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Transfection of the anti-apoptotic gene *bcl-2* inhibits oxidative stress-induced cell injuries through delaying of NF-κB activation

Research Article

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Abbreviations: artificial viral envelope, (AVE); Dulbecco's modified Eagle's medium, (DMEM); enhanced chemiluminescence, (ECL); electrophoretic mobility shift assay, (EMSA); post-ischemic reperfusion, (I/R); reactive oxygen species, (ROS); hemagglutinating virus of Japan, (HVJ); human Bcl-2, (*h*Bcl-2); terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labeling, (TUNEL); *tert*-butyl hydroperoxide, (*t*-BuOOH)

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Summary

We investigated the relation between endogenous NF-κB and exogenous overexpressed Bcl-2 in rat fibroblastic cells (Rat-1) in response to H_2O_2 after confirming the cytoprotective effect of Bcl-2 against oxidative stresses such as *in vitro* treatment with H_2O_2 and *in vivo* hepatic post-ischemic reperfusional (I/R) injury. Exogenous Bcl-2, which was expressed by hemagglutinating virus of Japan (HVJ)-artificial viral envelope (AVE) liposome-mediated gene transfer of human *bcl-2* that was incorporated into an SV2 vector, prevented I/R-induced hepatic injuries such as cellular DNA strand cleavages more effectively than the non-transfection treatment. The *bcl-2*-transfected Rat-1 fibroblasts exerted the cytoprotective effect against H_2O_2 of 50-250 uM more markedly than the SV2 vector-transfected or non-transfected counterpart cells. Immunocytochemical analysis and electrophoretic mobility shift assay (EMSA) showed that intracellular activation of NF-κB in *bcl-2*-transfectants was repressed more appreciably than in SV2-transfectants at a period as early as 30 min after H_2O_2 stimulation, but, at advanced periods of 90 and 120 min, was increasingly exhibited up to the similar and exceeding levels relative to those of SV2-transfetants, respectively. Thus the prevention by the anti-apoptotic gene *bcl-2* against oxidative stress-induced injury may be attributed at least partly to the repressed early activation and/or the delayed activation of NF-κB. The results provide the foundation for redox-mediated gene therapies using *bcl-2* gene directing at ameliorative effects against oxidative stress-induced injuries.

I. Introduction

Bcl-2, a mammalian homologue of the anti-apoptotic gene *ced-9* in *C. elegans*, is localized mainly to the mitochondrial membrane (Hockenbery et al, 1990; Akao et al, 1994), and is known to be a key regulator for apoptosis, functioning as an anti-apoptotic protein with the ability to protect against a variety of physiologic or pathologic insults and environmental stimuli (Vaux et al, 1988; Reed, 1994; Tsujimoto, 2003). A number of mechanisms have been proposed to explain the ability of Bcl-2 to suppress apoptosis (Oltvai et al, 1993; Yang et al, 1997; Shimizu et al, 1998). The localization of Bcl-2 at the loci of free radical generation such as mitochondria may correlate with its ability to protect the subcellular organization (Gross et al, 1999) and to function as an apparent anti-oxidant agent against oxidative stress that

may induce apoptosis (Hockenbery et al, 1993; Voehringer et al, 2000; Jang et al, 2003).

We previously showed that *in vitro* cytoprotective effects of human Bcl-2 (*h*Bcl-2) against oxidants such as the *tert*-butylhydroperoxide (*t*-BuOOH) or post-hypoxic anoxia-induced oxidative injury (Saitoh et al, 2003a,b). And we recently demonstrated that *in vivo* inhibitory effects of *h*Bcl-2 on ischemia-reperfusion (I/R) injury involve the repression of increased reactive oxygen species (ROS) (Yanada et al, 2004 and 2005). However, we have not demonstrated definite inhibitory mechanism against oxidative stress by Bcl-2, and it has been in a state of controversy.

As a hint to elucidate the mechanism underlying cell-death inhibition by Bcl-2, it is able to cite the ubiquitous eukaryotic transcription factor, NF-κB which regulates expression of numerous cellular genes that play important roles in mediating/regulating immune and stress responses, inflammation, apoptosis, proliferation and cell survival (Baeuerle et al, 1994 and 1996). In addition, NF-κB is known to be activated by oxidative stress, which is generated by ROS such as H_2O_2 and O_2^-, resulting in occurrence of apoptosis and/or necrosis in correspondence to balance with inherent anti-oxidative cellular defense (Wang et al, 2002).

