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FAST TRACK

Cytoprotection by *bcl-2* Gene Transfer Against Ischemic Liver Injuries Together With Repressed Lipid Peroxidation and Increased Ascorbic Acid in Livers and Serum

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Abstract The maximum gene exhibition was shown to be achieved at 48 h after transfection with human *bcl-2* (h*bcl-2*) genes built in an SV40 early promoter-based plasmid vector and HVJ-liposome for cultured rat hepatocytes. The similar procedure of h*bcl-2* transfection was therefore conducted for livers in rats via the portal vein, and after 48 h followed by post-ischemic reperfusion (I/R) operation for some hepatic lobes. The I/R-induced hepatic injuries were in situ observed as both cell morphological degeneration and cellular DNA strand cleavages around capillary vessels of the ischemic liver lobes as detected by HE stain and TUNEL assay, and were biochemically observed as release of two hepatic marker enzymes AST and ALT into serum. All the I/R-induced injuries examined were appreciably repressed for rats transfected with h*bcl-2*; h*bcl-2* was expressed in hepatocytes around the capillaries of ischemic regions such as the median lobe and the left lobe, but scarcely around those of non-ischemic regions. Thus cytoprotection against I/R-induced injuries may be attributed to the I/R-promoted expression of transferred h*bcl-2* genes. The possibility was examined firstly by methylphenylindole method, which showed that I/R-enhanced lipid peroxidation in the reference vector-transfected livers were markedly repressed in the h*bcl-2*-transfected livers. Contents of ascorbic acid (Asc) in serum and livers of h*bcl-2*-transfected rats were enriched, unexpectedly, versus those of non-transfected rats, and were as abundant as 1.90-fold and 1.95- to 2.60-fold versus those in the pre-ischemic state, respectively. After I/R, an immediate decline in serum Asc occurred in h*bcl-2*-transfectants, and was followed by prompt restoration up to the pre-ischemic Asc levels in contrast to the unaltered lower Asc levels in non-transfectants except a transient delayed increase. Hepatic Asc contents were also diminished appreciably at the initial stage after I/R in the ischemic lobes of h*bcl-2*-transfectants, which however retained more abundant Asc versus non-transfectants especially at the initial I/R stage when scavenging of the oxidative stress should be most necessary for cytoprotection. The results showed a close correlation between cytoprotection by exogenously transferred h*bcl-2* and repressive effects on the lipid peroxidation associated with Asc consumption or redistribution. J. Cell. Biochem. 93: 857–870, 2004. © 2004 Wiley-Liss, Inc.

Key words: Bcl-2; HVJ-liposome; ischemia-reperfusion; reactive oxygen species (ROS); ascorbic acid

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Ischemic state can be induced by some clinical situations such as the blood stream interception in the internal organ transplant, and the decreased pressure of the blood vessel along with the operation. There are many similarities in a case for the injury along with ischemia in most of organs such as liver, brain, kidney, and lung. Hepatic post-ischemic reperfusion (I/R) injuries occur during liver transplantation, surgical resections and shock. It is well known that this can lead to cell death (such as necrosis and apoptosis) induced by reactive oxygen species (ROS) which is produced in the period of reperfusion [McCord, 1985; Ferdinand et al., 2001], and ROS induces apoptosis in the I/R-subjected rat liver where ROS was explosively produced [Kohli et al., 1999].

Apoptosis involves the activation of a genetically determined programmed cell suicide that results in a morphologically distinct type of cell death characterized by cell shrinkage, nuclear condensation, DNA fragmentation, membrane reorganization, and blebbing [Kerr et al., 1972]. Among some genes controlling the apoptotic process, the *bcl-2* proto-oncogene 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 [Vaux et al., 1988; Reed, 1994; Tsujimoto, 1998]. Thus, it constitutes a prime target for therapeutic intervention in numerous disease states [Thompson, 1995; Kroemer, 1997]. Bcl-2 is known to be localized mainly at the outer mitochondrial membrane, as well as to the endoplasmic reticulum and nuclear membranes [Hockenbery et al., 1993]. 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. In addition, anti-apoptotic effects of Bcl-2 may correlate with repression of ROS because ROS is generated at an early stage during the process of apoptosis and mediates at least a part of the overall process [Jacobson, 1996].

Actually, we previously showed in vitro that inhibitory effects of hBcl-2 on the lipid hydroperoxide-induced subacute cytotoxicity was suggested to be attributed to suppressions of both lipid peroxidation and the subsequent cell death events such as caspase-3 activation and DNA cleavage, some of which may ensue at an earlier stage from partial enhanced antioxidant cellular ability including enrichment of intracellular ascorbate [Saitoh et al., 2003].

Within our knowledge, however, there has been no report proving that Bcl-2 may influence either contents or pathway of any antioxidant low-molecular agents in vivo. Therefore, in the present study, to elucidate the practical mechanisms involved in Bcl-2 cytoprotection with in vivo oxidative stress model; I/R operation of rat liver, we introduced the exogenous h*bcl-2* into rat livers by hemagglutinating virus of Japan (HVJ)-liposome (artificial viral envelope: AVE type)-mediated method, and subsequently examined whether I/R-induced hepatic injuries such as blood release of hepatic marker enzymes, morphological degeneration, and cellular DNA strand cleavages would be suppressed, and how the lipid peroxidation extent and the ascorbic acid (Asc) level that could be regarded as oxidative stress-related indexes would change following HVJ-liposome-mediated gene transfer.

MATERIALS AND METHODS

Vector for the Gene Transfection

Plasmid DNA. As the plasmid constructs, pCΔj-SV2 (12.5 kbp) which is a mammalian expression vector, and pCΔj-*bcl-2* (13.5 kbp) [Tsujimoto, 1989] were used. The pCΔj-SV2 contains SV40 early promoter, EBV *ori P*, SV40 enhancer, poly (A) signal, neomycin, and ampicillin-resistant genes. Human *bcl-2* cDNA (1.0 kbp) was inserted into the *Eco*RI sites of SV40 early promoter in the pCΔj-SV2. The pCΔj-SV2 and the pCΔj-*bcl-2* were amplified in *Escherichia coli* DH5a. The plasmids were kindly provided by Dr. Shoji Yamaoka of Tokyo Med. Dent. Univ. and Dr. Yoshihide Tsujimoto of Osaka Univ. Schl. Med.

