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Effect of Bcl-2 expression on morphological alteration of hepatic mitochondria in liver regeneration after a partial hepatectomy: An immunohistochemical and ultrastructural study

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ABSTRACT

We investigated the effects of Bcl-2 overexpression on the morphological changes of the mitochondria (MC) in the regenerating rat liver after a 70% partial hepatectomy (PH). Bcl-2 or control marker (LacZ) gene transfection to the regenerating liver was performed immediately after PH by a systemical injection of 1×10^9 pfu/body of recombinant adenovirus, which encodes either human Bcl-2 protein (ACalbBcl2pLpA) in group 1 (n=39) or E.Coli β-galactosidase (AxCALacZ) in group 2 (n=39). In group 3 (n=13), 1ml of normal saline was injected instead of the recombinant adenovirus. The rats were allowed to survive until the scheduled sacrifice at 0.5h, 2h, 4h, 12h, 24h, 2d, 3d, 5d, 7d, 9d, 11d, 14d, and 21d after PH (3 rats each). In the immunohistochemical analysis, the Bcl-2 protein expression showed a peak intensity at 12 to 72 hrs after PH in group 1, whereas it was delayed until 5 days after PH in groups 2 and 3 with a rather mild intensity, followed by a gradual decrease in all three groups. At its peak intensity, the Bcl-2 protein expression was recognized over a wide range of the parenchymal area as well as in the periportal area. The β-galactosidase activities in X-gal staining, showed a peak intensity at 48 hrs after PH followed by a gradual decrease until 21 days after PH in group 2. In the hepatocyte ultrastructure, MC shape was well preserved in group 1 until 7 days after PH with number of regular shaped mitochondria, well preserved cristae and dense matrix (RM) being 58±22% at 12hrs and 60±21% at 24 hrs, compared to groups 2 (36±23% at 12hrs and 40±15% at 24hrs, $p\leqq0.015$ and $\leqq0.004$ respectively) and 3 (29±11% at 12hrs and 35±12% at 24 hrs, $p\leqq0.0004$ and $\leqq0.0015$ respectively). In conclusion, Bcl-2 overexpression in the regenerating liver was important in preserving mitochondrial shape in the early stage of liver regeneration, thus contributing to an accelerated liver regeneration. *Ryukyu Med. J., 19(4)203~209, 2000*

Key words: Bcl-2, mitochondria, liver regeneration, electron-microscopy

INTRODUCTION

The regenerating liver after partial hepatectomy is the model for studying controlled growth in vivo[1]. Using this model, the liver regenerative response is now defined as a complex of responses induced by specific external stimuli, which are related to the sequential changes in gene expression, growth factor production, and morphologic structure[2]. Among them, molecular based analyses of such apoptosis regulating genes such as Bcl-2, Bcl-X and Bax have been performed during the process of liver regeneration[2,3]. These studies, however, have been done separately from the morphological changes of the hepatocyte organelle including the mitochondria (MC)[4,5].

The Bcl-2 protein has been found to block programmed cell death or even promote cellular proliferation, it therefore helps in maintaining the proliferation of the hepatocytes in the regenerating liver[6]. Moreover, Bcl-2 protein is known to be localized in the MC membrane, while topographically functioning in the antioxidant pathway, blocking the release of calcium from MC, and preventing the loss of MC potential. In these contexts, Bcl-2 plays an important role in the MC function[7].

MC alteration such as swelling has been reported to be associated with a partial hepatectomy, especially during the first 24 to 48 hrs[8]. A significant decrease in substrate-supported phosphate in the cellular hypertrophy has been suggested to induce mitochondrial swelling, hence a similar reduction of substrate-supported phosphate in liver regeneration might be related to mor-

Table 1 Immunohistochemical staining of the Bcl-2 expression during rat liver regeneration

	Time after partial hepatectomy				
	0.5~4h	12h~3days	5~7days	9days	11~21days
Group I	+/−	+++	+++	++	++
Group II	+/−	++	+++	+/−	++
Group III	+/−	++	+++	+/−	++