In the present study, after confirming the inhibitory effect of Bcl-2 on *in vivo* oxidative stress, to investigate the practical mechanisms involved in Bcl-2 cytoprotection against H_2O_2-induced oxidative stress, we focused on the relations between activation of endogenous NF-κB and exogenous overexpressing Bcl-2 in rat fibroblastic cells.

II. Materials and methods

A. Plasmid DNA

As a plasmid vector, pCΔj-SV2 and pCΔj-*bcl-2* (12.5 kbp, 13.5 kbp; [Tsujimoto, 1989]) was used as previously described (Yanada et al, 2004). Human *bcl-2* cDNA (1.0 kbp) was inserted into the *Eco*RI sites of SV40 early promoter in the pCΔj-SV2. The plasmids were amplified in *Escherichia coli* DH5a. Both plasmids were kindly provided by Dr. Shoji Yamaoka of Tokyo Med. Dent. Univ. and Dr. Yoshihide Tsujimoto of Osaka Univ., respectively.

B. *In vivo* oxidative stress model; I/R operation of rat livers

Male Wistar rats weighting 250 to 300 g (8-weeks old) were purchased from Japan SLC, Shizuoka, Japan, and were housed at 22 ± 2°C for 12 hr light-dark cycle with access to water and food. They were used in experiments following adjustment to these conditions for at least 3 days and were fasted overnight before the experiments. I/R operation was performed as previously described (Eguchi et al, 2003; Yanada et al, 2004 and 2005). An approximately 70% region of the whole liver was made ischemic by clamping both portal vein and the hepatic artery, and resultantly ROS was generated in the ischemic livers (Eguchi et al, 2003).

C. *In vivo* transfection of plasmids encoding *bcl-2* gene

For preparing gene transfection's vector, hemagglutinating virus of Japan (HVJ)-artificial viral envelope (AVE) liposome was prepared as described (Saeki et al, 1997; Yanada et al, 2005). Prepared HVJ-AVE liposome mixture (200 μg of plasmid DNA and 65 μg of HMG-1, 2 mixture [Wako Pure Chemicals Industries, Osaka]) was injected into the liver via the portal vein by cannulation. The transfection ratio into hepatocytes by this cannulation was approximately 20-30% on the second day after transfection. The transfected genes were expressed around the portal vein of median and left lobes at 2 days after transfection (Yanada et al, 2005). At the time, therefore, livers were quarried and analyzed.

D. TUNEL assay

Cell death associated with I/R-induced hepatic injuries was analyzed by terminal deoxyribonucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) assay using the *In situ* Apoptosis Detection Kit (TaKaRa, Shiga, Japan) according to the manufacture's protocol as described (Yanada et al, 2005). Sections of the caudal and median lobes in the I/R-receiving rat livers were prepared at 150 min after reperfusion, and were evaluated by TUNEL assays as compared with those of non-transfected and *bcl-2*-transfected rats. Sections were examined under a laser scanning confocal fluorescence microscope [MRS-600 Cosmos; Carl Zeiss, Oberkochen, Germany (Bio-Rad, Hercules, CA)] at a 100-fold magnification, and expressed in pseudo-color from red (scarcely stained) via yellow (weakly stained) to purple (most strongly stained) by processing of fluorescence intensity with an NIH-Image software for evaluation of the degree of apoptosis. And to elucidate degrees of I/R-induced DNA strand cleavages, images were analyzed and expressed by histogram.

E. Cell culture

Rat fibroblastic cells, Rat-1 (Topp, 1981) were used as a parent type, and were kindly provided by Dr. Shoji Yamaoka of Tokyo Med. Dent. Univ. Rat-1 cells (non-transfectants, WT) were cultured in complete medium, Dulbecco's modified Eagle's medium (DMEM, Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% heat-inactivated fetal bovine serum (FBS; GIBCO BRL, Grand Island, NY), 4 mM L-glutamine, 50 μg/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. To make stable *bcl-2*-overexpressed transfectants or SV2 (the empty vector without encoding *bcl-2*) transfectants, Rat-1-SV2 cells, pCΔj-SV2 or pCΔj-*bcl-2* was introduced into Rat-1 cells by the calcium phosphate precipitation method, respectively as described (Paker et al, 1979). Briefly, 1.5 x 10^5 cells of Rat-1 were seeded into a 35-mm dish. At 16 hr after seeding, 275 μl of 2 M Ca^{2+} solution containing plasmid DNA (pCΔj-*bcl-2* or pCΔj-SV2, 10 μg each) and 275 μl of 2 x HBS was mixed under supplying air. The DNA mixture solution was poured into the cells. After 1-2 days, the cells were washed twice with phosphate-buffered saline [PBS(-)], and subcultured into 100-mm dishes at appropriate cell concentration in complete medium containing 600 μg/ml Geneticin disulfate (G418; Wako) at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Medium was changed at every 3 days. After 2 weeks, single colony picked up using a cloning cup (Iwaki Co., Tokyo), and cultured to be grown up and become near-confluent. Bcl-2 expression levels of our prepared-*bcl-2*- transfectants were compared with the level of *bcl-2*-stable transfectants, b5 cells (a kind gift from Dr. Shoji Yamaoka of Tokyo Med. Dent. Univ.), and the cells which expressed the same level as that of b5 cells were selected out of some candidate colonies.

