BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **243,** 217–223 (1998)
ARTICLE NO. RC977925

Prevention of Hypoxic Liver Cell Necrosis by *in Vivo* Human *bcl-2* Gene Transfection

Kazuo Yamabe,* Shigeomi Shimizu,*,† Wataru Kamiike,* Satoshi Waguri,‡ Yutaka Eguchi,† Jun-ichi Hasegawa,* Shin-ichiro Okuno,* Yasuhiko Yoshioka,* Toshinori Ito,* Yoshiki Sawa,* Yasuo Uchiyama,‡ Yoshihide Tsujimoto,† and Hikaru Matsuda*

**The First Department of Surgery, †Department of Medical Genetics, Biomedical Research Center, and ‡The First Department of Anatomy, Osaka University Medical School, 2-2 Yamada-oka, Suita 565, Japan*

Received November 20, 1997

Prevention of hypoxic cell death is a key to successful liver transplantation. We developed a new method for preventing liver hypoxic cell death by introducing an anti-cell death gene directly into rat livers. When the human *bcl-2* gene (*hbcl-2*) was directly transfected into rat livers together with nonhistone chromosomal protein high mobility group 1 (HMG1) by the hemagglutinating virus of Japan (Sendai virus; HVJ) -liposome method, human Bcl-2 protein (hBcl-2) was efficiently expressed. Electron microscopy and fluorescence microscopy revealed that hepatocytes expressing exogenous hBcl-2 were almost completely protected the hypoxic cell necrosis. The expression of the hBcl-2 also inhibited activation of caspase-3 (-like) proteases and liver dysfunction. Thus, we conclude that transfection of the *hbcl-2* gene through HVJ-liposome method is useful to prevent liver cell necrosis induced by hypoxia. This finding could lead to new strategies to avoid the hypoxic cell death, the major problem in liver transplantation.

***Key Words: bcl-2; in vivo* transfection; HVJ-liposome; hypoxia; necrosis.**

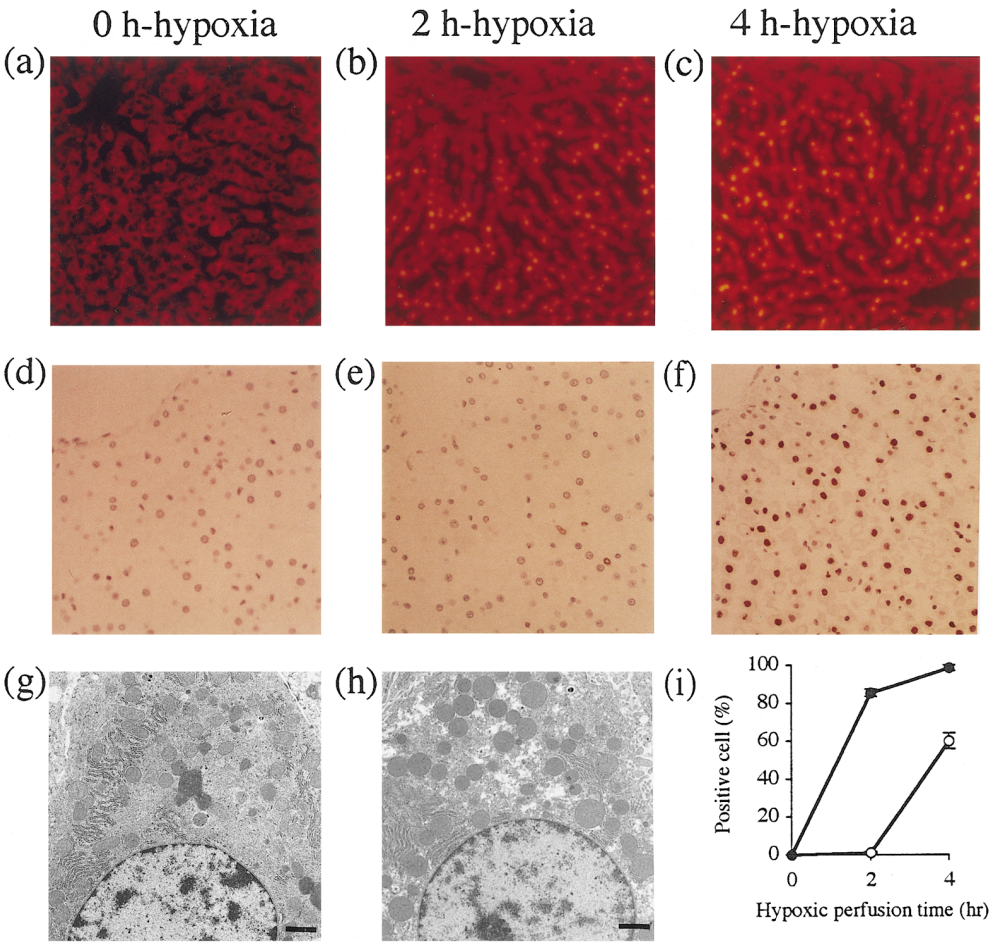
The high susceptibility of the liver to hypoxic damage is a major problem in liver transplantation leading to primary graft failure (1,2). Although hepatocytes briefly exposed to hypoxia lose their function transiently, longer exposure induces their cell death. Since the extent of hypoxic cell death determines the outcome of transplantation, its prevention is a key to success. Many attempts, such as changing the preserving solution (3) or the harvesting technique (4) and nutritional modification of the donor liver (5), have been tried to prevent hypoxic cell death, but these were not enough for complete success of transplantation. In a trial to solve this problem, we developed a new method involving transfection of an anti-cell death gene, *bcl-2*.