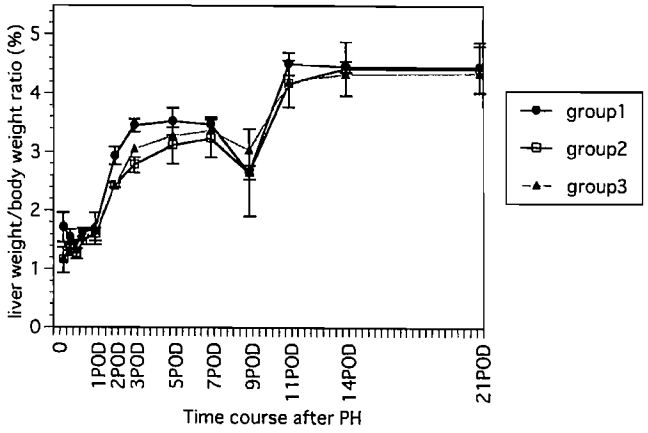


Fig. 1 Liver weight/body weight ratio.

phological changes in the hepatocytes[8]. In these contexts, Bcl-2 might also be related to both the morphological changes of the MC and hepatocytes.

In the present study, Bcl-2 protein expression was genetically enhanced in the regenerating liver, using adenovirus mediated gene transfer. The morphological changes of MC were then investigated electromicroscopically during liver regeneration, in relation to the changes in Bcl-2 expression.

MATERIALS AND METHODS

Construction of recombinant adenovirus

All recombinant adenovirus were constructed using an "Adenovirus Expression Vector Kit" (Takara Biomedical, Japan). LacZ cDNA was adjusted by the restriction enzyme and then were subcloned into the SwaI site of the Cosmid vector (pAxCAwt) which had a CAG promoter (cytomegalovirus enhancer, chicken β-actin promoter, rabbit β-actin polyA signal). The cosmid vector coding Bcl-2 (albumin promoter) was also similarly prepared. These cosmid vectors and restriction enzymes were cotransfected into the 1×10^5 of 293 kidney embryonal cells. The supernatant containing the recombinant adenovirus was collected by 4 freeze and thaw cycles. These steps of cotransfection and collection were repeated 4 times without significantly expanding culture volume in order to obtain a high virus solution. The viral titers were determined based on a 50% tissue culture infectious dose (TCID50), in which, the virus titer in TCID50 was approximated to the plaque-forming units (pfu)[9]. The solution of ACalbBcl2pLpA (1×10^9 pfu/ml) and AxCALacZ (1×10^9 pfu/ml), were stored at −80℃ until use.

Animals

Sixteen-week-old adult Wistar rats weighing 250-300g were purchased from Ryukyu Biotech and used for this study. The animals were fed a laboratory chow diet and had free access to food and water. All animals received human care according to the "Guide for the Care and Use of Laboratory Animals" prepared by the University of the Ryukyus.

Experimental Design

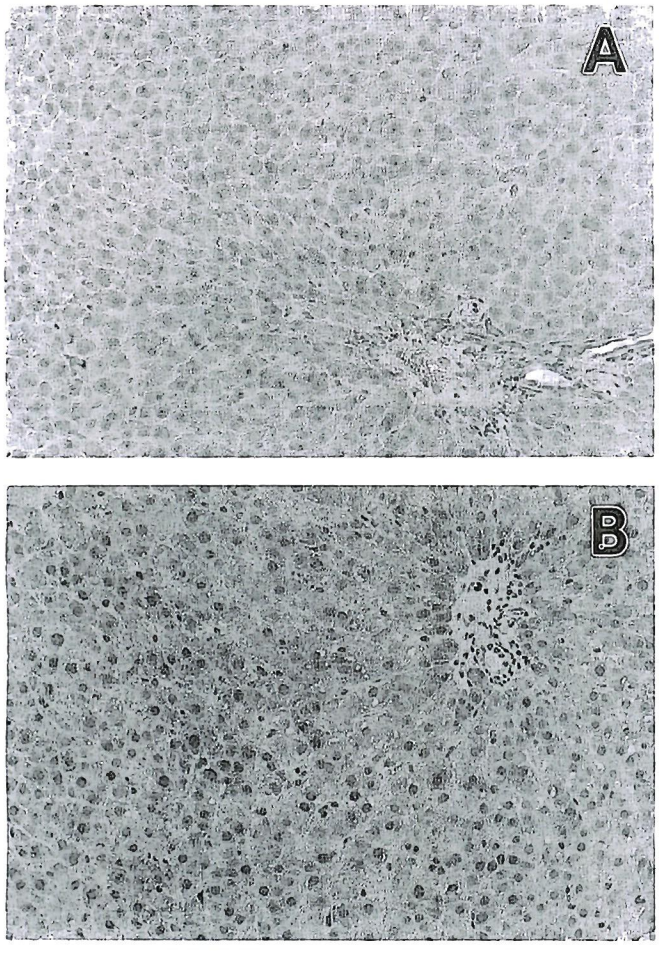
Under ether inhalation, a 70% partial hepatectomy (PH) was performed following the standard procedures[10] in which the median lobe was ligated at its root and then removed. Since more than 90% of the recombinant adenovirus is known to accumulate in the liver after an intravenous injection of the recombinant adenovirus[11], gene transfer to the liver was performed by injecting 1×10^9 pfu of either ACalbBcl2pLpA in group 1 (n=39) or AxCALacZ in group 2 (n=39), through the penile vein immediately after a partial hepatectomy. The group 3 (n=13) animals received 1ml of normal saline. The rats were allowed to survive until the scheduled sacrifice. Hepatectomies were performed between 9 AM and 12 AM to avoid any circadian variations on regeneration.