F. Western blotting

Western blotting was performed for analysis of *bcl-2* expression of Rat-1 and our picked up- SV2- or *bcl-2*-

transfectant, as previously described (Saitoh et al, 2003a,b). 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 DTT, 1 mM PMSF, 1% IGEPALCA-630, 1% SDS, 4 mM leupeptin, and 3 μM pepstatin A. After being three 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 thick polyvinylidene difluoride (PVDF) membranes (Millipore). Nonspecific binding was blocked by incubating the membranes for 2 hr 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 the 1: 2,500 diluted mouse monoclonal antibody against human Bcl-2 (product #sc-509; Santa Cruz Biotechnology, CA) in blocking buffer overnight at 48°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 the 1: 3,000 diluted horseradish peroxidase-conjugated anti-mouse IgG 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, England, UK), and blots were exposed to Hyperfilm MP (Amersham) for 0.5–2 min. Laser scanning densitometry was conducted for semiquantitative analysis of the data. Approximately equivalent amounts of loaded proteins were confirmed by the densitometric values of some randomly selected bands on the Coomassie Brilliant Blue-stained gel.

G. Cell viability assay

Cell viability of Rat-1 and SV2- or *bcl-2*-transfected cells was measured by WST-1 method as previously described (Saito et al, 2003a and b). Briefly, the cell layer in a dish was incubated with WST-1 (2-(4-iodophenyl)- 3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2 H-tetrazolium, monosodium salt) (Dojin Laboratories Co., Kumamoto, Japan) solution at 1:10 volume of phenol red-free culture medium for 3 hr 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 an absorbance plate reader (Bio-Rad), and the absorbance values detected have been demonstrated to be proportional to viable cell numbers. Since there was no difference in the basal viability (mitochondrial dehydrogenase activity) between non-transfectants and transfectants, the values obtained from control cultures (non-treated non-transfectants and transfectants) are represented as 100% viability. The values of treated cultures are expressed as a percentage of those versus the corresponding control cells.

H. Detection of the activation of NF-κB: Electrophoretic mobility shift assay

Nuclear protein extraction was performed as described previously (Yang et al, 1995). Electrophoretic mobility shift assay (EMSA) was performed with specific gel-shift assay system of NF-κB (Promega, Madison, WI). The double-stranded oligonucleotide probe containing the specific wild-type DNA binding domain for NF-κB was as follows:

5'-TTTCTAGGGACTTTCCGCCTGGGGACTTTCCAG-3'. The oligonucleotides were labeled with [α-^{32}P]dATP (Pharmacia) using the Klenow fragment of DNA polymerase I (Takara, Tokyo) and purified using a gel-filtration column (MicroSpin G-25, Pharmacia).

I. Visualized detection of activation of NF-κB: Immunocytochemical staining

Cells of 3.8 x 10^3 were seeded into each well of 8 well chamber slide (Nunc. Inc., Roskilde, Denmark), after 24-hr incubation, the cells were treated with H_2O_2 at 100 μM for 2 hr. After further 24-hr incubation, the intracellular activation of NF-κB in SV2-transfectants and *bcl-2*- transfectants was analyzed at 0, 15, 30 and 90 min. Briefly, the cells were washed twice in PBS(-), and fixed with 4.5% paraformaldehyde in PBS(-) for 15 min, and subsequently washed with PBS(-). Cells were then treated with 0.5% Triton X-100 in PBS(-) for 20 min, and were thereafter treated with anti-rat NF-κB, p65 subunit (c-20) rabbit polyclonal antibody (product # sc-372, Santa Cruz Biotechnology Inc., CA) at a final concentration of 0.5 μg/ml in 3% bovine serum albumin (BSA, Sigma) in PBS(-) at 37°C for 1 hr in humidified atmosphere. Cells were then washed with 0.05% Triton X-100 in PBS(-), and subsequently were incubated with the secondary antibody, an FITC-conjugated anti-rabbit IgG goat antibody (product #55646, Organon Technika Co.) at a final concentration of 0.1 μg/ml in 3% BSA in PBS(-) at 37°C for 40 min. The preparations were thereafter washed three times with PBS(-) for 10 min and mounted in PermaFluor aqueous mounting medium (Immnunon, Pittsburgh, PA). The slides were examined on a confocal laser scanning fluorescence microscope [MRS-600 Cosmos; Carl Zeiss (Bio-Rad)] equipped with an argon laser as the light source, and then were analyzed with Photoshop 4.0J and NIH Image softwares.