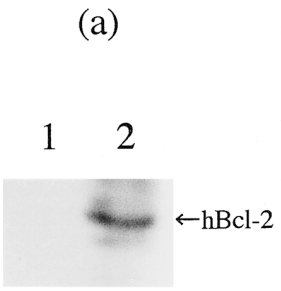
Although cell death due to hypoxia has been generally believed to occur as necrosis (6), we have showed that hypoxia can induce both apoptosis and necrosis in some cell lines, and that the proportion of these two modes of death is highly dependent on the cell type (7). Apoptosis is characterized by chromatin condensation, nuclear fragmentation, condensation and fragmentation of cytoplasm and formation of apoptotic bodies, whereas necrosis is characterized by cell membrane disruption, mitochondrial swelling, lose-condensed cytoplasm and cytosolic vacuoles without damages in nuclei. It is still uncertain which mode of cell death occurs in hypoxic liver *in vivo*.

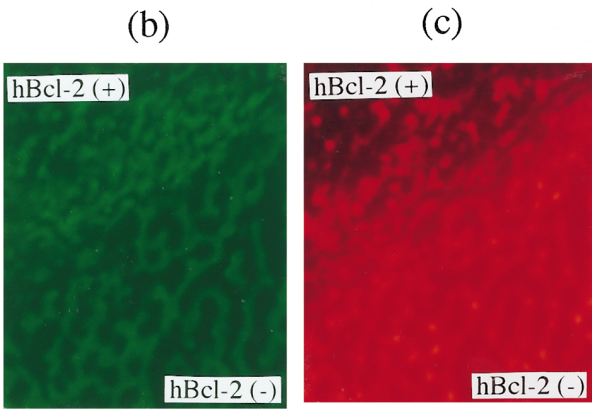
Apoptosis is well known to be prevented by Bcl-2 (8,9). We and others have recently shown that Bcl-2 is also effective to prevent some types of necrotic cell death (10, 11), including that of hepatoma cell line 7316A treated under hypoxic conditions (7, 12). These observations raise the possibility that hypoxic cell death in liver can effectively be prevented by overexpressing Bcl-2, regardless of the mode of cell death induced in hypoxic liver.

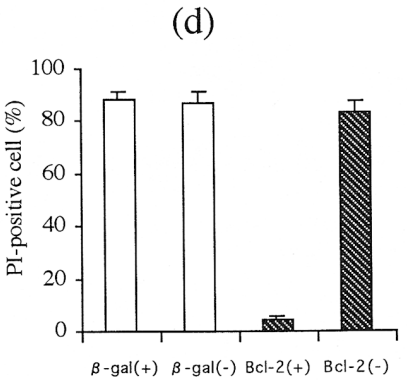
Considering the clinical application, we attempted to introduce *bcl-2* gene together with non-histone chromosomal protein high mobility group 1 (HMG1) into liver by *in vivo* gene transfection with the aid of the hemagglutinating virus of Japan (Sendai virus; HVJ)-liposome, which provides high efficiency for *in vivo* gene transfection into organs (13,14). Injection of HVJ conjugated with liposomes, in which the plasmid DNA and HMG1 were encapsulated, easily introduced the plasmid into the cytoplasm, and HMG1 assists the incorporation of exogenous DNA into the nucleus, resulting in a high level of expression of the gene products. The HVJ-liposome method is shown to deliver genes without severe cytotoxicity in several systems such as transfection into livers with hepatitis B virus (15), human insulin (14), and human renin (16) genes. Here, we studied the effect of exogenously transfected *hbcl-*

0006-291X/98 $25.00


0 h-hypoxia
2 h-hypoxia
4 h-hypoxia
(a)
(b)
(c)
(d)
(e)
(f)
(g)
(h)
(i)
Positive cell (%)
100
80
60
40
20
0
0
2
4
Hypoxic perfusion time (hr)


(a)
1
2
←hBcl-2


(b)
(c)
hBcl-2 (+)
hBcl-2 (-)
hBcl-2 (+)
hBcl-2 (-)


(d)
PI-positive cell (%)
100
80
60
40
20
0
β-gal(+)
β-gal(-)
Bcl-2(+)
Bcl-2(-)

2 gene on hypoxic liver cell death, and suggested *in vivo* transfection of *bcl-2* gene efficiently expressed its gene products and prevented not only hepatocyte necrosis but liver dysfunction induced by hypoxia.