Sampling

To obtain samples of the liver, 3 rats each were sacrificed at 0.5 h, 2 h, 4 h, 12 h, 24 h, 2d, 3d, 5d, 7d, 9d, 11d, 14d, and 21d after PH. After measuring the total body and liver weight of each rat at sacrifice time, all specimens were either fixed in 4 % paraformaldehyde and in 2 % glutaraldehyde, for electronmicroscopic and a histologic analysis. Frozen liver sections were also obtained for X-gal staining.

Immunohistochemistry of Bcl-2 protein

Four micro-thick serial tissue sections were cut from each tissue specimen and immersed in xylene followed by rehydration in graded alcohol. The sections were further immersed in citrate buffer solution (pH 6.0), kept at 120℃ in the autoclave (labo autoclave



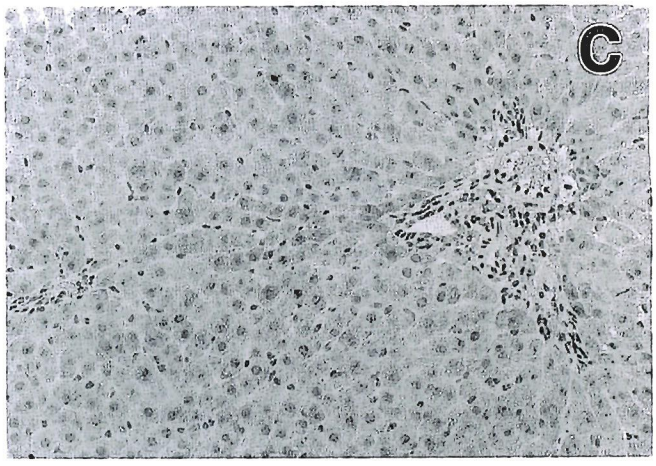


Fig. 2 The immunohistochemical detection of Bcl-2 protein in the regenerating livers. Intense immunoreactivity (+++) of Bcl-2 was recognized at 12 hrs after PH in group 1 (A), whereas only scattered (++) immunoreactivity was observed in group 2 (B) and 3 (C). (× 200).

Sanyo-Japan) for 15 min, and incubated with 3% H_2O_2 for 5 min to quench any endogenous peroxidase activity. Tissue sections were incubated overnight with monoclonal mouse anti-human Bcl-2 oncoprotein, 124 (Dako N-Series Dako Carpinteria, CA U.S.A) at 1:50 dilution. Subsequently the avidin-biotin-complex method was used[12] with AEC (AEC Substrate, DAKO corporation Carpinteria, CA 93013 U.S.A) as chromogen. The slides were finally counterstained with hematoxylin, dehydrated and mounted with glycerol. Bcl-2 protein staining was graded based on a 3 point scale grade, as 0; no staining, +/−; scattered weak staining, +; continuous weak staining, ++; scattered strong staining, +++; continuous strong staining. A breast cancer section was used as positive control that exhibited strong staining (+++) (data not shown). The cells demonstrating a positive Bcl-2 protein expression were also counted by digital imaging, using NIH imaging software (max cut off value; 50μm, minimum cut off value 30μm).

X-gal staining

Eight-micrometer-thick frozen sections were produced on a cryostat and fixed with 1.25% glutaraldehyde at 4℃ for 10 min. After removing the glutaraldehyde, the preparations were immersed in X-gal solution (1mg /ml) for 3 h, and then were counterstained with eosin. The cells expressing β-galactosidase turned blue in the presence of X-gal, while the non expressing cells demonstrated either a clear or a faintly yellow color. The stained sections were examined by light microscopy.

