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Original Article

Protective effects against hepatic ischemia-reperfusion injury after rat orthotopic liver transplantation because of BCL-2 overexpression

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Abstract: This study aims to investigate the protective effects and mechanism of recombinant adenovirus Ad.VSG-hBCL-2 towards ischemia/reperfusion injury in rat liver graft. Recombinant adenovirus Ad.VSG-hBCL-2 was injected into the donor rat liver of the experiment group through the portal vein, the laparotomy was performed for liver 36 h later, and the liver was save in lactated Ringer's solution at 4°C for 4 h, "two-cuff method" was used to perform the orthotopic liver transplantation. The bile secretion situations of two groups were observed 6 h after the portal vein reflow; the recipient rats were killed to detect the plasma levels of AST, ALT and LDH. And the expressions of Bcl-2 and TNF-α in liver tissue, and TUNEL assay was used to detect the apoptosis of liver tissue cells, electron microscopy was used to observe the changes of subcellular structures of liver tissue. 6 h after the surgery, the immunohistochemistry and Western Blot test showed that the Bcl-2 expression in the liver of the experiment group significantly increased than the control group, the bile secretion increased, the levels of AST, ALT and LDH were significantly lower, and the TNF-α expression increased significantly. The changes of cellular morphology of the experiment group were milder, and the apoptotic index was significantly lower than the control group. The portal vein-transfected recombinant adenovirus Ad.VSG-hBCL-2 could be effectively expressed in rat liver, and the high expressed Bcl-2 could reduce the ischemia/reperfusion injury in the transplanted liver.

Keywords: Liver transplantation, rat, ischemia/reperfusion injury, gene transfection, Bcl-2

Introduction

Orthotopic liver transplantation is an established treatment for patients with end-stage liver disease or acute liver failure [1]. Liver dysfunction or failure remains a significant clinical problem after transplantation surgery. Ischemia-reperfusion injury (IRI) is responsible for primary liver dysfunction and failure after transplantation.

Apoptosis of liver cells is the main mechanism of ischemia/reperfusion injury in the transplanted liver [2]. Before the organ transplantation, the transfection of the ectogenic apoptosis inhibiting gene into the graft to express the functional protein and inhibit the apoptosis of donor liver cells, thereby reducing the postoperative ischemia/reperfusion injury, would be of great significance to prolong the graft survival time and improve the liver functions [3, 4]. In the process of apoptosis, release of cytochrome c into the cytoplasm activates death-promoting proteolytic enzymes called caspases, which in turn cleave a set of cellular proteins and promote the death program [5]. The Bcl-2 family of proteins regulates these changes during both apoptosis and necrosis [6]. The protein encoded by the Bcl-2 gene has been implicated in the prolongation of cell survival by blocking apoptosis and necrosis [7, 8].

In this experiment, the improved "two-cuff method" was used to establish the rat orthotopic liver transplantation model [9, 10]. The recombinant adenovirus Ad.VSG-hBCL-2 was then transfected into the donor liver for the

observation of BCL-2 expression in rat liver and its protective effects in ischemia/reperfusion injury of rat orthotopic liver transplantation, we investigated whether Bcl-2 up-regulation could decrease ischemia-reperfusion injury and improves function of transplanted liver grafts, aiming to explore and provide theoretical basis for the protective method of ischemia/reperfusion injury in liver transplantation.

Materials and methods

Primer synthesis

PCRPLAN program in PCGENE software was used (synthesized by the Genecore Gene company). Upstream primer of h-BCL-2 gene: VT369 5'-GGA GAT AGT GAT GAA GTA C-3'; Downstream primer of h-BCL-2 gene: VT370 5'-AGA GAC AGC CAG GAG AAA T-3'.

Animals and grouping

56 healthy male SD rats were purchased from the Experimental Animal Center of Soochow University, weighed 220~260 g, the weights of the receptors were equal to or slightly heavier than the donors. 48 rats were paired into 24 pairs, and then randomly divided into three groups, with n = 8 in each group, and the rest 8 rats were set as the normal group. ① Normal group (NG): would be performed the liver transplantation, the liver and blood would just be obtained for the determination of relative indexes. ② A group (AG): recombinant adenovirus Ad.VSG-hBCL-2 (synthesized and provided by the Gene Laboratory of Shanghai Second Military Medical University) was used to pretreat the donor. ③ B group (BG): empty virus vector Ad-EGFP was used to pretreat the donor. ④ C group (CG): 0.9 % sodium chloride pretreated the donor. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal use protocol has been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Huai'an First People's Hospital, Nanjing Medical University.