III. Results

A. *In vivo* protective effect of Bcl-2 on ischemia-reperfusion (I/R) in rat non-transfected livers and *bcl-2*-transfected livers

Tissue sections of I/R-operated livers were made at 150 min after reperfusion, and comparison was made within two groups: *bcl-2*-transfected, and the non-transfected livers as analyzed by TUNEL method (**Figure 1**). Apoptotic TUNEL-positive cells, indicated by purple or deep blue dots, were markedly observed in the median lobes of non-transfected livers, concomitantly with the nuclear condensation in the vicinity of the portal vein (**Figure 1Ab**). In contrast, in the median lobes of *bcl-2*-transfected livers and the caudal lobes of non- and *bcl-2*-transfected livers, TUNEL-positive cells were scarcely observed (**Figures 1Aa, c and d**). The I/R-induced DNA strand cleavage 3'-OH terminals, indicative of a symptom for apoptotic cells, were also detected by histogram analysis (**Figure 1Bb**). Apoptotic cells were observed to be significantly diminished in the median lobes of *bcl-2*-transfected livers, and were not detected by histogram analysis (**Figure 1Bd**). Thus, exogenously transfected *bcl-2* is suggested to markedly prevent I/R-induced cellular DNA strand cleavages.

B. Expression of Bcl-2 in Rat-1 cells, SV2- or *bcl-2*-transfectants

To investigate the relationship between endogenous NF-κB and exogenous *h*Bcl-2 after stimulation of ROS,

we made *bcl-2*-transfectants, and analyzed Bcl-2 expression without ROS stimulation by western blotting (**Figure 2**). Bcl-2 expression in *bcl-2*-transfectants was markedly overexpressed when compared to their non-transfected and SV2-transfected Rat-1 cells. In addition, when compared to expression of Bcl-2 stable expressed cell line, b5 cells, it was confirmed that the Bcl-2 expression level of our picked up-*bcl-2* transfectants was similar to that of b5 cells (data not shown).

C. Protective effect of *bcl-2* genes against H_2O_2–induced cell death in Rat-1, SV2- or *bcl-2*- transfectants

To examine the role of *bcl-2* genes in the cytotoxic response to H_2O_2, Rat-1 and SV2- and *bcl-2*- transfected cells were exposed to the indicated concentrations of H_2O_2 (0–250 μM) for 2 hr. After the indicated exposure time, cells were incubated for 24 hr in fresh medium, and then were assessed for the cell viability by WST-1 assay. Treatment with H_2O_2 for 2 hr decreased the cell viability of both the parent and *bcl-2*-transfected cells in a dose-dependent manner (**Figure 3**). Cell viability of *bcl-2*-transfected cells was more markedly retained than that of the parent Rat-1 or SV2-transfected cells against H_2O_2 -induced injuries, and was slightly increased than the initiate level at 100 and 125 μM of H_2O_2.