Electron microscopy

Transmission electronmicroscopic (TEM) images were used to evaluate the MC morphology. Small pieces (1mm^3) of the regenerating liver tissues were obtained and fixed in buffered 2% glutaraldehyde, post fixed in 1% phosphate buffered osmium tetroxide, dehydrated in increasing concentrations of alcohol, and embedded in Epon-812 resin. Sections (0.5m thick) were cut and stained with alkaline toluidine blue for the light microscopic analysis. Next ultrathin sections (approximately 65 to 70nm) were cut from three blocks per animal on a Porter-Blum MT ultra microtome with a diamond knife. The sections were mounted on copper grids, double stained with uranyl acetate and lead citrate and examined with a JEM 2000 Ex electron-microscope.

To evaluate the ultrastructure changes of the hepatocyte organelles such as MC, electromicrographs were taken at a primary magnification of ×6000 and printed at a final magnification of ×10,000. Five hepatocytes for each field were selected and the fields containing less than 50% cytoplasm were discarded. A total of 15 electron micrographs per time-interval were evaluated: 5 micrographs from each of 3 tissue block from each of 3 animals. In those samples at 12 h, 24 h and 7 days after PH, a total of 10 MC in each hepatocyte were evaluated and classified according to the morphological alterations observed. In the hepatocyte ultrastructure,

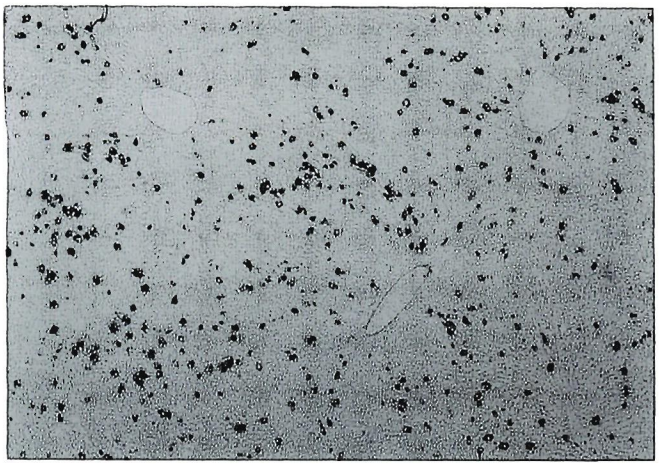


Fig. 3 X-Gal staining, for detecting the β-galactosidase activity from the transfected LacZ genes, was performed on frozen sections, which were obtained from regenerating rat livers in group 2 after a systemic injection of 1×10^9 plaque forming unit of AxCALacZ, more than 90% of the hepatocytes were found to be transduced with the LacZ gene at 48 hrs after a partial hepatectomy. (×40)

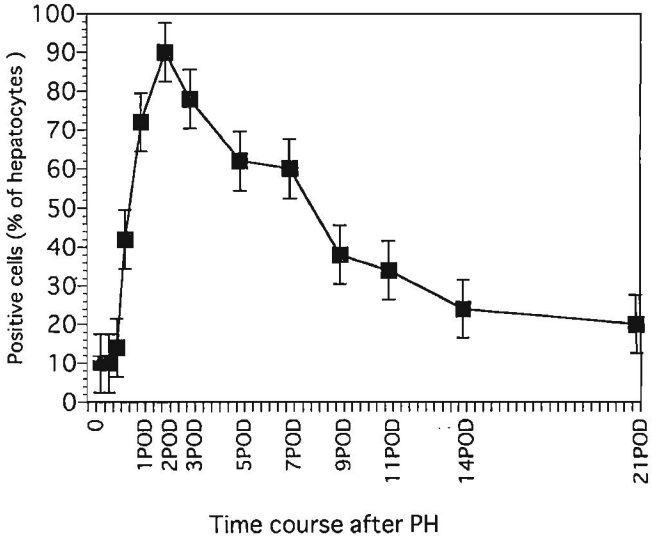


Fig. 4 Average LacZ positive cells in 5 fields (×40) continued to be detected until 21 days after PH, with a gradual decrease.

the mitochondria shape has been classified into three categories, including: 1. RM; regular shaped MC with well preserved cristae and a dense matrix, 2. SM; round shaped and swollen MC with poorly developed cristae and a lucent matrix, 3. IM; Irregular shaped MC with poorly developed cristae and a lucent matrix. All results were subjected to a statistical analysis, which included a calculation of the mean, standard deviation, percentage and the Mann-whitney method was used to evaluate comparisons between the groups.