Donor treatment

AG: ① 12 h fasting and 4 h water-inhibition was performed preoperatively, then performed intraperitoneal anesthesia with ketamine (80 mg/kg). ② After anesthesia, an incision was made on the middle abdomen to open the abdomen, push the intestine to the left lower quadrant, exposed the portal vein and mesenteric vein. ③ Used a fine needle to penetrate the mesenteric vein, slowly injected 2 ml suspension of Ad.VSG-hBCL-2 (1.5×10^{12} pfu/L), performed the compression hemostasis for 1min, then closed the abdomen. ④ 1 h after awok from anesthesia, the rats were free for water and food.

BG: The same procedure as AG was performed to BG with 2 ml empty virus vector suspension of Ad-EGFP (1.5×10^{12} pfu/L) to pretreat the donor rats.

CG: 2 ml 0.9% sodium chloride solution was used to pretreat the donor rats, with the same method as AG.

Receptor treatment and specimen collection

AG: ① Ad.VSG-hBCL-2 pretreated the donor rats, then the donor liver was obtained and weighed (g) 36 h later. The donor liver was preserved with lactated ringer perfusion, the cold ischemia time was 4 h. ② The "two-off method" was used to perform the orthotopic liver transplantation [4, 5], and 6 h after the receptors restored the portal vein reflow, the liver was put into the abdomen under anesthesia. The epidural anesthesia catheter was placed into the common bile duct, calculated the bile volume per minute according to the length of bile in the catheter, which was then divided by the weight of the donor liver to obtain the bile secretion per liver gram per minute (μl/min/g liver). The measurement method of bile secretion in the epidural catheter: the epidural catheter was placed into the proximal common bile duct, measured the catheter length of biliary drainage per minute. Determination of catheter volume: 50 μl colored liquid was injected with a micro syringe into the catheter to measure its length for 6 repeated times, and the result was 1 mm = 0.65±0.26 μl. ③ 1.0 ml blood was obtained from the hepatic inferior vena cava for the determination of AST, ALT and LDH. ④ The left lobe of liver was cut and preserved in liquid nitrogen for Western Blot determination (NEB Beijing Co. kit). The right anterior lobe of liver was excised, placed in 10% formalin, and embedded in paraffin for immunohistochemical detection (Wuhan Boster immunohisto-

Table 1. Bile secretion volume after 6 h of portal vein reflow

Group	n	Bile secretion (μl/min.g liver)
NG	8	9.52±1.38
AG	8	7.17±1.25*
BG	8	2.50±0.64**,#
CG	8	2.73±0.62**,#

NG: Normal control; AG: recombinant adenovirus Ad.VSG-hBCL-2; BG: empty virus vector Ad-EGFP; CG: 0.9 % sodium chloride. Note: Compared with NG, *P < 0.05, **P < 0.01; compared with AG, #P < 0.01.

chemistry kit) and TUNEL assay (Shanghai Roch company kit). The right anterior lobe of liver was also fixed with 4% glutaraldehyde liquid, and sent for the TEM observation (Hitachi H-600).

The specimen collection method of BG and CG was the same as AG.

Specimen detection

The biochemical assay was performed for the detection of plasma AST, ALT and LDH levels, Western Blot for the detection of Bcl-2 expression in rat liver, immunohistochemistry for the detection of Bcl-2 and TNF-α expression in liver tissue, TUNEL assay for liver tissue apoptosis, and TEM for the changes of subcellular structures of liver tissue.

Apoptotic index (AI) and immunohistochemical quantitative analysis

① Image-Pro Plus 6.0 image analysis software was used, and the sliced tissue was magnified with × 400 optical microscope, the images were taken into the analysis system, and the positively stained area and field area were selected and measured. Five fields were randomly selected in each slice, the positive area of Bcl-2 and TNF-α expression was divided by the field area, and the mean (%) was set as the "positive area". ② AI 5 fields (magnified × 400 times) of each specimen slice under optical microscope were randomly selected, and calculated the average apoptotic cells number out of 100 cells, and AI was expressed as the percentage form.