MATERIALS AND METHODS

Preparation of HVJ-liposomes. HVJ-liposomes were prepared as reported (13). Briefly, a dried lipid mixture (phosphatidylcholine, phosphatidylserine, and cholesterol) was hydrated in balanced salt solution (140 mM NaCl, 5.4 mM KCl, 10 mM Tris-Cl, pH 7.5) containing HMG1 and either pAct-LacZ (17), β-galactosidase (β-gal) control plasmid or pUC-CAGGS-*bcl-2*, which was constructed by insertion of *hbcl-2* cDNA into a pUC-CAGGS vector (18). The mixture was agitated and sonicated to prepare unilamellar liposomes. Then, the liposomes were conjugated with HVJ, which had been inactivated with ultraviolet irradiation.

In vivo gene transfection. Wistar male rats (250-300g) were anesthetized with an intraperitoneal injection of sodium pentobarbital. During ligation of the portal vein for 15 min, HVJ-liposome mixture carrying pUC-CAGGS-*bcl-2* or pAct-LacZ (200μg) was injected into the right lobe of the liver. The liver was analyzed four days after transfection.

Hypoxic perfusion of rat liver. Rat livers were perfused at 37°C with hemoglobin-free Krebs-Henseleit buffer saturated with 95% oxygen and 5% carbon dioxide for 10 min, and then with 95% nitrogen and 5% carbon dioxide for 1 h -4 h. Propidium iodide (PI, 250 μM) was added to the perfusate for the final 15 min of perfusion. After perfusion, the livers were fixed with 4% paraformaldehyde containing 0.1 M phosphate buffer (pH 7.4).

Western blotting. Liver specimens were homogenized in buffer containing 50 mM Tris-Cl (pH 8.0), 20 mM EDTA, 1 % SDS, and 100 mM NaCl. The lysates were cleared by centrifugation. The lysates (15μg protein) were boiled with reducing SDS sample buffer for 5 min, and analyzed by SDS-PAGE on 12.5% gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes and hBcl-2 protein was detected with anti-human Bcl-2 monoclonal antibody (19).

Immunofluorescence. After changing the fixation buffer to 20% sucrose in PBS, PI-stained livers were frozen and sectioned on a cryostat, and specimens were incubated overnight with an FITC-labeled anti-human Bcl-2 antibody (DAKO A/S, Glostrup, Denmark). The expression of hBcl-2 and cell death (shown by PI staining) were assessed by fluorescence microscopy (Olympus BHS-RFL-LSM) using a narrow interference blue abstraction (NIBA) filter with excitation at 480 nm and emission at 530 nm, and a wide interference green (WIG) filter with excitation at 520 nm and emission at 580 nm, respectively.

DNA end-labeling assay. Apoptosis was estimated with DNA end-labeling assay using ApopTag™ (ONCOR).

β-gal staining. The frozen sections of PI-stained livers were stained with 5-bromo-4-chloro-3-indolylβ-D-galactoside (X-Gal) for identification of individual cells expressing β-galactosidase (20).

Electron microscopy. The section adjacent to that used for immunofluorescence or β-galactosidase staining was fixed with 2.5% sodium phosphate-buffered glutaraldehyde (pH 7.4), and postfixed for 1 h with 1 % veronal acetate-buffered OsO_4. Ultrathin sections were cut and stained with ethanolic uranyl acetate and lead citrate, and analyzed using electron microscopy.

Measurement of caspase-3 (-like) protease activity and ketone body ratio. At the indicated times after hypoxia, a part of the liver tissue transfected with *hbcl-2* or *lac-Z* genes was homogenized in 50 mM sodium phosphate buffer (pH 7.4) with 0.5 mM EDTA. After addition of 1 % Triton-X100, the homogenates were incubated at 37°C for 10 min, and the resulting lysates were cleared by centrifugation at 15000 r.p.m. for 15 min at 4°C. Caspase-3 (-like) protease activity was measured by incubation of lysates (40 μg protein) with 50 nmol enzyme substrates acetyl-L-aspartic-L-glutamic-L-valyl-L-aspartic acid 4-methyl-coumaryl-7-amide (Ac-DEVD-MCA) (Peptide Institute) at 37°C for 1 h as described (10, 21, 22). One unit was defined as the amount of enzyme required to release 1 pmol 7-amino-4-methylcoumarin (AMC) per min at 37°C. The ketone body ratio (acetoacetate/β-hydroxybutyrate) in the lysates was measured as described (23-25).