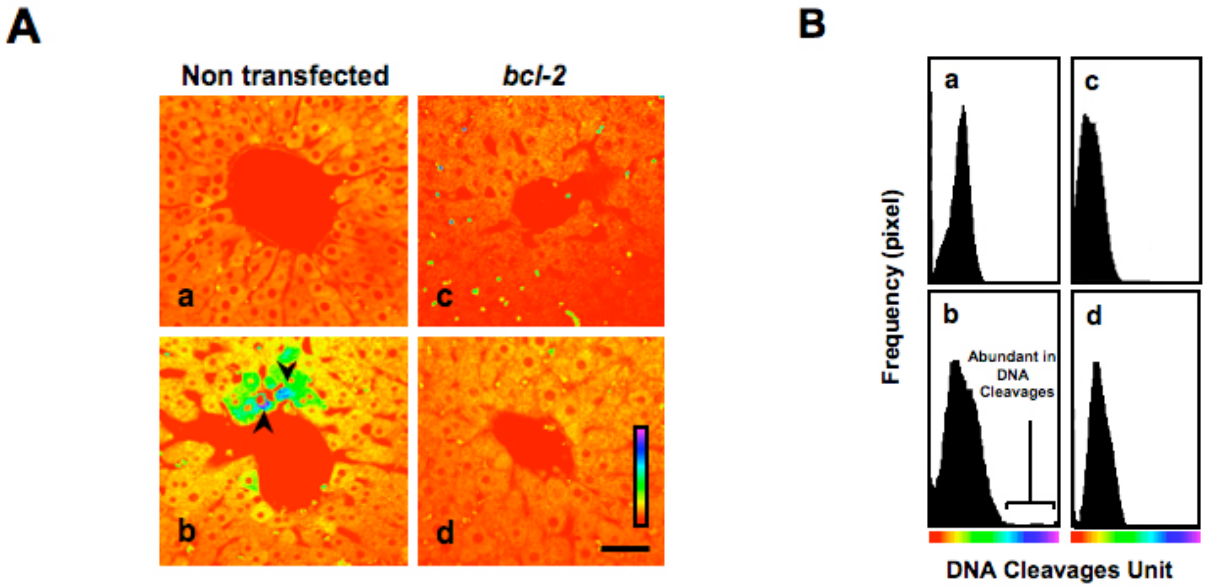

Figure 1. Cellular DNA cleavages in paraffin-embedded tissue sections of non-transfected and *bcl-2*-transfected livers after post-ischemic reperfusion (I/R) as assayed by TUNEL method (**A**). The sections of the caudal (non-ischemia; a, c) and median lobes (ischemia treatment; b, d) of the I/R-receiving rat livers were prepared at 150 min after the beginning of reperfusion, and were evaluated for non-transfected rats (a, b), and *bcl-2*-transfected rats (c, d) by TUNEL assays. Sections were examined under a confocal fluorescence microscope at a 100-fold magnification, and expressed in pseudo-color from red (scarcely stained) via yellow (weakly stained) to purple (most strongly stained) by processing of fluorescence intensity with an NIH-Image software for evaluation of the degree of DNA 3'-OH cleavage terminals as an indicator for apoptosis. The scale indicates 50 μm. To detect I/R-induced DNA strand cleavages, images were analyzed to be expressed in histograms (**B**). All data shown are typical of 3-4 sheets of micro-slices per each examined groups that showed the same staining degree among three independent experiments.

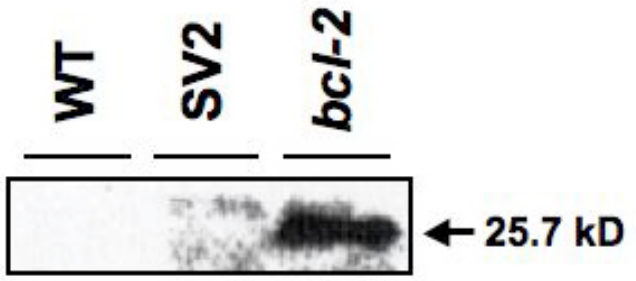

Figure 2. Expression of exogenous human Bcl-2 in wild type (Rat-1 cells; WT), SV2- and *bcl-2*- transfected cells. After establishment of transfectants, each cell population was analyzed for expression of Bcl-2 without oxidative stimulation by western blotting using an anti-*h*Bcl-2 antibody.