Preparation of HVJ-liposome. The HVJ-liposome (AVE type) was prepared as described [Saeki et al., 1997]. Briefly, plasmid DNA of 200 μg, HMG-1, 2 Mixture (Wako Pure Chemicals Industries, Osaka, Japan) of 65 μg or BSS and 15 mM of a dried AVE lipid mixture (cholesterol), L-α-phosphatidylcholine (egg yolk), L-α-dioleoylphosphatidylethanolamine, sphingomyeline (chicken egg yolk) (Sigma-Aldrich, St. Louis, Missouri), phosphatidylserine sodium salt (Funakoshi, Tokyo, Japan) were mixed at a molar ratio of 50:13.3:13.3:13.3:10, respectively,

were shaken vigorously, and then sonicated to unilamellar liposomes. Purified HVJ (Z strain, 20,000 hemagglutinating units/ml) was inactivated with a UV irradiation device (ABEX; HN-600, 198 mJ/cm^2) and then added to the liposome. To form fusigenic HVJ-liposome, the mixture were incubated at 37°C by shaking (120 rpm/min) for 1 h, and then was kept on ice for 10 min. After 30 and 60% sucrose density gradient ultracentrifugation, the HVJ-liposomes were visualized in a layer between BSS and 30% sucrose solution, whereas free HVJ sediments were at 60% sucrose solution. The HVJ-liposomes were harvested, kept at 4°C, and used as a vector for gene transfection within 24 h.

Transfection With h*bcl-2* into Rat Liver Epithelial Cells RL-34, and Expression of hBcl-2

Cell culture. Rat liver epithelial cell line RL-34 was kindly provided by Dr. Tohru Okigaki of Shigei Med. Research Center. Cells was 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, 1.2 g/L $NaHCO_3$, 0.5 mM L-proline, 50 μg/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. As a cultivation cell matrix, type I collagen (Nitta gelatin, Osaka, Japan) was diluted with 0.05 M Tris-HCl buffer containing 0.5 M NaCl (pH 7.5), and was placed on the surface of a culture dish. Cells were subcultivated by treatment with 0.05% trypsin plus 0.02% EDTA-4Na in PBS(−), and the cell suspension obtained was enumerated with an electric particle counter ZBII (Beckman Coulter, Tokyo, Japan), and seeded at a density of 1.0×10^5 cells per 100-mm dish.

Transfection With h*bcl-2* into RL-34 Cells

RL-34 cells of 5×10^4 cells were seeded on the cover glass (18 × 18 mm) within a 35-mm dish to conform expression of hBcl-2 proteins with immunocytochemical assay. HVJ-liposome solution containing 1 mM $CaCl_2$, plasmids of 200 μg and HMG-1,2 mixture of 65 μg was poured into 2 ml of complete culture medium. Cells were subjected to h*bcl-2* transfection for 24 h, and the transfectants were incubated in the complete culture medium for 48 h.

Expression of hBcl-2 Proteins in the h*bcl-2* Gene Transfectants RL-34-bcl2 (Immunocytochemical Assay)

After h*bcl-2* transfection, cells were washed, and fixed 4.5% paraformaldehyde in PBS(−) for 15 min, and subsequently washed three times with PBS(−). Cells were then treated with 0.5% Triton X-100 in PBS(−) for 20 min, and were thereafter treated with anti-human Bcl-2 mouse monoclonal antibody (Santa Cruz Biotechnology Inc., CA) at a final concentration of 0.5 μg/ml in 3% bovine serum albumin (BSA, SIGMA)–PBS(−) at 37°C for 1 h in humidified atmosphere. Cells were then washed twice with 0.05% Triton X-100 in PBS(−), and subsequently were incubated with the secondary antibody, FITC-conjugated anti-mouse IgG goat antibody (Santa Cruz Biotech.) at a final concentration of 0.1 μg/ml in 3% BSA–PBS(−) at 37°C for 40 min. The preparations were thereafter washed three times with PBS(−) and mounted in mounting medium (Dakopatts, Japan). The coverglasses were examined on a laser scanning confocal fluorescence microscope MRS-600 Cosmos (Bio-Rad, Hercules, CA) equipped with argon laser as the light source, and then were analyzed with Photoshop4.0J.

The h*bcl-2* Transfection Into Rat Livers and the In Situ Detection of hBcl-2 Proteins

Animals. Male Wistar rats weighing 250–300 g (8-weeks old) were purchased from Japan SLC, Shizuoka, Japan, were housed at 22 ± 2°C under artificial light for 12 h light/dark cycle and 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.

Transfection With h*bcl-2* Into the Rat Livers

Rats were anesthetized with an intraperitoneal injection of sodium pentbarbital (20 mg/kg body mass), and exposed the ileocolic vein by ventral midline incisions. HVJ-liposome mixture carrying pcΔj-*bcl-2* (containing plasmids: 200 μg) was injected slowly into the liver via the ileocolic vein using 30G needle. The liver was analyzed after transfection in 2 days.

Expression of the hBcl-2 in the Rat Livers (Immunohistochemical Assay)

Rat livers that were transfected with h*bcl-2* were fixed with 10% formalin, and subsequently

embedded in paraffin. To make a tissue section, paraffin-embedded livers were cut in 5 μm with Microtome (Yamato Kohki Industrial Co., Japan). After de-paraffinization, tissue sections were washed once with distilled water for 5 min, was incubated with 3% BSA in PBS(−) for the blocking, and were incubated for reaction at 37°C for 1 h in a humidified atmosphere. The tissue sections were washed three times with PBS(−), and subsequently were incubated with anti-human Bcl-2 mouse monoclonal antibody (Santa Cruz Biotech.) in 3% BSA–PBS(−) at a final concentration of 0.5 μg/ml at 37°C for 1 h. The sections were then washed three times with PBS(−), and subsequently were incubated with FITC-conjugated anti-mouse IgG goat antibody (Santa Cruz Biotech.) in 3% BSA–PBS(−) at a final concentration of 0.1 μg/ml at 37°C for 40 min. The preparations were thereafter washed three times with PBS(−) and mounted in a mounting medium (Dakopatts). After being dried in the dark and kept in 4°C for overnight, the coverglasses were examined on a Ratio imaging system AquaCosmos fluorescence microscope (Hamamatsu Photonics Inc., Shizuoka, Japan).

In Vivo Oxidative Stress Model; I/R Operation of Rat Liver

Rats were anesthetized by intraperitoneal injection of sodium pentobarbital. An abdominal midline of the rat was incised, and ischemic treatment was conducted by clamping both the portal vein and the hepatic artery resulting in interruption of blood supply to the left lateral and median lobes of the rat liver. Ischemic treatment was carried out for 30 min. Livers were reperfused by declamping, and the rat was allowed to awaken. Sham-operated rats were regarded as the control experimental group. The approximately 70% region of the whole liver was made ischemic by clamping, but stasis of portal blood stream was avoided.

Evaluation of the Cell Death Along With the Hapatic I/R Injuries

Activities of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in the rat serum after the hapatic I/R operation

Serum sampling. Rats were anesthetized as same above mentioned. Blood (0.5 ml) was rapidly obtained by puncturing the femoral vein before ischemia and during the period of I/R. The blood was transferred to chilled tubes containing heparin, and immediately centrifuged at 4°C. Serum samples were stored at −30°C until the measurement of aminotransferases.