Statistical analysis

SAS 9.1.3 statistical software package was used for the analysis of variance, the test data were expressed as $\bar{x} \pm s$, with the test level $\alpha = 0.05$; $P < 0.05$ was considered as statistical significance.

Results

Bile secretion amount

The bile secretion was measured after 6 h of portal vein reflow. There was a significant increasing in AG when compared with BG and CG, with statistically significant difference ($P < 0.01$), while there was no statistically significant difference between BG and CG ($P > 0.05$). The bile secretion volume in AG was also statistically different from NG ($P < 0.05$) (**Table 1**).

Serum ALT, AST and LDH detection in each group

6 h after portal vein reflow, the serum ALT, AST and LDH in AG were significantly lower than BG and CG, with significant difference ($P < 0.01$), while there was no statistically significant difference in the indicators between BG and CG. The liver functions of AG group showed significant differences when compared with NG ($P < 0.01$) (**Table 2**).

Expression of Bcl-2 protein in rat liver

Western Blot detection was performed, and the results revealed that there was no expression of Bcl-2 protein in normal liver of NG, in BG and CG, only trace amount of Bcl-2 was expressed, while in AG, the Bcl-2 protein was expressed significantly (**Figure 1**).

Detection of immunohistochemistry and apoptosis

There was no obvious Bcl-2 protein expression in normal rat liver cells. The most liver cytoplasm in AG was stained brown, while weak expression could be seen in the hepatocytes of BG and CG occasionally, there was significant difference in the positive expression percentage among AG, BG and CG ($P < 0.01$), while there was no statistically significant difference between BG and CG ($P > 0.05$) (**Table 3**).

There was no expression in normal rat liver, a large number of hepatocytes and sinusoidal endothelial cells in BG and CG were observed to be stained brown, while the pale brown

Table 2. Liver function comparison of receptors in each group 6 h later (μ/L)

Group	n	ALT	AST	LDH
NG	8	32.32±8.76	198.67±15.49	2563.58±246.27
AG	8	851.25±206.91*	1289.63±183.92*	3661.25±375.40*
BG	8	2653.75±441.17*,#	3865.68±336.87*,#	7971.25±739.45*,#
CG	8	2321.25±398.23*,#	3577.83±269.39*,#	7883.75±640.96*,#

Note: Compared with NG, *P < 0.01; compared with AG, #P < 0.01.

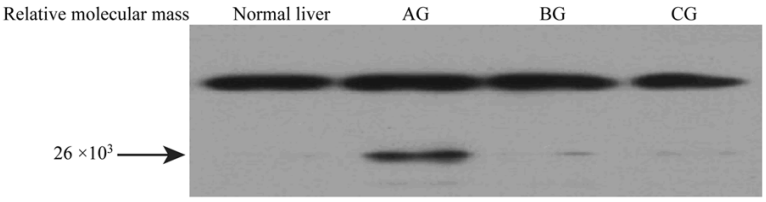

Figure 1. Expression of Bcl-2 in the receptor liver tissues 6 h after portal vein reflow.

Table 3. Comparison of Bcl-2, TNF-α and AI on the postoperative 6th h in each group (%)

Group	AI	Bcl-2	TNF-α
NG	1.87±0.63	0.17±0.05	3.12±0.95
AG	10.76±2.79*	85.45±11.35*	16.52±2.81*
BG	61.71±11.30*,#	1.84±0.12*,#	85.59±10.67*,#
CG	60.22±12.38*,#	1.76±0.35*,#	83.10±10.32*,#

Note: Compared with NG, *P < 0.01; compared with AG, #P < 0.01.

weak-positive cells in AG could be seen only in a few local areas, there was significant difference in the positive expression percentage among AG, BG and CG (P < 0.01), while there was no statistically significant difference between BG and CG (P > 0.05) (**Table 3**).

Occasional apoptosis could be seen in normal rat liver cells. The TUNEL assay was performed 6 h after portal vein reflow to detect the apoptosis of transplanted hepatocytes, and the results revealed that the brown apoptotic cells could be seen in the 3 groups, while the apoptosis in AG was significantly less than BG and CG. The AI of AG was significantly different from BG and CG (P < 0.01), while there was no statistically significant difference between BG and CG (P > 0.05) (**Table 3**).