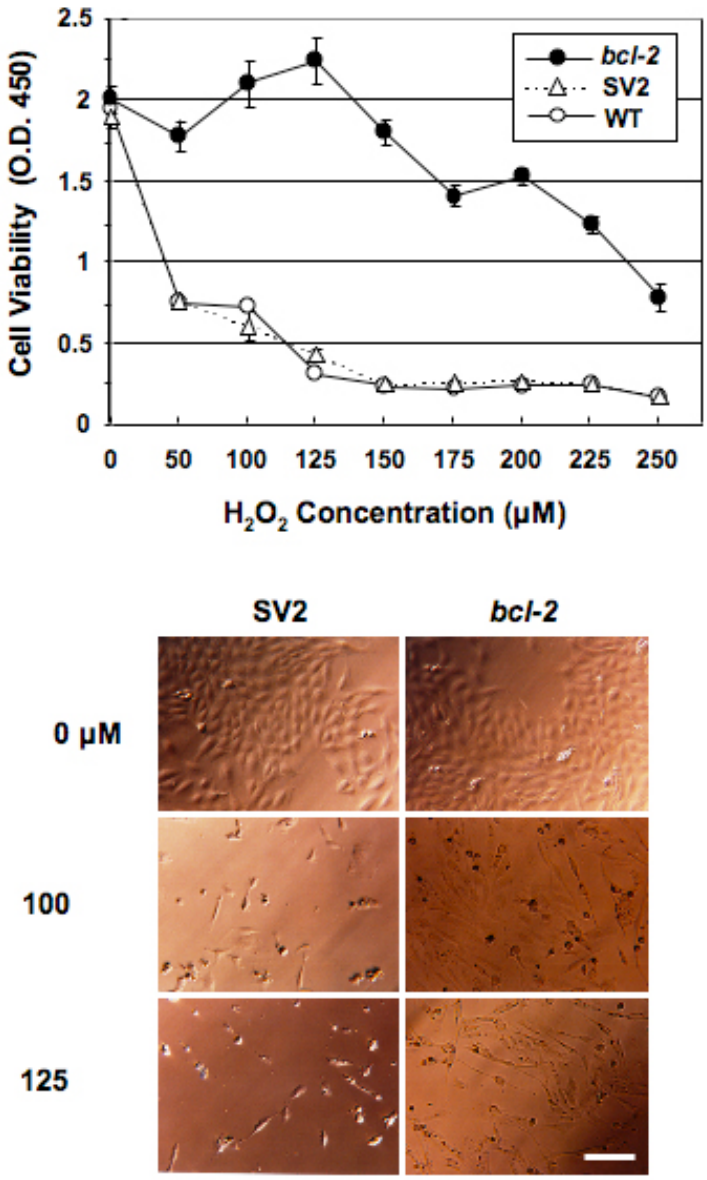

Figure 3. Dependences of cell viability of Rat-1, SV2- and *bcl-2*-transfected cells on treatment with H_2O_2. At 24 hr after stimulation of different H_2O_2 concentration (0-250 μM), cell viability in each cell population was evaluated by mitochondrial dehydrogenase-based WST-1 assay. And microscopic views of SV2- and *bcl-2*-transfected cells with treatment of H_2O_2 (0, 100 and 125 μM) show together. The scale in the image indicates 50 μm.

D. Bcl-2 affects intracellular activation of NF-κB at initiate period after stimulation of H_2O_2

To elucidate the cytoprotective mechanism of Bcl-2, we examined whether activations of NF-κB in non- or *bcl-2*-transfectants would be occurred when both cells were exposed to 100 μM of H_2O_2. The intracellular activation of NF-κB in SV2- and *bcl-2*-transfectants was analyzed at 0-120 min by EMSA system. In SV2-transfectants, intracellular activations of NF-κB were rapidly and strongly occurred at 30 min, but, in *bcl-2*-transfectants, were detected weakly at 0 and 30 min (**Figure 4**). However, at 90 and 120 min after stimulation, intracellular activation of NF-κB was strongly detected in both cells (**Figure 4**). Moreover, interestingly, NF-κB activation in *bcl-2*-transfectants was markedly occurred than that in SV2-transfectants (**Figure 4**). These results suggest that Bcl-2 affected intracellular activation of NF-κB at an initiate period after stimulation of H_2O_2. And to visualize the expression and intracellular distribution of NF-κB under stimulation of H_2O_2, immunocytochemical analysis using polyclonal antibody which recognized a p65 subunit of NF-κB was performed. At 15 and 30 min after the stimulation, intracellular activation of NF-κB was appreciably detected in SV2-transfectants, but weakly observed in *bcl-2*-transfectants. At 90 min after the stimulation, in both SV2-transfectant and *bcl-2*-transfectant, translocation of NF-κB into the nucleus was observed (**Figure 4**), showing the consistence with results of EMSA.

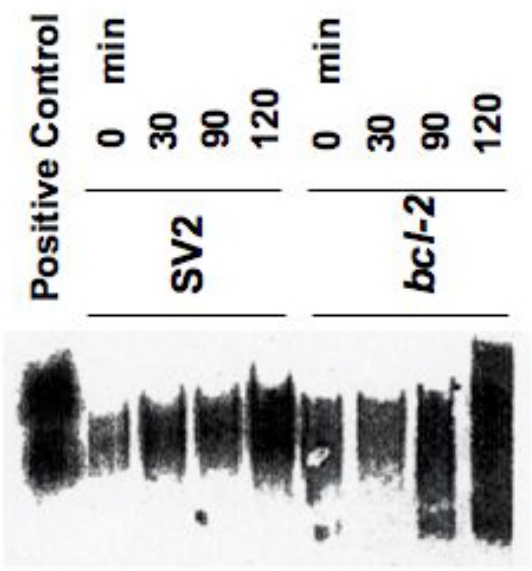

Figure 4. Time course analysis of DNA binding activities of NF-κB in SV2- and *bcl-2*-transfected cells by electrophoretic mobility shift assay (EMSA) after stimulation of H_2O_2. The cells were treated with H_2O_2 at 100 μM for 2 hr. The intracellular activation of NF-κB in SV2- and *bcl-2*-transfectants was analyzed at 0-120 min. Nuclear protein extraction of each cell was analyzed at different time after stimulation of H_2O_2 by EMSA using specific gel-shift assay system.