Measurement of AST and ALT Activities in the Rat Serum

As an index of the liver cell death in hepatic I/R injuries, rat serum AST, and ALT activities were measured by using a Transaminase CII test kit (Wako Pure Chemicals Industries) according to the manufacture's protocol. In the case of AST, the rat serum was diluted 10 times with saline, and was transferred into a test tube. The AST standard solution was diluted, and transferred into a test tube. Blank and each dilution serum samples were added to AST standard enzyme solution, then mixed vigorously, and were incubated at 37°C for 5 min. Samples were added to Color reagents solution, and then mixed. The AST standard solution for standard curve preparation was added to AST standard enzyme solution. All the samples were incubated at 37°C for 20 min, were then added to Reaction terminate solution, and were measured for absorbance at 555 nm with a Jasco spectrophotometer. The measurement of ALT activities was also carried out in the same manner as that of AST activities.

Histological Analysis of HE Staining

The tissues were fixed with 10% formalin, and embedded in paraffin, deparaffinized, and soaked in distilled water for 5 min. The tissue sections were further soaked in Maiyer's Haematoxiline solutions for 7 min. Then the sections were soaked in water at 50°C, and were soaked in Eosin solutions for 7 min. After it was slightly soaked in distilled water, excessive Eosin solutions were removed. Thereafter, it was soaked in ethanol and xylene in each leaf for dehydration, and subsequently mounted in a rapid mounting medium Entellan® New (Merck, Germany). After it was dried up and kept at room temperature overnight, the coverglasses were examined on a Nicon light microscope.

In Situ Labeling of Nuclear DNA Fragmentation (TUNEL Assay)

The cell death along 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, Kyoto, Japan) according to the manufacture's protocol. The tissue sections were embedded in paraffin, deparaffinized, and were soaked in PBS(−) for 15 min. The sections were permeabilized with Proteinase K (final concentration: 20 μg/ml) in 0.1% Tween-20 in PBS(−), were left for 15 min at room temperatures, and washed three times with PBS(−). To label the terminals of DNA fragments, the sections were incubated with TUNEL reaction mixture containing TdT and fluorescein-dUTP in the binding buffer for 90 min at 37°C in a humidified atmosphere. To examine on a Nicon light microscope, the sections were washed three times with PBS(−), and were incubated with anti-FITC HRP conjugates for 40 min at 37°C in a humidified atmosphere. Then, the sections were washed three times with PBS(−) and were incubated for reaction with DAB reagents under light-sheltering at the room temperature for 15 min. To perform nuclear counterstain, the sections were soaked in the distilled water, were applied to methyl green solution (Vector Lab., Burlingame, CA) at the room temperature, and were incubated at 60°C on a metal plate in an oven for 5 min. After incubation, the sections were washed with deionized water until rinsing water became lucid. Then, the sections were washed with deionized water for 1 min, tapped to eliminate excess water, and were dipped 5–10 times in acetone containing 0.05%(v/v) acetic acid. They were thereafter, immediately dehydrated, clarified, and mounted on the medium Entellan® New. After it was dried up and kept at the room temperature for overnight, the coverglasses were examined on a Nicon light microscope.

Evaluation of Oxidative Stress Along With the Hapatic I/R Injuries

Preparations of liver tissue and serum samples. Rats were anesthetized by as same above mentioned, and immediately perfused with saline in a non-recirculating mode via the portal vein, and the livers were cut with razor. Isolated liver preparations (wet weight: 2 g) were soaked with 5 mM butylated hydroxytoluene (BHT) in 20 mM Tris-HCl buffer (pH 7.0), and were sonicated with a homogenizer Physcotron (Microtec Nition, Chiba, Japan) for 40 s. After homogenization, preparations were centrifuged at 3,000*g* and 4°C for 10 min, and the resultant supernatants were stocked at −80°C until measurements for lipid peroxidization. On the other hand, to determine Asc in the I/R-operated livers and the serum, the samples was isolated and collected. In case of liver tissue samples preparation, the I/R-operated livers were isolated as same, and separated into ischemia lobes (median lobe and left lobe) and non-ischemia lobes (right lobe). After addition of 10% metaphosphoric acid, each 1 g (wet weight) of sample was sonicated, then they were centrifuged at 1,300*g* and 4°C for 5 min, and the supernatant was collected. In case of serum preparation, the blood (0.5 ml) was rapidly obtained by puncturing the femoral vein under pentobarbital anesthesia. The blood was transferred to chilled tubes containing heparin, and immediately centrifuged at 4°C. These samples were stored at −80°C until determination for Asc concentration.

Determination of lipid peroxidation by *N*-methyl-2-phenylindole method. Lipid peroxidation in the rat liver along with I/R was analyzed by *N*-methyl-2-phenylindole method using the Bioxytech LPO-586 kit (Oxis International, Inc., Portland, Oregon) according to the manufacture's protocol. Out of diverse products resulting from lipid peroxidation, 4-hydroxynonenal (4-HNE), and malonedialdehyde (MDA) were selected as the target compounds for measurement. The supernatants that were stocked, 4-HNE and MDA standard solutions (each 2.5–20 μM) were added to 0.5 M BHT in acetonitrile in order to inhibit auto-oxidation, and were added to R1 reagent (10.3 mM *N*-metyhl-2-phenylindole in acetonitrile:100% methanol = 3:1). They were mixed with a vortex-mixer, and were added to R2 Reagent (15.4 M methanesulfonic acid). Then, samples were incubated at 45°C for 45 min. After incubation, samples were placed on ice, and were centrifuged at 15,000*g* for 10 min. The supernatants were measured for both spectrum and absorbance at 586 nm with a Jasco spectrophotometer.

Determination of Asc in the I/R-operated livers and the serum. The samples and Asc standard solution were added to 5% metaphosphoric acid containing 20 mM deferoxamine mesylate, then centrifuged at 3,000*g* and 4°C for 10 min, and the supernatant was added to distilled water and then to 50% metaphosphoric acid, and centrifuged at 3,000*g* and 4°C for 10 min. Samples were filtrated with a 0.2-μm-pore YMC Duo-filter (YMC, Kyoto), and

an aliquot of 20 μl was injected on an Eicompak SC-5DS column of 3.0 × 150 mm (Eicom, Kyoto) connected into a Gilson HPLC system 302, followed by development with the mobile phase of 99% 0.1 M KH_2PO_4–H_3PO_4–1% methanol buffer (pH 2.5) containing 50 mg/L octanesulfonic acid and 5 mg/L EDTA-2Na at a flow rate of 1.5 ml/min at 25°C. Asc was detected with a coulometric electrochemical detector (ECD) Coulochem 2 (ESA Co., Bedford, MA) equipped with a dual analytical cell Model 5010 operated at 600 mV. For the measurement of total Asc, the same sample as for Asc measurement received dithiothreitol (final 3.6 mM), resulting in reduction of dehydroascorbic acid (DehAsc) to Asc. By subtracting the Asc amount from the total amount, DehAsc was determined. The standard Asc (Sigma) solutions were prepared at graded concentrations of 0–10 μM in the HPLC buffer and freshly diluted just before use. Each result was represented as the mean of duplicate injections on HPLC. Asc was eluted at a retention time of 3.0 min under chromatographic conditions employed.