RESULTS

Morphologic features of hypoxic liver cell death. To evaluate the injury of hepatocytes caused by hypoxia, we examined with hypoxically perfused livers, which were commonly used as ischemia model, and facilitated analysis of the cell death by addition of propidium iodide (PI) into perfusate. Because PI only stained nuclei of cells with a disrupted membrane, the extent of cell death including necrosis and terminal apoptosis which is characterized by cell membrane disruption, was conveniently estimated by PI staining. When livers were perfused with normoxic condition, none of nuclei was stained with PI (FIG.1 a, i). Although cells showed red, it was due to the non-specific attachment of PI to cell

FIG. 1. Representative morphology of hypoxic perfused livers. Livers were perfused with Krebs–Heiseleit buffer saturated with 95 % N_2 and 5 % CO_2, and stained with PI (250 μM) at the end of perfusion for 15 min. (a-c) Fluorescent microscopy of livers after hypoxic perfusion for 0 h–4 h ($\times$ 400) subsequent to normoxic perfusion for 10 min. Frozen liver sections were observed by fluorescence microscopy using WIG filter. (d–f) DNA end-labeling assay of livers after hypoxic perfusion for 0 h–4 h ($\times$ 400). Frozen liver sections were stained by using *in situ* apoptosis detection kit. (g,h) Ultrastructural findings of the liver after hypoxic perfusion for 0 h–2 h (bar=1 μm). (i) Time course of the rate of PI- and DNA end-labeling assay positive cells. Both PI-positive and DNA end-labeling assay-positive cells were quantified by counting the positive hepatocytes / all hepatocytes in the three sliced sections of the liver. Data represent means ± SD (n=3). Open circle, DNA end-labeling assay positive cells; closed circle, PI-positive cells.

FIG. 2. Expression of hBcl-2 in the transfected livers and effect of *hbcl-2* on cell death in hypoxic perfused livers. (a) Western blot analysis of *lac-Z* (lane 1) and *hbcl-2* (lane 2) transfected right lobe of the liver. The right lobe of the liver was transfected with these genes and homogenized after 4 days. Expression of hBcl-2 was detected with anti-human Bcl-2 monoclonal antibody. (b, c) Transfected livers were subjected to 2 h hypoxia and stained with PI and with FITC-labeled anti-hBcl-2 antibody. Expression of hBcl-2 (b, $\times$ 400 with NIBA filter) and cell death (c, $\times$ 400 with WIG filter) were assessed in the same field using fluorescent microscopy. (d) Quantitative analysis of the anti-necrotic effect of exogenously transfected *lac-Z* and *hbcl-2* after 2 h hypoxia. PI-positive cells were calculated by counting PI-positive hepatocytes / β-gal (+), β-gal (−), Bcl-2 (+), or Bcl-2 (−) hepatocytes in three sliced sections of the transfected right lobe from one rat. All data represent means ± SD (n=3).

membrane (Fig. 1 a). After 2 h hypoxia, about 80 % of nuclei in hepatocytes were positively stained with PI (FIG. 1 b, i) and showed round without shrinkage, indicating hepatocytes die with necrosis. The absence of apoptosis in hypoxic condition for 2 h (FIG.1 e) was confirmed by *in situ* DNA end-labeling assay, which stained 3′-OH DNA cleavage associated with apoptosis. Although longer hypoxia for 4 h produced positive-staining cells in DNA end-labeling assay, it is probably the consequence of random DNA cleavage due to the necrotic cell death (FIG.1 f, i). To examine the morphology of hypoxic liver cell death, electron microscopy was used. As shown in FIG.1 h, almost all the hepatocytes treated with 2 h hypoxia showed the characteristic features of necrosis, i.e., mitochondrial swelling, electron-lucent cytoplasm, a cytosolic vacuoles with apparently normal nucleus. Apoptotic cells characterized with condensed chromatin, nuclear and cellular fragmentation were hardly observed. All these findings suggested that hypoxia mainly induced necrosis of liver cells.

Expression of transfected bcl-2. HVJ-liposome mixture (300 μl) with human *bcl-2* gene (*hbcl-2* ; 200 μg) or the *lac-Z* gene (200 μg) (as a control) was injected into the right lobe of the liver during ligation of the portal vein for 15 min. Four days after transfection, the expression of hBcl-2 protein was identified in the lobe of the liver where *hbcl-2* was transfected but not *lac-Z* by Western blotting with anti-human Bcl-2 antibody (FIG. 2 a). Endogenous Bcl-2 was not detected using anti-mouse Bcl-2 antibody which crossreacts with rat Bcl-2 (data not shown), consistent with extremely low levels or no *bcl-2* mRNA in liver (26). Immunostaining with FITC-labeled anti-human Bcl-2 antibody also showed the expression of hBcl-2 in the liver surrounding the *hbcl-2* injection site (FIG. 2 b, left upper portion). Slight staining with FITC in hBcl-2(−) cells (FIG. 2 b, right lower portion) should be the nonspecific staining of FITC, because normal livers, in which Bcl-2 was not expressed, were also slightly stained with anti-hBcl-2-FITC (data not shown). As was expected, *lac-Z* transfected lobe of the liver was negative for human Bcl-2 by immunostaining with anti-human Bcl-2 monoclonal antibody (data not shown). The efficiency of *hbcl-2* and *lac-Z* gene expression into transfected lobes (which was calculated by counting the positive hepatocytes / all hepatocytes in the three sliced sections of the transfected lobe) were 28.0 $\pm$ 5.6 % assessed by immunostaining, and 41.1 $\pm$ 3.6 % by β-gal staining (n=3) respectively. These results indicated that exogenously transfected *hbcl-2* using HVJ-liposome method efficiently expressed in the rat liver.