RESULTS

Liver weight/body weight ratio

The liver weight/body weight ratio showed biphasic increase in all the groups after a partial hepatectomy, reaching the plateau level at post-operative day (POD) 14. Although no statistical difference was observed between the groups at any time point, the liver weight /body weight ratio reached the first peak at POD 3 to 5 in group 1, but it did not reach the peak until POD 5 to 7 in groups 2 and 3 (Fig. 1).

Immunohistochemistry for Bcl-2 protein

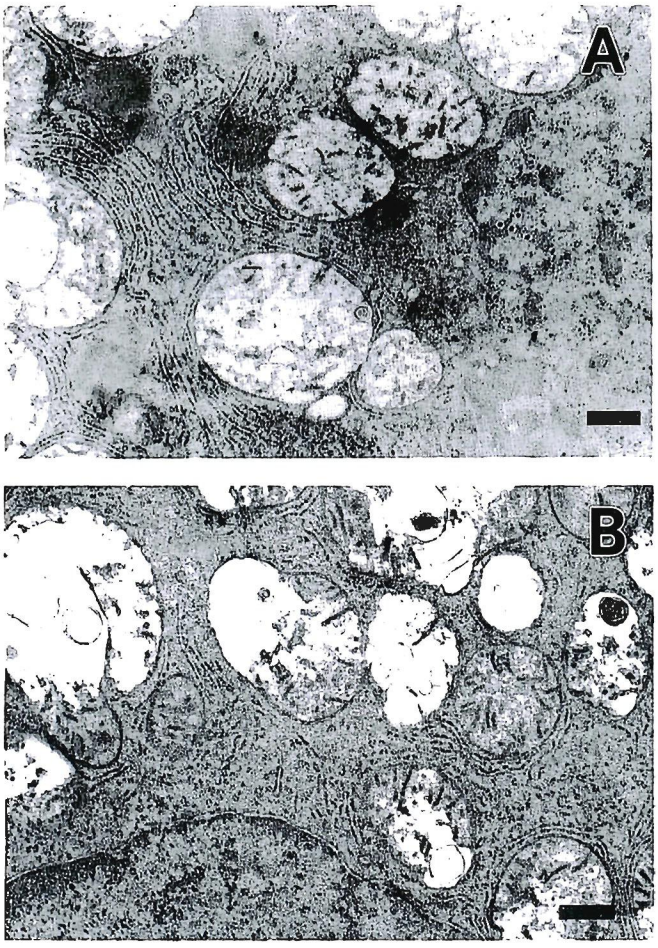
The expression of Bcl-2 protein in the liver after a 70% hepatectomy was serially evaluated by an immunohistochemical study. Weak Bcl-2 protein immunoreactivity (+/−) was recognized around the periportal area at 2 hrs after PH in all the groups. Immunoreactivity of Bcl-2 protein increased thereafter and reached a peak intensity (+++) at 12 to 72 hrs after PH in group 1 (Fig. 2 A). However in groups 2 and 3, the levels of immunoreactivity reached its peak intensity (+++) 5 days after PH. The Bcl-2 protein expression decreased thereafter in all the groups (+/−) until the second increase (++) at 11 to 21 days after PH. The expression of Bcl-2 in groups 1 to 3 is summarized in Table 1.

X-gal Staining

In group 2, the expression of marker LacZ was also evaluated by X-gal staining, to confirm the transgene expression by an adenovirus mediated gene transfer. Positively stained hepatocytes started to be recognized at 12 hrs after PH, and thereafter increased in number until 48 hrs after PH with more than 90% of the hepatocytes showing LacZ expression (Fig. 3). Average LacZ positive cells in 5 fields (×40) continued to be detected until 21 days after PH, with a gradual decrease (Fig. 4).