IV. Discussion

In the present study, exogenous Bcl-2 prevented I/R-induced apoptosis in rat livers. And our prepared-*bcl-2*-transfectants have the preventive effect against cell injuries induced with 50 -250 μM of H_2O_2. Additionally, the *in vitro* examination in *bcl-2*-transfectants by immunocytochemical analysis and EMSA showed that Bcl-2 repressed intracellular activation of NF-κB at an initiate period after stimulation of H_2O_2, although NF-κB activation was quickly and strongly occurred in SV2-transfectants. And at 90 and 120 min after stimulation, NF-κB activation in *bcl-2*-transfectants were more remarkably detected than that in SV2-transfectants, suggesting that the transient repressive effect of exogenous Bcl-2 on an early NF-κB activation might be attributed to the avoidance from the subsequent destiny to oxidative injuries.

We showed using TUNEL method with histogram analysis that exogenous Bcl-2 has the *in vivo* preventive potential against I/R injury (**Figure 1**). In our previous study, it has firstly reported that inhibitory effect of Bcl-2 in combination with the Bcl-2-associated athanogene 1 protein, BAG-1 can be evaluated by the same method (Yanada et al, 2005). These results suggest histogram analysis with by the same method can easily and usefully quantify I/R-induced apoptosis. Furthermore, it suggests that transfection by Bcl-2 alone is a useful strategy for gene therapy against I/R injury.

Regarding the *in vitro* examination to investigate the relation between Bcl-2 and NF-κB, we took a notice of H_2O_2 as an ROS stimulant, which is known to less difficultly penetrate through the living membrane and be generated when the cells are exposed to oxidative stress such as I/R. The cytoprotective effect of exogenous Bcl-2 against H_2O_2-induced injuries was obtained, and accentuated for H_2O_2 as low as 100 and 125 μM, as shown by the hormesis-like enhancement in cell viability of *bcl-2*-transfectants over the initiate level (**Figure 3**). It seems that this increase depends on the increase in viable cells with activity of mitochondrial dehydrogenase by the stimulation of H_2O_2, which is known at higher doses to lower the electric potential at the mitochondrial membrane through a depolarization effect. Recently, it has been reported by our laboratory that treatment with H_2O_2 of lower concentrations enhances the maximum cell population doubling level of human skin keratinocytes together with slow-down of age-dependent shortening of telomeric DNA (Yokoo et al, 2004), suggesting a trace H_2O_2-induced benefit effects such as telomere protection and enhanced *bcl-2* expression in common through a feeble oxidant-induced bottom-up effect on the emergent antioxidant ability.

And exogenous Bcl-2 repressed intracellular activation of NF-κB at an initial period after stimulation of H_2O_2, although activation of NF-κB was occurred in SV2-transfectant (**Figure 4**). Activation of NF-κB has been reduced under the existence of in the presence of the intracellular antioxidant in Rat-1 cells after stimulation of ROS as previously reported (Nagao et al, 2000). Additionally, we have previously detected intracellular ROS accumulation in Rat-1 cells when the cells were exposed to the alkyl hydroperoxide *t*-BuOOH or operated with hypoxia-reoxygeneration which occurred accumulation of ROS such as H_2O_2 resulting in cell death (Saitoh et al, 2003b). At this time, in b5 cells (*bcl-2*-stable transfectants), intracellular accumulation of ascorbic acid was enhanced than in the parental cells, Rat-1, suggesting that intracelluar anti-oxidants may be indirectly related with overexpression of *bcl-2* (Saitoh et al, 2003a) assumedly owing to lowering of demand for scavenging of cell-death-derived secondarily generated extra ROS. Moreover, at 90 and 120 min after stimulation of H_2O_2, although activation of NF-κB was detected in both *bcl-2*- and SV2-transfetants, the activation in *bcl-2*-transfectants remarkably increased than that of SV2-transfectants in particular (**Figure 5**). High constitutive DNA binding and transcriptional activities of NF-κB were observed in rat pheochromocytoma PC12 cells overexpressing *bcl-2* gene after stimulation of H_2O_2 (Jang et al, 2004), which mostly supports our results in spite of difference in cell lines.