Prevention of hypoxic cell death by transfected hbcl-2. To examine the effect of transfected *hbcl-2* on hypoxic liver cell death, transfected livers were perfused under hypoxic conditions for 2 h, and stained with PI added to the perfusate. The frozen sections of PI-stained livers were immunostained with FITC-labeled anti-human Bcl-2 antibody. The effect of hBcl-2 expression on necrosis was assessed in the same field with different bandpass filters on the fluorescence microscope. As shown in FIG. 2 b, c, few of the nuclei of hBcl-2 expressing hepatocytes (FIG. 2 b, hBcl-2(+)), which was identified as FITC-positive cells, were stained with PI (FIG. 2 c, hBcl-2(+)), whereas, almost all nuclei of hepatocytes which did not express hBcl-2 (FIG. 2 b, hBcl-2(−)), were positively stained with PI (FIG. 2 c, hBcl-2(−)).

Quantitative analysis using immunostaining and β-gal staining showed that *hbcl-2* transfection significantly prevented hypoxia-induced necrosis (rate of necrosis : 4.1 $\pm$ 2.1 % in Bcl-2 positive cells, 82.9 $\pm$ 5.0 % in Bcl-2 negative cells), and *lac-Z* transfection did not prevent it (rate of necrosis : 88.6 $\pm$ 2.8 % in β-gal positive cells, 87.1 $\pm$ 4.5 % in β-gal negative cells) (FIG. 2 d). To confirm the anti-necrotic activity of transfected *hbcl-2,* electron microscopy was used. Serial sections were made, and one was used to identify hBcl-2 expression by immunostaining and β-gal expression by β-gal staining respectively, and the other was for electron microscopy. Interestingly, the hepatocytes transfected with *hbcl-2* and *lac-Z* contained electron-dense round vesicles (FIG. 3 a, b, arrows), which should be the phagocytosed liposomes. After 2 h hypoxia, the *bcl-2* transfected hepatocytes including electron-dense round vesicles were apparently viable without mitochondrial swelling and electron-lucent cytoplasm (FIG. 3 a), whereas the *lac-Z* transfected hepatocytes including the vesicles showed necrotic feature with electron-lucent spaces in the cytosol and mitochondrial swelling in the absence of nuclear change (FIG. 3 b), which was equal to the findings of the non-transfected hepatocytes (FIG. 3 c). All these findings suggested that *hbcl-2* transfection can rescue hepatocytes from necrotic cell death induced by hypoxia *in vivo.*

Transfection of hbcl-2 prevents caspase-3 (-like) protease activation. We assessed the effect of transfected *hbcl-2* on caspase-3 (-like) protease activity, which are known to have an important role in many types of cell death at the downstream of the target of Bcl-2 (22,27,28). As shown in FIG. 4 a, hypoxia induced activation of caspase-3 (-like) proteases in the part of the liver where *lac-Z* was transfected. In contrast, the increase was strongly repressed in the part of the *hbcl-2* transfected lobe of livers (FIG. 4 a). These findings suggested that introduced human Bcl-2 inhibited the activation of caspase-3 (-like) proteases in *in vivo,* resulting in prevention of necrosis induced by hypoxia.

Transfection of hbcl-2 prevents liver dysfunction. Finally, effect of *hbcl-2* transfection on liver function was examined. We have previously shown that mitochondrial dysfunction is an early event in the process of cell