Statistical Analysis

In measurement of AST and ALT activities and determination of lipid peroxidation, Bonferroni/Dunn procedure (Dunn's procedure as a multiple comparison procedure), and Fisher's PLSD procedure (Fisher's protected least significant difference) were used to evaluate the significance of differences between groups and the criterion of statistical significance was taken as $P < 0.05$. Unpaired *t*-test was used in determination of Asc, and the criterion of statistical significance was taken as $P < 0.05$.

RESULTS

Time-Dependency of Expression of hBcl-2 Proteins in Rat Liver Epithelial Cells RL-34 That Were Transfected With h*bcl-2* Gene by HVJ-Liposome Method

Rat hepatocytes RL-34 were transfected with the plasmid vector pCΔj-*bcl-2* encoding h*bcl-2* gene by the HVJ-liposome method. The resultant cells underwent the immunocytochemical stain, which showed that hBcl-2 proteins were exhibited in the cellular organera mitochondria at 12–48 h after the transfection (Fig. 1Ab–d) in comparison with the non-transfected cells (Fig. 1Aa). We decided therefore that a period of in vivo transfection with the h*bcl-2* gene in the next experiment is attempted to be at 48 h before outset of the ischemia treatment so as to be fully cytoprotective against post-I/R-induced hepatic injuries (Fig. 1B).

Expression of hBcl-2 Proteins in Rat Livers That Were Transfected With h*bcl-2* Gene by HVJ-Liposome Method

Human *bcl-2* gene was transferred with the use of HVJ-liposome into rat liver at 48 h before outset of the ischemia treatment that was followed by reperfusion for 4 h. Each lobe of the transfected liver received immunohistochemical stain, which showed that hBcl-2 proteins was exhibited around the portal vein of the transfected liver, especially in the median and left lobes (Fig. 1C ML, LL), but scarcely exhibited in right and caudal lobes (Fig. 1C RL, CL). We adopted the route of injection of the h*bcl-2* containing HVJ-liposome via the peripheral portal vein (the ileocolic vein) (Fig. 1B). The target regions of gene transfection into rat liver were therefore suggested to be selectively restricted around the portal vein of the hepatic median and left lobes.

Repressive Effects of hBcl-2 on I/R-Induced Cell Death in Rat Liver as Quantified by Serum AST and ALT Activities

As an index of the I/R-induced hepatic injuries, activities of hepatic enzymes AST and ALT in serum were measured (Fig. 2A). In the "Sham-bcl2" rats that were transfected with h*bcl-2* genes and did not subjected to I/R but only to incision of the abdomen, AST and ALT activities were maintained within normal ranges (AST activities at 0, 3, 6, and 12 h after reperfusion, 157, 138, 143, and 154 IU/L; ALT activities at 0, 3, 6, and 12 h after reperfusion, 28.5, 26.5, 26.2, and 25.1 IU/L, respectively). The AST activities in the serum of the "non-transfected" rats receiving the I/R treatment were raised gradually until reperfusion for 6 h (828 ± 177 IU/L), and were raised appreciably thereafter (after reperfusion for 12, 24 h (not shown): $1{,}200 \pm 118$, 523 ± 9 IU/L, respectively). The similarly increased AST activities were also observed in the "SV2" rats that were transfected with the reference vector pCΔj-SV2 containing no h*bcl-2* gene. In contrast, in rats transfected with h*bcl-2* genes at 48 h before outset of the ischemia treatment, the AST activities were repressed to levels as low as approximately 57,