Hepatic pathological changes

TEM revealed that the organelles in AG had integrated structures, the mitochondria arranged in neat rows, without significant change, rough endoplasmic reticulum expansion could be seen occasionally. In BG, the hepatic mitochondria swelled, the cytoplasm was loose, lysosomes increased, the intranuclear chromosomes gathered marginally, the liver cell membrane and nuclear membrane deformed, the apoptotic cells and apoptotic bodies could also be seen. The pathological changes of CG and BG were basically the same as each other.

Discussion

Injury caused by ischemia/reperfusion can be a limiting factor in the clinical settings of liver surgery and transplantation. The aim of this study was to test the hypothesis that Bcl-2 decreases reperfusion injury after rat liver transplantation.

Our study shows that the portal vein-transfected recombinant adenovirus Ad.VSG-hBCL-2 could be effectively expressed in rat liver, and the high expressed Bcl-2 could reduce the ischemia/reperfusion injury in the transplanted liver. Specifically, we evaluated the hypothesis that up-regulation of the Bcl-2 gene is a mechanism of protective effects against hepatic ischemia-reperfusion injury after rat orthotopic liver transplantation. Specifically, we evaluated the hypothesis that up-regulation of the Bcl-2 gene is a mechanism of diazoxide cytoprotection.

The current studies showed [11] that the early apoptosis after the transplantation was the important reason which would lead to the liver preservation-reperfusion injury. Apoptosis could not only be found in liver cells, but also found in cholangitis epithelial cells and vascular endothelial cells [12, 13].

The hepatic parenchymal cell loss induced by apoptosis could delay the function-appearing time of the transplanted organs, which, in severe situation, would result in the early dysfunction of graft and liver failure [14, 15]. Ischemia/reperfusion injury-induced liver apop-

tosis is closely related to such factors as mitochondrial function damage, energy metabolism disorder, oxygen free radicals generation, intracellular Ca^{2+} overload and the generation of a large number of cytokines (TNF-α, IL-8) [16-19]. Different pathological factor-induced apoptosis would be regulated by P53, Fas, Myc and Bcl-2 family, and the receptor-mediation is the main pathway of hepatocyte apoptosis, which could inhibitly regulate the apoptosis, and also mean the effective protection against the ischemia/reperfusion injury of the transplanted liver, even further reduce acute or chronic organ transplantation rejections [20-22]. Because Bcl-2 gene is in the terminal part of apoptotic regulation, and its protein expression could reduce the formation of oxygen free radicals and lipid peroxide, inhibit the transmembrane flow of Ca^{2+}, the activation of apoptosis protease (Caspase) and its function as the organelle-stabilizer, thereby preventing various factors-mediated apoptosis, and therefore Bcl-2 is the most important anti-apoptosis gene in human body [23-25].

In view of the important position of Bcl-2 in the regulation of apoptosis, there are a lot of research reports about its roles in cancer in recent years [26], but very few was reported about the Bcl-2 gene transfection therapy in the organ transplantation in the past decade. Bilbao *et al*. [27] blocked partial liver blood vessels with microvascular clamp to establish liver ischemia-reperfusion model, and found that Bcl-2 gene had the protective role in liver transplantation. However, because the hepatic-blood-vessels-blocking time was very short (20~40 min), which was likely to cause serious liver ischemia/reperfusion injury under normal circumstances, and the incidence of ischemia/reperfusion injury is not only related with the damages caused by ischemia and reflow, but also closely linked with a series of pathological changes occurred after portal vein occlusion [28]. So it would be much more reasonable to establish the animal orthotopic liver transplantation model to pretreat the donor and study the mechanism and the protective effects of ischemia-reperfusion injury.

We established the rat orthotopic liver transplantation model, the donor liver experienced a long cold ischemic time and reperfusion injury after transplantation, while the portal-vein-transfected Bcl-2 gene could still be effectively expressed. The postoperative liver function damage significantly reduced, and the subcellular morphology was basically normal, indicating that the functional proteins expressed by Bcl-2 gene had a significant protective effect towards the transplanted liver.

In addition, we also found that the normal liver tissue would not express Bcl-2, but trace amount of Bcl-2 was expressed in the control group, suggesting that liver tissues could regulate the expression of apoptosis inhibitory Bcl-2 gene in ischemia/reperfusion injury, and play a role of self-protection towards the cell damages. This finding suggested that the donor pretreatment with the transfected recombinant adenovirus, which carried Bcl-2 gene, to protect the transplanted liver was in line with the physiological regulatory mechanism in which the body would act in the case of damages.

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Disclosure of conflict of interest

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