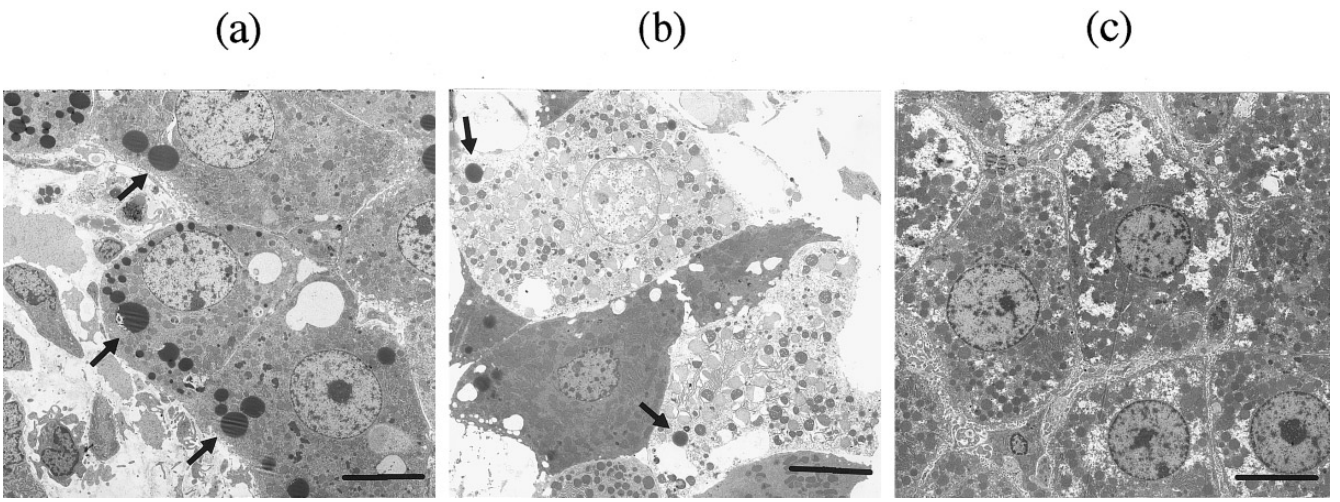


FIG. 3. Ultrastructural findings of *hbcl-2* (a), *lac-Z* (b) gene, and non (c) -transfected livers treated with 2 h hypoxia. Livers were treated with hypoxic perfusion for 2 h, and were cut into two serial sections, one to identify hBcl-2 and β-gal expression, and the other for electron microscopy (a–c, bar=10 μm). Arrows, electron-dense round vesicles.

death and is prevented by Bcl-2 (21). This finding raises the possibility that *hbcl-2* transfection might affect the liver function. Since the ketone body ratio (acetoacetate/β-hydroxybuthyrate) indicated the liver function, especially the mitochondrial function (25), we measured acetoacetate and β-hydroxybuthyrate in the part of *hbcl-2* and the *lac-Z* transfected lobe of the liver. As shown in FIG. 4 b, ketone body ratio decreased rapidly within 60 min in control livers, whereas it was maintained near a initial level in *bcl-2* transfected livers,