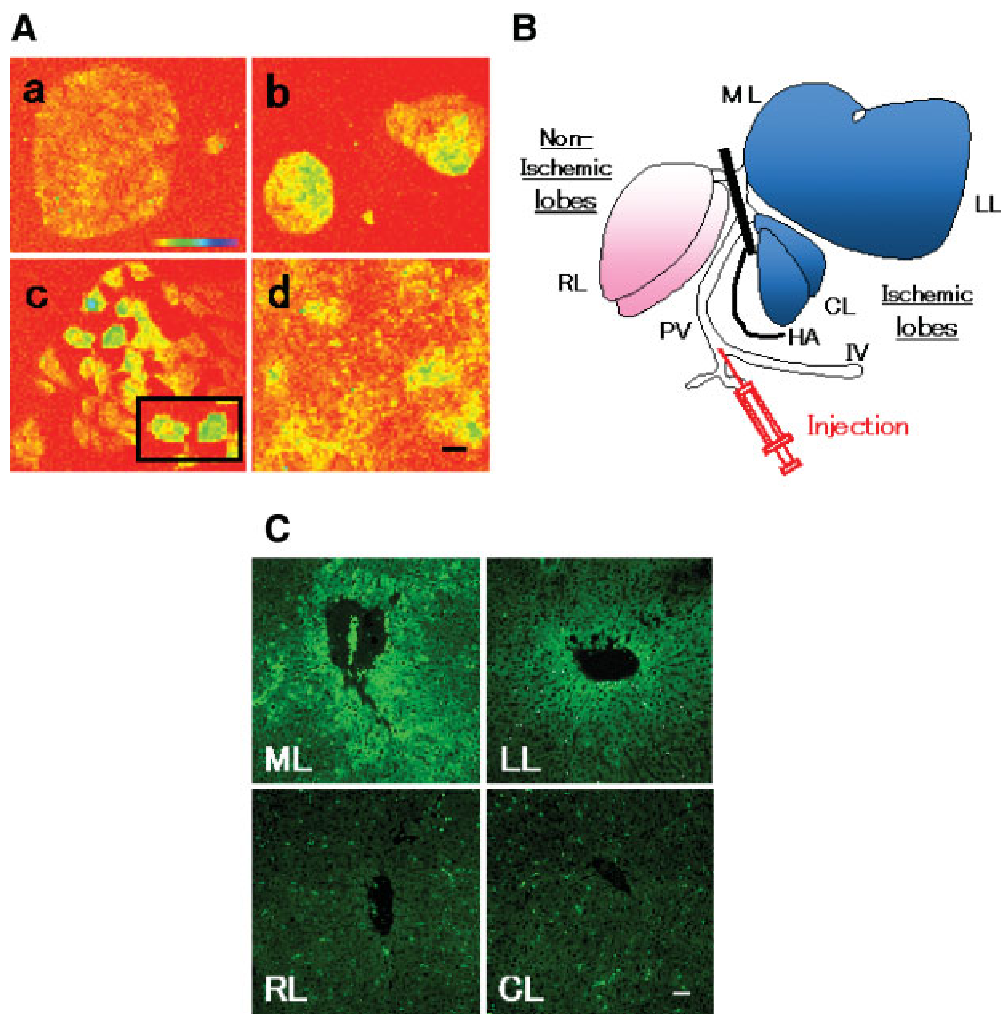


Fig. 1. Gene expression and gene transfection method. **A**: Expression of hBcl-2 proteins in rat liver epithelial cells RL-34 that were subjected to transfection with h*bcl-2* gene by HVJ-liposome method. **a**: before transfection, **b**–**d**: at 12, 24, 48 h after transfection, c: a small photograph shows the one which magnified the transfectants RL-34-bcl2. RL-34 cells received h*bcl-2* transfection for 24 h, and were incubated in the complete culture medium for 12–48 h. Meanwhile, hBcl-2 proteins expressed in the transfectants RL-34-bcl2 were detected by immunocytochemical stain with anti-hBcl-2 antibody and FITC-conjugated anti-IgG, and expressed in pseudo-color from red (scarcely stained) via yellow (weakly stained) to green (mediumly stained) by processing of fluorescence intensity with an NIH-Image software. The hBcl-2 proteins were confirmed to be abundantly localized in the cellular organelles such as mitochondria from 12 to 48 h after the transfection. The data shown are typical of three independent experiments. Magnification was 100-fold. The scale indicated 50 µm. **B**: Schema of an in vivo post-ischemic reperfusion (I/R) model in rat livers that receive the in vivo gene transfection. Ischemic treatment was conducted by clamping both the portal vein and the hepatic artery for 30 min. The bold line indicates a clamping locus. Livers were then reperfused by declamping both the blood vessels, and the rat was thereafter allowed to awaken. The approximately 70% region of the whole liver was made ischemic by clamping, but stasis of portal blood stream was avoided. HVJ-liposome mixture carrying pCΔj-*bcl-2* (containing plasmids of 200 µg) was injected slowly into the liver via the ileocolic vein using 30G needle. RL, right lobe; ML, median lobe; LL, left lobe; CL, caudal lobe; HA, hepatic artery; PV, portal vein; IV, ileocolic vein. **C**: Expression of hBcl-2 proteins in each lobe of the rat liver at 4 h after reperfusion. Rat liver received h*bcl-2* transfection, and, after 48 h, was subjected to hepatic ischemia for 30 min and the subsequent reperfusion for 4 h. The hBcl-2 proteins expressed in the transfected livers were detected by immunohistochemical stain with anti-hBcl-2 antibody and FITC-conjugated anti-IgG with a Ratio Imaging System AquaCosmos fluorescence microscope at a 100-fold magnification. The scale indicates 50 µm. The hBcl-2 proteins were detected around the portal vein of the rat liver, especially at the median and left lobes. RL, right lobe; ML, median lobe; LL, left lobe; CL, caudal lobe.

63, and 50% versus those of "non-transfected" rats following reperfusion for 3, 6, and 12 h, respectively, and were thereafter repressed to the lower levels (after reperfusion for 12, 24 h (not shown): 617 ± 200, 355 ± 5 IU/L). On the other hand, the ALT activities reached a peak in the "non-transfected" rats following reperfusion for 6 h (254 ± 9 IU/L), and were thereafter

