that caspase-1 activity precedes the activation of caspase-3, indicating that caspase-1 is the upstream regulator of caspase-3 activation [Enari et al., 1996; Fraser and Evan, 1997]. Thus, our results suggest that Bcl-2 participates in control of *t*-BuOOH-induced cell death between caspase-1 and caspase-3.

A number of mechanisms have been proposed to explain the ability of Bcl-2 to suppress apoptosis [Oltvai et al., 1993; Kluck et al., 1997; Yang et al., 1997; Shimizu et al., 1998]. One of the proposed mechanism is a function of Bcl-2 as an apparent anti-oxidant [Hockenbery et al., 1993; Kane et al., 1993], which seems to be important because there are growing evidences that ROS plays a key role in the regulation of apoptosis [Buttke et al., 1994; Bonfoco et al., 1995; Salgo et al., 1995; Lin et al., 1997]. In the normal metabolism, *t*-BuOOH is detoxified by the GSH peroxidase–GSH reductase system, leading to oxidation of GSH and NAD(P)H

[Flohe and Schlegel, 1971]. When the *t*-BuOOH-induced cell injury exceeds beyond the upper threshold ability of the GSH peroxidase–GSH reductase system, *t*-BuOOH induces an array of cellular dysfunctions including depletion of intracellular GSH [Geiger et al., 1993; Korytowski et al., 1995], excessive oxidation of NAD(P)H [Bellomo et al., 1984; Livingston et al., 1992], and peroxidation of membrane lipids [Masaki et al., 1989]. The GSH peroxidase–GSH reductase system is dependent on the availability of GSH and NAD(P)H, both of which appear, therefore, to serve a pivotal role in the cellular outcome after *t*-BuOOH exposure. There are some interesting observations for some diverse systems that the Bcl-2-expressing cells have higher levels of intracellular GSH, primarily existing as the reduced form [Kane et al., 1993; Ellerby et al., 1996; Mirkovic et al., 1997], but, notably, levels of intracellular Asc have not been reported within our knowledge (Fig. 5). Additionally, NAP(P)H pools are expanded in Bcl-2-expressing cells [Esposti et al., 1999]. Bcl-2-expressing cells are, therefore, suggested to have higher levels of overall reducing equivalents (in the form of GSH, NADPH, and putatively Asc). Although, the function of Bcl-2 as an apparent anti-oxidant has been so long controversial for truth, it may be exerted substantially by enhancing the intracellular anti-oxidant potential but not by its gene product itself. From these observations, we investigated whether the function of Bcl-2 involves the inhibitory actions against the generation of intracellular ROS and *t*-BuOOH-induced apoptosis or not.

The present study showed that *t*-BuOOH caused a large increase of ROS generation, which preceded onset of cell death as estimated by CDCFH assay (Fig. 4A). Transfection with *bcl-2* genes were found not to suppress the *t*-BuOOH-induced increase of ROS production. This agrees with a previous report that peroxide formation in appearance cannot be inhibited by Bcl-2, which, however, blocks its damaging effects [Hockenbery et al., 1993]. We also confirmed that *bcl-2* transfectants can remarkablly suppress the *t*-BuOOH-induced lipid peroxidation (Fig. 4B). These results indicate that Bcl-2 prevented lipid peroxidation that may occur sequentially after *t*-BuOOH-induced ROS generation at an early stage.

Recently, there has been an interensing report using microarray analysis [Voehringer et al., 2000] that *bcl-2*-transfected cells express the enhanced level of fatty acid binding proteins (FABP), which might act as molecular scavengers through binding the cytotoxic oxidized forms of fatty acids that are generated by oxidative stress at the early stages of apoptosis [Mei et al., 1997]. It is also interesting that overexpression of Bcl-2 suppresses lipid peroxidation significantly [Hockenbery et al., 1993; Myers et al., 1995], and, in addition, the increased lipid peroxidation is observed in *bcl-2* knockout mice [Hockenbery et al., 1993]. The increased lipid peroxidation is speculated to be inhibited by functions of Bcl-2 in some direct manners [Hockenbery et al., 1993].

Another study showing the elevated FABPs in Bcl-2-expressing cells, however, suggests that Bcl-2 may function, through nuclear GSH compartmentalization, as a transcriptional regulator at an upstream stage prior to lipid peroxidation [Voehringer et al., 1998; Voehringer, 1999], thereby enhancing a cellular capacity to buffer and terminate these destructive pathways.

Furthermore, overexpression of *bcl-2* may enhance the potential anti-oxidative ability to overcome oxidative stress. So, we paid attention to Asc, which is known to defend the living tissues against damage by free radicals because of its anti-oxidant properties [Antonenkov and Sies, 1992; Sinclair et al., 1992; Makar et al., 1994]. Asc reacts readily with a variety of ROSs including superoxide anion radical, singlet oxygen, hydroxyl radical, and water-soluble peroxyl radicals [Halliwell and Gutteridge, 1990]. Moreover, it has been reported that Asc and alpha-tocopherol act synergistically in the prevention of lipid peroxidation, as Asc readily reduces the tocopheroxyl radical at the membrane interface [Niki, 1991]. We demonstrated that Asc uptake amount was larger into the b5 than into Rat-1 constantly regardless of conditions for preculture and uptake itself. These results suggest that Bcl-2 may modulate the mechanism for transport of the anti-oxidant such as Asc into cells to protect themselves from oxidative stress that are generated during the initiation phase of many apoptotic pathways. Accordingly, the increase of Asc uptake in *bcl-2*-transfectants b5 may be correlated with one of protective responses in cell against oxidative stress.

Since there are some reports that cell protection is often correlated with Bcl-2 regulation

[Rowe et al., 1994; Akbar et al., 1996; Katoh et al., 1996; Lock and Stribinskiene, 1996; Nor et al., 1999], we analyzed Bcl-2 expression time-dependently after *t*-BuOOH application. We demonstrated that treatment of b5 cells with the pro-oxidant *t*-BuOOH resulted in increased expression, assumedly not of endogenous scarce Bcl-2, but of exogenously transferred Bcl-2, which is closely correlated with the prevention of apoptosis, and, therefore, *t*-BuOOH-induced Bcl-2 upregulation is one of the important events for cell survival. Moreover, the increased expression of Bcl-2 immediately after *t*-BuOOH exposure (Fig. 7) indicates that upregulation of Bcl-2 involves not only an increase in intracellular anti-oxidative potentials, but also the suppressions of both lipid peroxidation and caspase-3 activation. Thus, our study provides an evidence for a close relationship between the suppression of cell death and upregulation of Bcl-2. From these reasons, we suggest that oxidative stress triggers the upregulation of Bcl-2, which is one of the important physiological reaction to protect the cells from oxidative stress-induced apoptosis.

In conclusion, *t*-BuOOH-induced cell death in rat fibroblasts Rat-1 and their *bcl-2* transfectants b5 is achieved by an apoptosis-like mechanism involving the lipid peroxidation, emergence of DNA-3′-OH cleavage terminals and caspase-3 activation as the signaling of apoptosis. The counteractive mechanism underlying the cytoprotection by Bcl-2 against oxidative stress-induced cell death may be associated with Bcl-2 expression-induced partial enhanced anti-oxidant cellular ability including suppression of lipid peroxidation, enrichment of intracellular Asc, and oxidative stress-induced upregulation of Bcl-2. Thus, a gene therapy using Bcl-2 may be a crutial tool to prevent oxidant-induced apoptosis that may contribute to cell death under in vivo disease conditions.

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