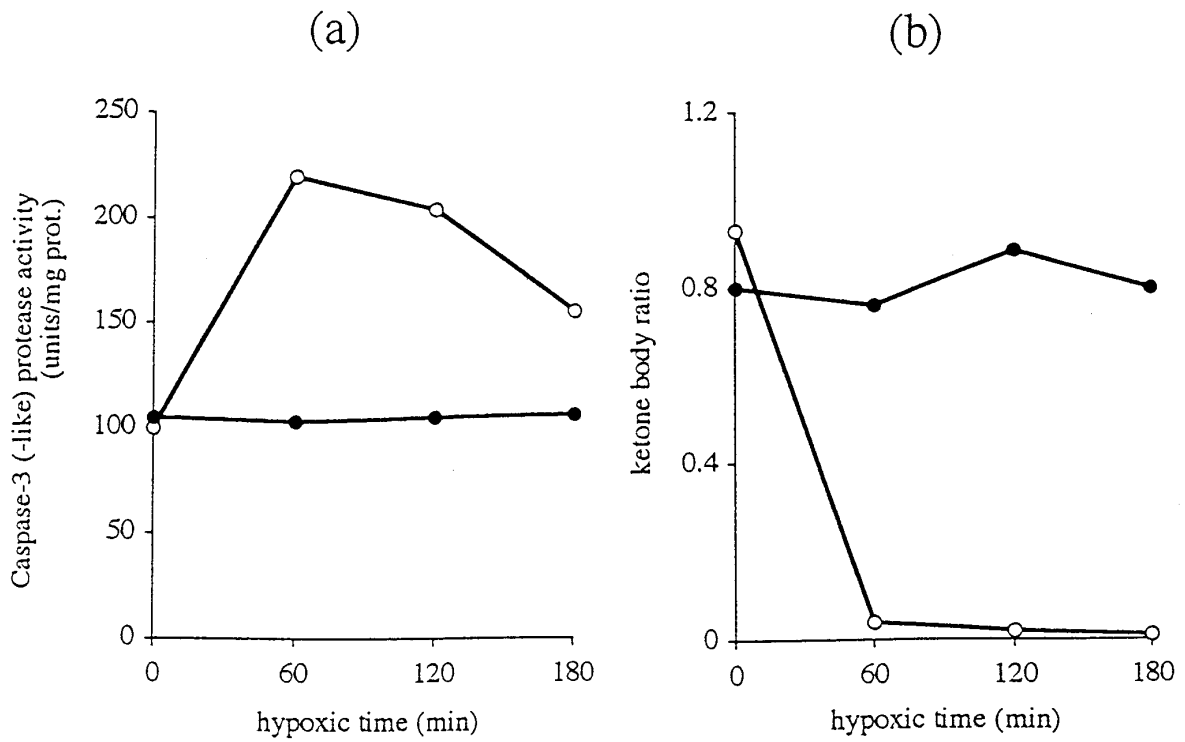


FIG. 4. Effect of transfected *hbcl-2* on caspase-3 (-like) protease activity and liver function. The *lac-Z* or *hbcl-2* transfected livers were hypoxically perfused, and the liver specimens were withdrawn from the transfected portion. (a) Time course of caspase-3 (-like) protease activity. (b) Time course of ketone body ratio during hypoxic perfusion. Acetoacetate and β-hydroxybutyrate were estimated at indicated times, and ketone body ratio was calculated. Open circle, *lac-Z* transfected liver; closed circle, *hbcl-2*-transfected liver. Data are representative of three independent experiments.

indicating exogenously introduced Bcl-2 prevents not only cell death but liver dysfunction.

DISCUSSION

We previously showed that hypoxia induces both apoptosis and necrosis, and its proportion was highly dependent on cell type (7). We tested the mode of hypoxic cell death using several cell lines derived from hepatomas and primary cultured rat hepatocytes, and found that all cell lines, HepG2, 7316A, Hep3B, and primary cultured hepatocytes mainly died by necrosis. Consistently, hypoxia-induced liver cell death showed apparently necrotic features (FIG. 1 b, e, h).

We have reported that Bcl-2 inhibits apoptosis as well as some forms of necrosis (10, 11) in tissue culture systems. Here we show that exogenously transfected *hbcl-2* effectively prevented hypoxia-induced necrosis in livers (FIG. 2c, d, and 3 a). These observations made sure the ability of Bcl-2 against some forms of necrosis, and suggested the possibility that Bcl-2 can rescue some types of liver injury involving necrosis, such as hypoxia, endotoxemia and viral infection.

In the hypoxic perfused liver, both oxidative phosphorylation and glycolysis are blocked by lack of oxygen and substrates, resulting in a decrease of ATP levels (29,30) and loss of mitochondrial function (31). Subsequently, disturbance of Ca^{2+} homeostasis (32) and of the cytoskeletal system (33) leads to necrotic cell death. Because loss of mitochondrial function precedes hypoxic cell death (34,35) and protection of mitochondrial function prevents it (31), mitochondria plays an essential role in hypoxic cell death pathway. Recently, we showed that Bcl-2 acts directly on the mitochondria to maintain their membrane potential (21). Consistently, the present data show that Bcl-2 prevents *in vivo* mitochondrial dysfunction in the hypoxic liver, indicating prevention of mitochondrial dysfunction *in vivo* appears to be not the consequence but the cause of cell survival.

In this study, we used the HVJ-liposome method to introduce the *hbcl-2* gene into liver cells, because of good transfection efficiency without host cell injury (13,14). This method is known to be a useful tool for *in vivo* gene transfection in some organs (16,36). We tried some modifications of transfection method, such as injection via the portal vein and transient portal vein ligation, with the plasmid carrying *lac-Z* gene. As β-gal was most efficiently expressed in liver cells by the method of direct injection with portal vein ligation, we applied *hbcl-2* gene transfection with this method. Expectedly, hBcl-2 was efficiently expressed in a part of the liver (FIG. 2 a, b), and prevented not only hypoxia-induced necrotic cell death (FIG. 2 c), but liver dysfunction (FIG. 4 b). Taken together, our findings raise the possibility that *bcl-2* gene transfection is a new strategy to inhibit preservation injury by gene transfection.