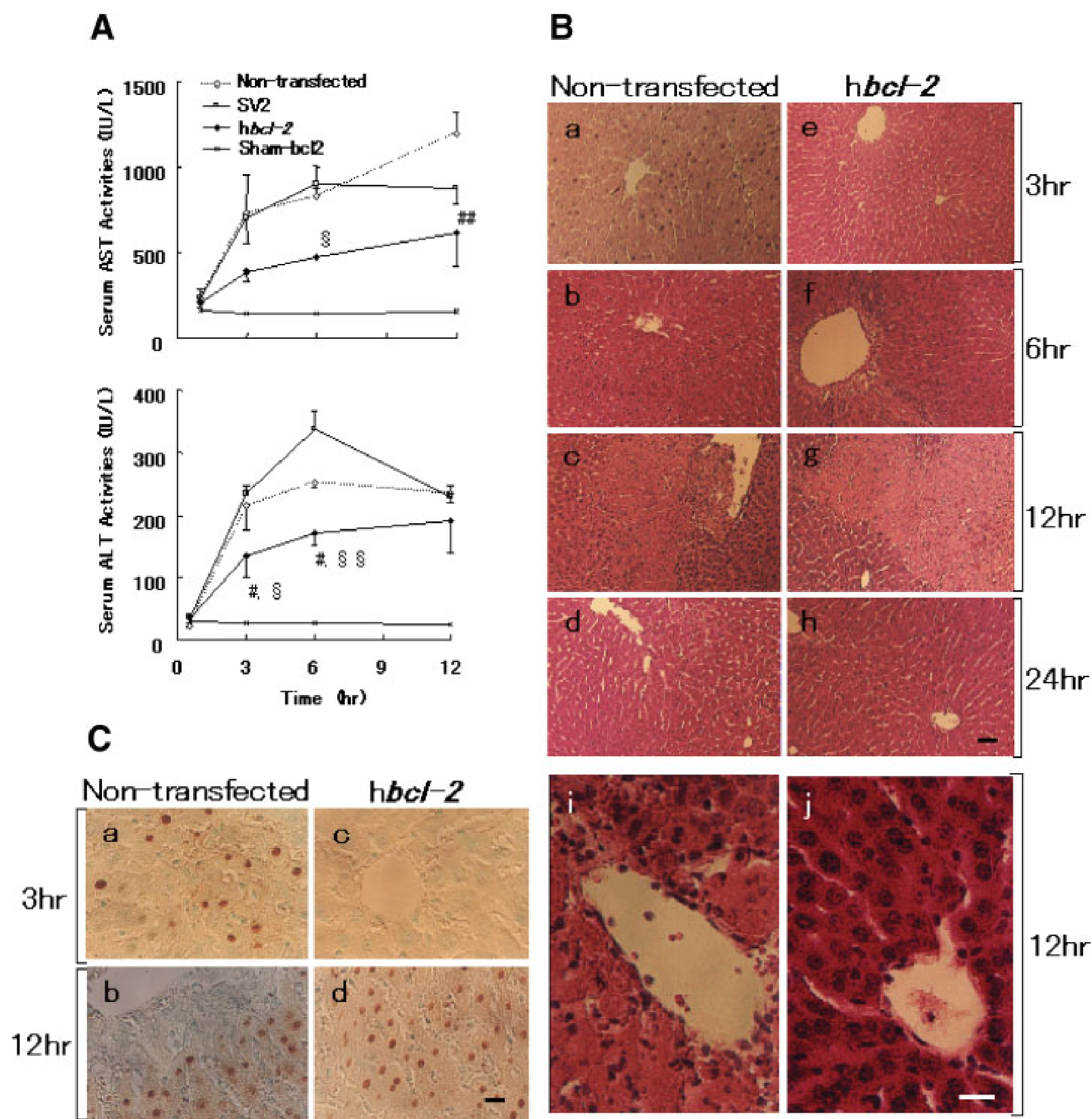


Fig. 2. Cell and DNA injuries. **A**: Effects of hBcl-2 on AST and ALT activities in the rat serum after I/R operation. As an index of I/R-induced hepatic injuries, rat serum AST and ALT activities were measured. The data shown are typical of three independent experiments, each of which contained several examined groups in triplicate. The bar represents SD of each group in triplicate ($^{\#}P<0.05$, versus non-transfected; $^{\#\#}P<0.001$, versus non-transfected; $^{\S}P<0.05$, versus SV2; $^{\S\S}P<0.001$, versus SV2). **B**: Haematoxylin–eosin (HE) staining of paraffin-embedded non-transfected and h*bcl-2* transfected liver sections after I/R. **a**–**h**: The sections of I/R-receiving liver tissues were prepared at 3, 6, 12, and 24 h after reperfusion, and were visualized for the corresponding region (the median lobe) of h*bcl-2* transfected rats and non-transfected rats by HE staining. The cover glasses were examined under a light microscope at a 100-fold magnification. The scale indicates 50 μm. **i**, **j**: Expanded views at 12 h after reperfusion. Magnification is 400-fold. The scale indicates 10 μm. The data shown are typical of three independent experiments. **C**: Cellular DNA cleavages of paraffin-embedded tissue sections in non-transfected and h*bcl-2* transfected livers after I/R as assayed by TUNEL method. **a**–**d**: The tissue sections of the median lobe in the I/R-receiving rat livers were prepared at 3 and 12 h after reperfusion, and were evaluated for the median lobe of the h*bcl-2* transfected livers and non-transfected livers by TUNEL assays. The cover glasses were examined under a light microscope at a 400-fold magnification. The scale indicates 10 μm. The data shown are typical of three independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

maintained in either the "non-transfected" or "SV2" rats. In the h*bcl-2* transfected rats, the ALT activities were repressed to 50, 72, and 75% versus those of "non-transfected" rats following reperfusion for 3, 6, and 12 h, respectively (after reperfusion for 12, 24 h (not shown): 190 ± 52, 31.4 ± 3.7 IU/L). Thus the I/R-induced releases of two hepatic enzymes were shown to be appreciably repressed in rats with the h*bcl-2* transfected livers.

Inhibitory Effects of h*bcl-2* Transfection on Morphological Degeneration of I/R-Operated Liver Cells

The tissue sections of I/R-operated livers were made at 3, 6, 12, and 24 h after reperfusion, and were compared with the h*bcl-2* transfected and the non-transfected groups as evaluated by HE stain (Fig. 2B). In the non-transfected livers, degenerative aspects such as nuclear condensation and vacuolation around the portal vein at 3 h after reperfusion were observed concurrently with a marked increase in apoptosis-like dead cells, and, after reperfusion for 6 h, nuclei flowed out from the hepatocytes together with emergence of necrosis-like dead cells (Fig. 2Ba). In contrast, a significant scanty of degenerated cells were observed in h*bcl-2* transfected livers (Fig. 2Be). At 12 h after reperfusion, the area with necrosis-like dead cells was repressed to 40% versus the total area in the h*bcl-2* trasfected liver sections (Fig. 2Bg,j) in contrast to 70% in the non-transfected liver sections (Fig. 2Bc,i). Thus, transfection with h*bcl-2* was suggested to inhibit both I/R-induced apoptosis- and necrosis-like cell mortalities.

Preventive Effects of hBcl-2 Proteins on I/R-Induced DNA Strand Cleavages

In the same manner as the results with HE stain (Fig. 2B), apoptotic TUNEL-positive cells were observed markedly in the non-transfected livers concomitantly with the nuclear condensation in the vicinity of the portal vein following reperfusion for 3 or 12 h (Fig. 2Ca,b), whereas a significant decrease in apoptotic cells was observed in h*bcl-2* transfected livers following reperfusion for 3 h (Fig. 2Cc). The repressive effects on I/R-induced DNA cleavages were not confirmed following reperfusion for 12 h around the portal vein in the h*bcl-2* transfected livers (Fig. 2Cd), which were rather prevented from drastic cell degeneration that may occur for a period as short as cannot afford to be reversibly repaired or compensated by proliferation of the neighboring cells.

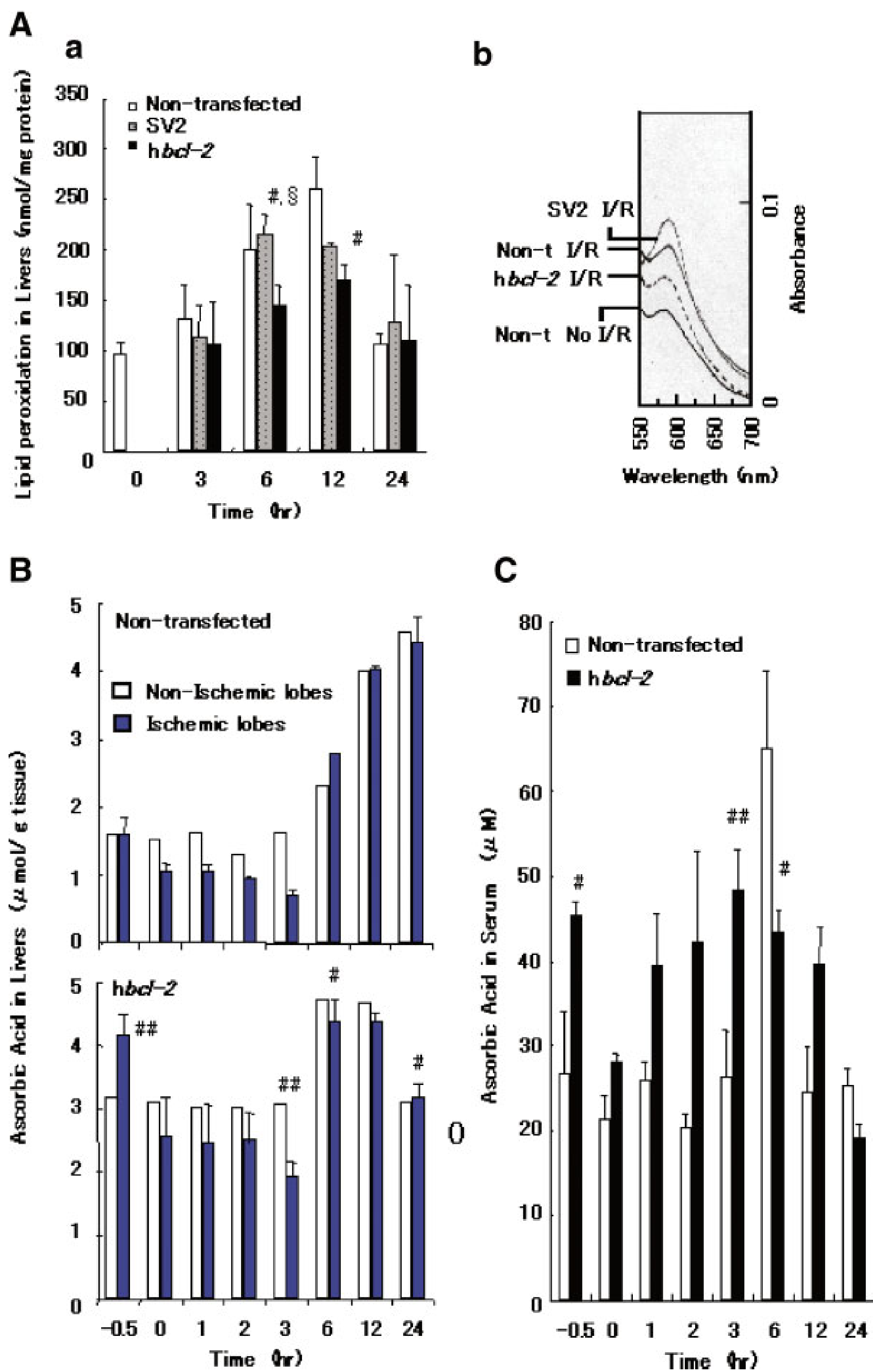
Increases in Intracellular ROS Along With the Hepatic I/R Operation