Ultrastructural findings

Electron microscopic examination was conducted to elucidate the role of Bcl-2 protein expression on the mitochondrial morphology. Thus during the first 24 hrs, the following ultrastructure alterations of the MC were noted. In the control groups, MC showed various characteristic shapes such as regular shape and swollen MC with poorly developed cristae and lucent matrix. Rough endoplasmic reticulum was often wrapped around MC (Fig. 5A). Some MC appeared irregular and markedly swollen with focal disruption of the outer membrane and vacuolization in the lucent matrix. (Fig. 5B). In the Bcl-2 transfected group 1, most of the MC retained a condensed configuration, with regular shaped MC, well-preserved cristae and dense matrix. Inner and outer membrane appeared intact (Fig. 5C). The prevalence of these ultrastructure findings was statistically significant among the groups during the first 24 hrs



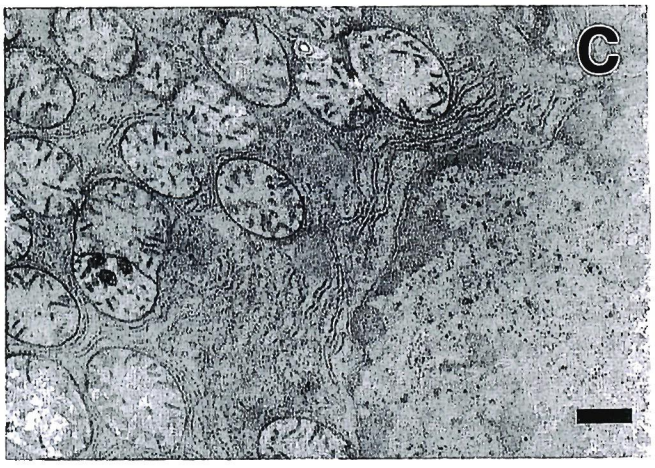


Fig. 5 Ultrastructural findings in the control groups (2 and 3), showed various alterations of the mitochondria including regular shape and swollen MC with poorly developed cristae and lucent matrix (A), and irregular shape MC with considerable fragmentation of the cristae architecture and focal disruption of the outer membrane and vacuolization in the lucent matrix. (B) bar: 500nm (×10,000)

In Bcl-2 groups, most of the MC showed a regular shape with a well preserved cristae and dense matrix (C) bar: 500nm (×10,000).

after a PH, therefore, in the Bcl-2 transfected group 1, the MC shape was well preserved with a significant increase in the number of regular shaped MC (RM) being 58±22% at 12 hrs and 60±21 % at 24 hrs, compared to groups 2 (36±23 % at 12 hrs and 40±15 % at 24 hrs, $p \leqq 0.015$ and $\leqq 0.004$ respectively) (Fig. 6A) and 3 (29 ±11 % at 12 hrs and 35±12 % at 24 hrs, $p \leqq 0.0004$ and $\leqq 0.0015$ respectively) (Fig. 6B).

DISCUSSION

In the present study, adenovirus mediated gene transfer was used to deliver the Bcl-2 or marker LacZ genes to the regenerating liver. Adenovirus vector, known to accumulate in the liver after systemic injection[11], was found to effectively express marker LacZ genes in more than 90% of the hepatocytes at 48 hrs after PH. In the present study, no increased hepatic damage or inhibition of liver regeneration was observed in those livers transfected with recombinant adenovirus (groups 1 and 2) compared to the control livers treated with normal saline (group 3).

These data directly suggested that adenovirus mediated gene transfer itself did not have any adverse effects on liver regeneration. Moreover, the LacZ expression could initially be recognized as early as 12hrs after PH, peaked at 48 hrs, with more than 90% of the hepatocytes being LacZ positive, and thereafter continued to be recognized until 21 days after PH. Compared to previous reports in which systemic injection was used for the gene transfer to the whole liver[13], the marker gene expression was more intense in its expression and continued for a longer period in the regenerating liver. After adenovirus mediated gene transfer, the Bcl-2 protein overexpression was thought to be achieved throughout the liver regeneration process with a maximum expression from 12 to 72 hrs after PH, corresponding to the G0 phase of liver regeneration. Since the albumin promoter was used for the adenovirus encoding Bcl-2 protein, the Bcl-2 transgene could only have produced protein in the hepatocytes[14].