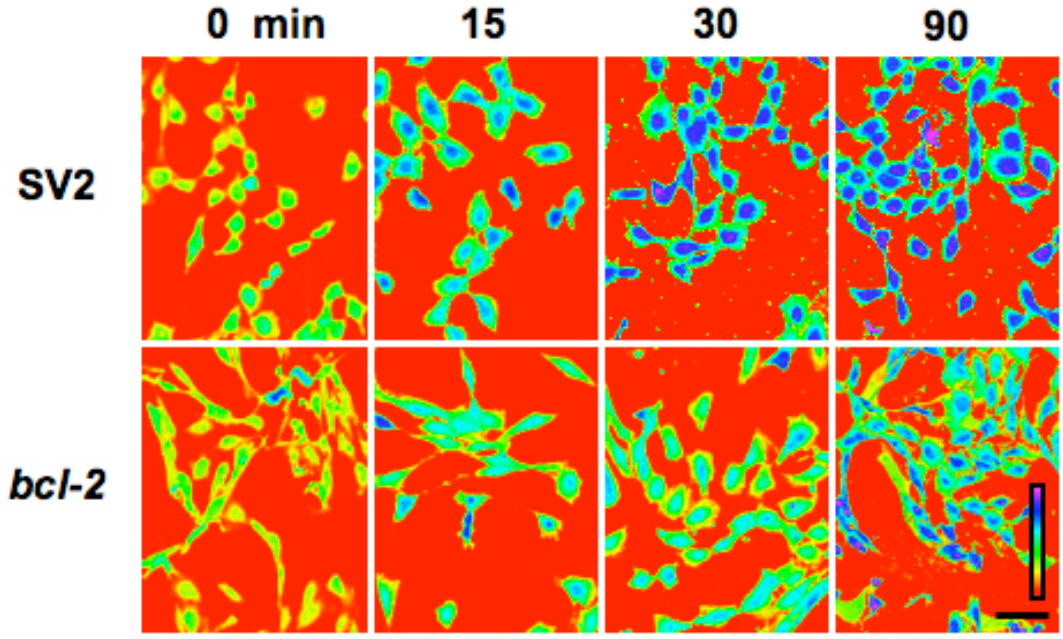

Figure 5. Immunocytochemical analysis of the transcriptional factor NF-κB in SV2- and *bcl-2*-transfected cells after stimulation with H_2O_2. The intracellular activation of NF-κB in SV2- and *bcl-2*-transfectants was analyzed at 0, 15, 30 and 90 min after 2 hr treatment with H_2O_2 at 100 μM. The slides were examined on a confocal laser scanning fluorescence microscope ([MRS-600 Cosmos] equipped with an argon laser as the light source), and expressed in pseudo-color similarly as in Fig. 1. The scale indicates 50 μm. Data shown are typical of 3-4 sheets of micro-slices per each group that showed the similar staining degree among three independent experiments.

These results suggest the possibility that constitutive activation of redox-sensitive transcription factor NF-κB acts as survival signal in *bcl-2*-overexpressing cells.

Currently, NF-κB has been attempted as a target of gene therapy in several diseases such as nephritis, liver failure and glioblastomas (Tomita et al, 2000; Robe et al, 2004; Higuchi et al, 2006). On the other hand, an experimental gene therapy using mitochondrial superoxide dismutase gene is reported to significantly reduce acute liver damage and be associated with redox activation of NF-κB, suggesting a benefit effect against oxidative stress-induced hepatic injuries (Zwacka et al, 1998). We have demonstrated the possibility of gene therapy against oxidative stress-induced injuries using Bcl-2 as a putative function as an antioxidant, which prevents apoptosis by controlling ROS through increase of intracellular antioxidant (Yanada et al, 2004 and 2005). And in the present study, showed that transfection of *bcl-2* repressed intracellular activation of NF-κB at an initiate period after stimulation of H_2O_2, resultantly H_2O_2 induced-cell death was inhibited. Taken together, exogenous Bcl-2 may be able to control indirectly the transcription factor NF-κB, because Bcl-2 acts as a multiplier or consumption-saver for intracellular antioxidants. Thus, it is possible that an *in vivo* transfection of *bcl-2* is useful as one of some strategies for gene therapy against oxidative stress-induced injury together with gene therapy using mitochondrial superoxide dismutase gene, which controls the intracellular redox state after stimulation of oxidative stress.

Acknowledgments

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