The lipid peroxidation in the livers of I/R-operated rats was raised after reperfusion and reached the maximum level at 12 h after reperfusion for non-transfected livers and h*bcl-2*-transfected livers, or at 6 h for the reference vector-transfected livers ("SV2") (Fig. 3Aa,b). The lipid peroxidation at the maximum time for h*bcl-2*-transfected livers was a level 1.6-fold higher than just after ischemia in contrast to a level 2.6- or 2.2-fold higher for non-transfected livers or the "SV2" livers, respectively (Fig. 3Aa). Thus, the production of intracellular ROS in the hepatic I/R injuries was suggested to be repressed by hBcl-2.

Decreases in Intracellular Ascorbic Acid (Asc) Concentration in the Rat Liver Along With the Hepatic I/R

In the evaluation of the oxidative stress along with the hepatic I/R injuries, it was technically difficult to evaluate ROS that may occur in the liver, especially at stages as early as 3 h after reperfusion, by *N*-methyl-2-phenylindolamine method that determines the lipid peroxidation in the I/R-induced livers. The methodological difficulty may ensue also from a putative phenomenon that ROS is produced along with the I/R and simultaneously is erased rapidly by the endogenous anti-oxidant substances. We attempted therefore to quantify the hepatic contents of Asc that can scavenge ROS most rapidly out of diverse anti-oxidant substances that exist naturally in the mammals, by HPLC technique, and thereafter we indirectly evaluated the oxidative stress that may oxidize Asc and resultantly decompose it in the liver. The livers were divided into the non-ischemic lobes (the right lobes: as the control) and ischemic lobes (the median and the left lobes), and Asc contents in both the lobe fractions were quantified for the h*bcl-2*-transfected livers and the non-transfected livers. The Asc contents in h*bcl-2*-transfected livers were markedly higher, unexpectedly, than those in non-transfected livers, especially in the ischemic lobes that were transfected abundantly with h*bcl-2* genes, before I/R operation (Fig. 3B). After I/R operation, the Asc concentrations in ischemic liver lobes were gradually decreased in the early

stage of reperfusion in the h*bcl-2* transfected group and the non-transfected group, in contrast to no appreciable decrease in Asc in non-ischemic lobes (Fig. 3B). The results suggested that ROS along with the I/R were produced immediately after post-ischemic reperfusion in the ischemic lobes but scarcely the non-ischemic lobes. At 6-h reperfusion when I/R-induced ROS generation may be persistently continued yet, Asc concentrations in both the lobes of



the h*bcl-2*-transfected livers were increased more markedly than in non-transfected livers (Fig. 3B). Thus the h*bcl-2* transfection may achieve both the enhancement of Asc contents in the usual or pre-ischemic state, and the prompt Asc-recruiting in response to I/R-induced ROS generation.

Enhancement of Asc Concentrations in the Serum by the h*bcl-2* Transfection

Asc concentrations in the rat serum were also determined in order to examine a possibility that Asc which circulates all over the body may be supplied from several Asc-synthesizing/accumulating organs, and may be delivered to Asc-deficient/demanding organs. The Asc concentration in the serum of h*bcl-2*-transfected rat was enhanced approximately twice as high as that in the non-transfected rat serum before the I/R operation (Fig. 3C). The abundant Asc in h*bcl-2*-transfected rat serum was drastically declined immediately after post-ischemic reperfusion. At 1–3 h reperfusion, the serum Asc levels of the h*bcl-2*-transfected rats were consistently higher than those of non-transfected rats (Fig. 3C). In the same manner as the results with Asc concentrations in the h*bcl-2* transfected livers (Fig. 3B), the sera of h*bcl-2* transfected rats were shown to contain Asc of concentrations about twice as high as those of non-transfected rat sera either upon the pre-ischemic period or during most of I/R period. The increased or retained Asc in the serum was therefore suggested to ensue from abundance or consumption-sparing of hepatic Asc that caused by h*bcl-2* transfection.

DISCUSSION

In the present study, we demonstrated that reactive oxygen species (ROS)-associated indexes such as lipid peroxidation extent and the Asc level were closely correlated with hepatic cell death in I/R injuries and its prevention. In brief, ROS-induced lipid peroxides were produced in the I/R-operated livers concurrently with the promoted decline in ROS scavengers, resulting in an increased oxidative stress in the liver. The hepatocytes subjected to accumulated oxidative stress finally reached cell death such as apoptosis and necrosis. Thus, it seems that oxidative stress may be substantially responsible for cell death. We demonstrated, however, that any I/R-induced hepatic injuries such as release of hepatic marker enzymes, cell morphological degeneration, and cellular DNA strand cleavages (chiefly, apoptosis at 3 h after reperfusion) can be suppressed by h*bcl-2* gene transfer with HVJ-liposome methods.

Involvement of ROS in I/R-induced cell injuries has been shown by some previous studies [McCord, 1985; Ferdinand et al., 2001], and preventive effects against I/R injuries have been demonstrated to be exerted by antioxidants such as Asc and 2-O-alpha-D-glucosylated Asc (Asc2G) [Eguchi et al., 2003]. One of causes for I/R injuries can be thought to be the promoted decline in ROS scavengers such as Asc, glutathione (GSH), and manganese superoxide dismutase (Mn-SOD). The mechanism of hepatic I/R injuries can be explained according to the knowledge of the past researches as follows: Firstly, in the period of ischemia, intracellular ATP is rapidly diminished by the lowering of