REFERENCES

1. Clavien, P. A., Harvey, P. R. C., and Strasberg, S. M. (1992) *Transplantation*. **53,** 957–978.
2. Jennings, R. B., Ganote, C. E., and Reimer, K. A. (1975) *Am. J. Pathol.* **81,** 179–198.
3. Belzer, F. O., and Southard, J. H. (1988) *Transplantation* **45,** 673–676.
4. Starzl, T. E., Hakala, T. R., Shaw, B. W. J., Hardesty, R. L., Rosenthal, T. J., Griffith, B. P., Iwatsuki, S., and Bahnson, H. T. (1984) *Surg. Gynecol. Obstet.* **158,** 223–230.
5. Morimoto, Y., Kamiike, W., Nishida, T., Hatanaka, N., Shimizu, S., Huang, T., Hamada, E., Uchiyama, Y., Yoshida, Y., Furuya, E., and Matsuda, H. (1996) *Gastroenterol.* **111,** 1071–1080.
6. Gores, G. J., Herman, B., and Lemasters, J. J. (1990) *Hepatology* **11,** 690–698.
7. Shimizu, S., Eguchi, Y., Kamiike, W., Itoh, Y., Hasegawa, J., Yamabe, K., Otsuki, Y., Matsuda, H., and Tsujimoto, Y. (1996) *Cancer Res.* **56,** 2161–2166.
8. Vaux, D. L., Cory, S., and Adams, J. M. (1988) *Nature* **335,** 440–442.
9. Tsujimoto, Y. (1989) *Oncogene* **4,** 1331–1336.
10. Shimizu, S., Eguchi, Y., Kamiike, W., Waguri, S., Uchiyama, Y., Matsuda, H., and Tsujimoto, Y. (1996) *Oncogene* **12,** 2045–2050.
11. Kane, D. J., Ord, T., Anton, R., and Bredesen, D. E. (1995) *J. Neurosci. Res.* **40,** 269–275.
12. Shimizu, S., Eguchi, Y., Kosaka, H., Kamiike, W., Matsuda, H., and Tsujimoto, Y. (1995) *Nature* **374,** 811–813.
13. Kaneda, Y., Iwai, K., and Uchida, T. (1989) *Science* **243,** 375–378.
14. Kaneda, Y., Iwai, K., and Uchida, T. (1989) *J. Biol. Chem.* **264,** 12126–12129.
15. Kato, K., Kaneda, Y., Sakurai, M., Nakanishi, M., and Okada, Y. (1991) *J. Biol. Chem.* **266,** 22071–22074.
16. Tomita, N., Higaki, J., Morishita, R., Kato, K., Mikami, H., Kaneda, Y., and Ogihara, T. (1993) *Circ. Res.* **73,** 898–905.
17. Kato, K., Nakanishi, M., Kaneda, Y., Uchida, T., and Okada, Y., *J. Biol. Chem.* **266,** 3361–3364.
18. Niwa, H., Yamamura, K., and Miyazaki, J. (1991) *Gene* **108,** 193–199.
19. Pezzella, F., Tse, A. G., Cordell, J. L., Pulford, K. A., Gatter, K. C., and Mason, D. Y. (1990) *Am. J. Pathol.* **137,** 225–232.
20. Harlow, E., and Lane, D. (1988) *in* Antibodies (Lane, D., Ed.), p. 408, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
21. Shimizu, S., Eguchi, Y., Kamiike, W., Waguri, S., Uchiyama, Y., Matsuda, H., and Tsujimoto, Y. (1996) *Oncogene* **13,** 21–29.
22. Shimizu, S., Eguchi, Y., Kamiike, W., Matsuda, H., and Tsujimoto, Y. (1996) *Oncogene* **12,** 2251–2257.
23. Mellanby, J., and Williamson, D. H. (1974) *in* Methods of Enzymatic Analysis (Bergmeyer, H. V., Ed.), pp. 1840–1843, Academic Press, New York.
24. Williamson, D. H., and Mellanby, J. (1974) *in* Methods of Enzymatic Analysis (Bergmeyer, H. V., Ed.), pp. 1836–1839, Academic Press, New York.
25. Tanaka, J., Ozawa, K., and Tobe, T. (1979) *Gastroenterol.* **76,** 691–696.
26. Negrini, M., Silini, E., Kozak, C., Tsujimoto, Y., and Croce, C. M. (1987) *Cell* **49,** 455–463.
27. Cory, S. (1995) *Ann. Rev. Immunol.* **13,** 513–543.
28. Tsujimoto, Y. (1997) *Cell Death Differ.* **4,** 429–434.
29. Gasbarrini, A., Borle, A. B., Farghali, H., Bender, C., Fran-

cavilla, A., and Thiel, D. V. (1992) *J. Biol. Chem.* **267,** 6654–6660.

30. Kamiike, W., Watanabe, F., Hashimoto, T., Tagawa, K., Ikeda, Y., Nakao, K., and Kawashima, Y. (1982) *J. Biochem.* **91,** 1349–1356.
31. Okabe, H., Kurosawa, K., Hatanaka, N., Yoshitaka, J., and Tagawa, K. (1991) *Biochim. Biophys. Acta.* **1098,** 41–48.
32. Hatanaka, N., Kamiike, W., Shimizu, S., Miyata, M., Inoue, T., Yoshida, Y., Tagawa, K., and Matsuda, H. (1995) *J. Surg. Res.* **58,** 485–490.
33. Koseki, M., Nishida, H., Kamiike, W., Nakao, K., Kawashima, Y., Dan, C., Wake, K., Yoshida, Y., and Tagawa, K. (1988) *Biomed. Res.* **9,** 47–57.
34. Pastorino, J. G., Snyder, J. W., Serroni, A., Hoek, J. B., and Farber, J. L. (1993) *J. Biol. Chem.* **268,** 13791–13798.
35. Watanabe, F., Kamiike, W., Nishimura, T., Hashimoto, T., and Tagawa, K. (1983) *J. Biochem.* **94,** 493–499.
36. Suzuki, K., Sawa, Y., Kaneda, Y., Ichikawa, H., Shirakura, R., and Matsuda, H. (1997) *J. Clin. Invest.* **99,** 1645–1650.