MC is known to be the main source of cellular energy which is produced by the oxidative phosphorylation system[7]. In the regenerating liver after a partial hepatectomy, the cellular energy metabolism initially decreases in the prereplicative phase (G0 phase, 0-24 h after a PH), which accompanies a decreased oxidative phosphorylation capa bility, an increased production of oxygen radicals, and a decreased MC gluthatione level[7]. Following the prereplicative phase, a progressive recovery of MC is observed in the G1 phase (from 24 to 72 hrs after PH). Both the anti-apoptosis protein Bcl-2 and Bcl-X_L were reported to be localized in the MC membrane, and function in the anti-oxidant pathway and preventing the release of Ca^{+2} from

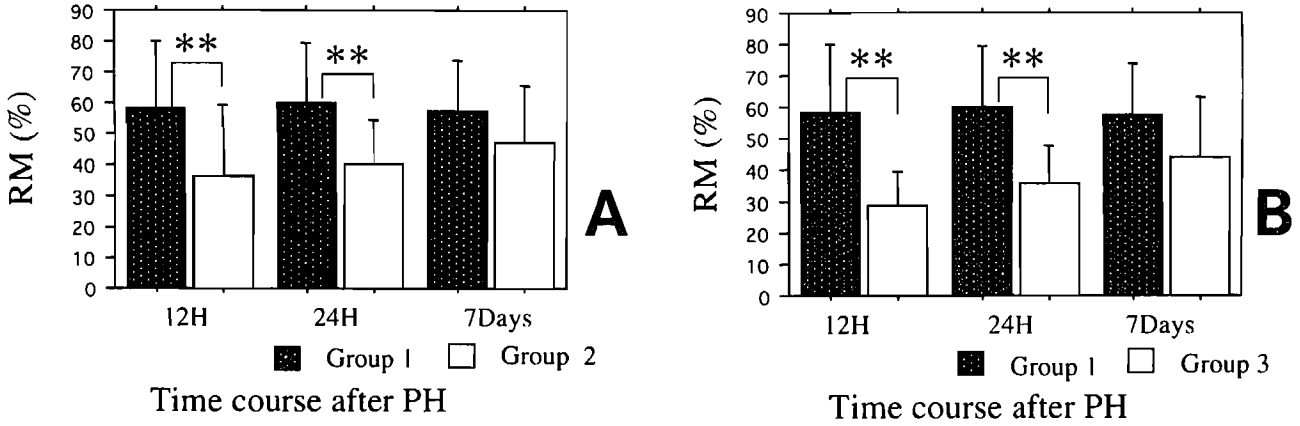


Fig. 6 Percentage of RM, during the time course of liver regeneration in the rat after gene transfer. The black bars indicate the percentage of RM in the Bcl-2 transfected group 1 and the white bars represent the percentage of RM in the control group 2 (A) and group 3 (B). Asterisks (**) show the data which have significant differences between the groups. The groups are compared according to the Mann-Whitney method.

the MC. These features of Bcl-2 family proteins might prevent the opening of the MC permeability transition pores and the release of apoptogenic proteins from the MC, which otherwise can result in either apoptosis or necrosis[15, 16]. It is also known that the loss of MC potential leads to morphological changes in the MC, and the Bcl-2 protein which acts upstream of these MC changes thus preventing morphological and functional changes in the MC. Based on these findings, Bcl-2 protein in the regenerating liver plays a central role in preventing MC damage during the early phase of liver regeneration and thus help supply the energy demand for liver regeneration.

In an electronmicroscopic study, hepatocyte MC showed various morphological changes during the early phase of liver regeneration, which corresponded to the G0 to G1 phase. These morphological changes were identical to those reported in previous reports and were characteristically accompanied by a marked swelling of the hepatocyte MC[8]. On the other hand, Bcl-2 transfection markedly suppressed these morphological changes in hepatocyte MC. Bcl-2 transfected liver also showed an accelerated regeneration based on the liver weight/body weight ratio up to 72 hrs after PH. These facts directly indicated that the morphological preservation of the hepatocyte MC and the accelerated regeneration correlate with each other, and the Bcl-2 protein, which is known to exist in the MC membrane, plays a central role in maintaining MC homeostasis. Although Bcl-2 protein expression is topographically restricted to the progenitor cells and long-life cells in adults, the widespread expression of Bcl-2 protein in the developing embryo has been reported which suggests that Bcl-2 may play a role in proliferation[3]. Moreover, the B cell lineage from the Bcl-2 transgenic mice, is known to show an extended cell survival, when progressing to high grade lymphoma[17]. These findings may support the notion that Bcl-2 plays a possible role in hepatocyte regeneration as suggested in the present study.

In conclusion, the Bcl-2 expression in the regenerating liver is thought to help preserve the hepatocyte mitochondrial morphology, and thereby help accelerate liver regeneration.

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