Fig. 3. ROS and Asc as an ROS scavenger. **A**: Effects of hBcl-2 on lipid peroxidation in rat livers after I/R. **a**: Lipid peroxidation products such as 4-hydroxynonenal (4-HNE) and MDA in the I/R-treated rat liver were analyzed by *N*-methyl-2-phenylindolamine method using methanesulfonic acid as the solvent. The data shown are typical of three independent experiments, each of which contained several examined groups in triplicate. The bar represents SD of each group in triplicate ($^{\#}P < 0.01$, versus non-transfected; $^{\S}P < 0.01$, versus SV2). **b**: The absorption spectra in the visible region around 586 nm of lipid peroxides produced by 6 h reperfusion. SV2 I/R, SV2 transfected livers after I/R; non-t I/R, non-transfected livers after I/R; h*bcl-2* I/R, h*bcl-2* transfected livers after I/R; non-t No I/R, non-transfected livers without operation of I/R. **B**: Ascorbic acid (Asc) concentrations in the non-transfected and h*bcl-2* transfected rat livers after I/R. Asc concentrations in the non-transfected and h*bcl-2* transfected rat livers after I/R were determined by HPLC technique and coulometric electrochemical detection. The data shown are typical of three independent experiments, each of which contained several examined groups in triplicate. The bar represents SD of each group in triplicate ($^{\#}P < 0.001$; $^{\#\#}P < 0.01$). **C**: Asc concentrations in the serum of rats with the non-transfected or h*bcl-2* transfected livers after I/R. In a manner similar to that for hepatic Asc contents (B), Asc concentrations in the rat serum were determined by HPLC technique and coulometric electrochemical detection. The data shown are typical of three independent experiments, each of which contained several examined groups in triplicate. The bar represents SD of each group in triplicate ($^{\#}P < 0.05$, $^{\#\#}P < 0.01$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

oxygen supply, and it causes both a continuous cell injury and destruction of an ionic balance, resulting in the flow of extracellular Na^+ into the intracellular space. Then, in the period of reperfusion following ischemia, intracellular Na^+ is exchanged with Ca^{2+}, and the enzymes which induce a tissue injury are activated. It was however reported that a direct cause for cell injuries occurring during reperfusion is the activation of neutrophils and macrophages, but not the activation of the enzyme xanthine–xanthine oxidase which potently induces a tissue injury [Marubayashi et al., 1991; Marubayashi and Dohi, 1996]. It is alternatively therefore suggested that the promoted decline in ROS scavengers becomes the trigger of I/R injury rather than the activation of the injurious enzyme, and it is followed by the subsequent increase in intracellular ROS, lipid peroxidation, and finally induction of cell death.

Actually, involvements of both ROS and the promoted decline in ROS scavengers in I/R injuries have been also demonstrated in the present studies. In the non-transfected livers in our experiments, the content of Asc in the ischemic lobe (the median lobe) lowered by 42% (0.69 μmol/g tissue) versus the non-ischemic lobe (the right lobe) at 3 h after reperfusion (1.6 μmol/g tissue) (Fig. 3B). The lipid peroxidation was raised at 3 h after reperfusion (Fig. 3A), and from the viewpoint of cell morphological observation, apoptosis occurred near the portal vein (Fig. 2Ca), and moreover necrosis happened in hepatocytes at 6 h following reperfusion (Fig. 2Bb). In the SV2 transfected livers, the lipid peroxidation reached the maximum level at 6 h after reperfusion (Fig. 3A). (The content of Asc was not determined, because difference between non-transfected liver and SV2 at stages as early as 3 h after reperfusion could not be conformed by the analysis of lipid peroxidation.) In contrast, in the h*bcl-2* transfected liver, the Asc concentration in the ischemic lobe lowered by 62% (1.9 μmol/g tissue) versus the non-ischemic lobe at 3 h after reperfusion (3.06 μmol/g tissue) (Fig. 3B). The lipid peroxidation at 3 h after reperfusion was repressed (Fig. 3A), and apoptosis near the portal vein at 3 h after reperfusion was inhibited by h*bcl-2* transfection (Fig. 2Cc).

Thus, it is vital for cytoprotection to retain contents of Asc in ischemic lobes, especially at a period immediately after reperfusion when explosive generation of ROS occurs. In fact, this vital requisite was accomplished either in serum or ischemic and non-ischemic lobes of *bcl-2*-transfected rats. It is suggested that an inverse correlation between amounts of ROS and ROS scavengers was involved in cell death control in I/R-operated livers, and that, in h*bcl-2* transfected livers, the sparing of ROS scavengers is secured and results in apoptosis inhibition.

Within our knowledge the present study is the first one in which gene transfection with h*bcl-2* by HVJ-liposome method is demonstrated to be cytoprotective against "warm" hepatic ischemic injuries by clamping although h*bcl-2* transfection is known to be cytoprotective also against perfusion-induced hypoxic liver cell [Yamabe et al., 1998] and the rabbit heart [Subhasis et al., 2002]. Unexpectedly, our result (Fig. 3B, C) of elevations of Asc in the serum and liver due to transfection with h*bcl-2* gene in the rat liver can secondarily ensue from sparing of Asc necessary for scavenging of ROS which was diminished by h*bcl-2* transfection in the liver rather than from the h*bcl-2*-induced enhancement of Asc synthesis in the liver and kidney.

Since an anti-apoptotic protein, Bcl-2 was found by Tsujimoto et al. [1985], 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]. Our unexpected results of repressive effects on Asc diminishments can be explained by a function of Bcl-2 as an apparent antioxidant [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 and Sandstrom, 1994; Bonfoco et al., 1995; Lin et al., 1997]. It is controversial whether cytoprotection by *bcl-2* against ischemic injuries is attributable to its putative antioxidant ability that may be indirectly exerted through multiple stepwise cascade stages, such as enhancement of redox signal potential.

It was reported that the intracellular GSH concentration in *bcl-2* transfectant-GT1-7 neural cell line is increased approximately twofold in comparison with the parent cell line, and suppressed both cell death and depletion of GSH [Kane et al., 1993]. Moreover, our laboratory reported recently that the uptake of Asc into the *bcl-2* transfected b5 fibroblastic cell line

was more markedly efficient than the parent Rat-1 cells [Saitoh et al., 2003]. Consequently, intracelluar anti-oxidants may be indirectly related with overexpression of exogenous *bcl-2* gene. It is suggested that an intracellular anti-oxidant pool may be raised in the cell by the exogenous *bcl-2* gene transfer, and that intra-cellular redox condition is shifted toward the reductive direction in the *bcl-2* transfected hepatocytes in the present study. Further studies using either Bcl-2 knockout or Bcl-2 transgenic animals are still required to elucidate the mechanisms of the anti-oxidant functions of Bcl-2.

Finally, a number of the gene modification studies for defense against I/R injuries have been proposed [Zwacka et al., 1998; Bilbao et al., 1999; Suzuki et al., 2002]. We chose HVJ-liposome methods as the gene modification, so that safety as vector is very high because there is no anxiety that a gene is incorporated into the chromosome of the host. We propose a novel molecular medicine approach against hepatic I/R injuries, namely the h*bcl-2* gene transfer by some reliable transfection methods such as HVJ-liposome method, because it is suggested that hepatic I/R-induced apoptosis and necrosis can be defended efficiently by h*bcl-2* gene transfer together with enhancement of intra-cellular anti-oxidant potential. And, we hope that our present experimental data are used as the fundamentals for the gene technological treatment aiming at the I/R defense.

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