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ORIGINAL ARTICLE

Improvement of recipient survival after small size graft liver transplantation in rats with preischemic manipulation or administering antisense against nuclear factor-κB

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Summary

The survival rate of small size graft liver transplantation (SSGLT) in rats is inversely related to graft volume. The present study aims to evaluate the protective effects of preischemic manipulation (PIM) and oligodeoxynucleotide (ODN) antisense against NF-κB on graft failure and animal survival. The protective effects of PIM and NF-κB ODN antisense were investigated in a rat SSGLT model. The graft function and survival of recipient animals over 3 weeks were monitored, and *in situ* staining for apoptotic cells in the graft tissue was examined. Both PIM and NF-κB antisense treatment significantly improved the survival of small graft-transplanted rats compared with the SSGLT group, lowered serum levels of alanine and aspartate aminotransferases, as well as tumor necrosis factor-α (TNF-α) levels, and minimized apoptotic cell counts in the liver sections. Moreover, the enhanced activation of NF-κB in the SSGLT group was diminished in both PIM and NF-κB antisense-treated groups. The findings suggest that enhanced NF-κB activation and TNF-α production may be involved in the ischemia/reperfusion-associated small size graft injury, and that PIM and antisense against NK-κB are effective in the attenuation of the small size graft injury, and improve the recipient animal survival.

Introduction

Living donor liver transplantation (LDLT) is an alternative practice to ease the increasing donor organ demand, and to shorten the time on the waiting list; in term, it may also yield a better survival rate in recipients. LDLT takes advantage of the tremendous regenerative capacity of the liver, and represents a major advance in transplantation medicine. In man, LDLT is often associated with the 'small-for-size syndrome' characterized by synthetic dysfunction, elevated aminotransferase and prolonged cholestasis [1]. Hundreds of successful LDLTs were performed each year in the US [2], but the number of LDLT has decreased during the last 3 years due to ethical issues related to donor safety and other concerns. One of these concerns is what factors affect the graft function and recipient survival, and how we can improve the survival and function of small size grafts in recipients? Few studies are available which explore the cause and pathogenesis of ischemia/reperfusion (I/R)-associated small graft injury, and it appears that the recipient survival is inversely related to graft volume [3]. Therefore, there is a huge demand to seek answers to remaining questions, and to improve the graft survival and function in recipients. In this context, the aim of the present study was to assess the protective effects of preischemic manipulation (PIM) and oligodeoxynucleotide (ODN) antisense against NF-κB on graft failure and animal survival in a rat model of

small size graft liver transplantation (SSGLT). We hypothesized that the PIM imposed to the donor liver before the harvest would elicit protection for subsequent I/R-associated injury to the small size grafts, and that NF-κB is a critical transcription factor that is involved in the mediation of I/R-induced graft damage and failure. Our results support the notion that both PIM and the treatment of NF-κB antisense were effective in improving recipient animal survival and graft function.

Materials and methods

Animal experiments

Male out-bred SD rats (250–300 g) were obtained from the Nanjing Medical University Animal Care Center (Nanjing, China). The protocol of animal experiments was approved by the Institutional Ethical Committee of Animal Experimentation, and the experiments were performed strictly according to governmental and international guidelines on animal experimentation. Eight pairs of rats were used for whole liver transplantation according to a method described previously [4,5]. SSGLT was performed according to the method described by Kamada N *et al.* [4] and both donor and recipient rats were anesthetized with ketamine (65 mg/kg, i.p.) and xylazine (4 mg/kg, i.p.) for the surgical procedure. Donor livers were harvested with a rapid perfusion of 20 ml of Ringer's balanced solution through a catheter placed in the portal vein. All donor livers were dissected and prepared beyond the enterocoelia, taking care of protecting the blood supplying and avoiding hurting inferior vena cava and portal vein. The common bile duct was cannulated with a polyethylene cuff. The portal vein was dissected, and a polyethylene cuff was inserted into the portal vein and infrahepatic vena cava. The isolated graft was put in a container filled with ice-cold saline for graft reduction. The left lateral lobe, left portion of the median lobe, and two caudate lobes were separately removed by ligation with 5-0 silk suture to achieve a graft size of 45% volume. The reduced graft was composed of the right portion of the median lobe and right lobe. The small size graft was transplanted into a body weight-matched rat following a two-cuff method described by Kamada N *et al.* [4,6]. Briefly, the suprahepatic vena cava was reconstructed using continuous 7-0 polypropylene sutures. The portal vein was re-anastomosed using a two-cuff technique [6,7]. When the anastomosis of portal vein and suprahepatic vena cava was completed, the liver was reperfused. After that the anastomosis of infrahepatic vena cava was completed by the same cuff method. The common bile duct was connected by inserting the cuff in the donor bile duct into the recipient bile duct. After the completion of the surgical procedure, recipient animals were under an intensive postoperation care for recovery.

The recipient animals with SSGLT were divided into three groups, including SSGLT controls, SSGLT plus PIM; and SSGLT plus NF-κB ODN antisense treatment by infusion with perfusate containing antisense NF-κB. For PIM, the portal vein and hepatic artery were blocked for 5 min to cause donor liver ischemia, followed by reperfusion for 5 min, and repeated for one more cycle before the donor livers were harvested. Phosphorothioate modified 5′-GGGAAACAGATCGTCCATGGC-3′anti P65 subunits ODN antisense was synthesized by BioAsia Company, Shanghai, China, and dissolved in Ringer's balanced solution. For administering NF-κB ODN antisense, after the portal vein blood flow was re-established, NF-κB ODN antisense in 2–3 ml of Ringer's balanced solution (0.5 mg/ml) was infused into the portal vein twice. Each rat received 2–3 mg of NF-κB ODN in total 4–6 ml Ringer's balance solution. Blood samples were collected at various time points from the penial vein for serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), tumor necrosis factor-α (TNF-α) determination. Serum ALT and AST levels were determined with an automatic Biochemical Analyzer from Olympus Inc., Tokyo, Japan, in a clinical laboratory of the University Hospital. Serum TNF-α levels were determined with an ELISA kit from R&D Systems, Inc (Minneapolis, MN, USA) according to the manufacturer's instruction and expressed as ng/ml.

In situ staining of apoptotic cells in liver graft tissue

Part of liver specimens was collected with an additional surgical procedure at one day of transplant. Liver specimens were fixed in 10% neutral formalin, embedded in paraffin and sectioned routinely. Liver sections were deparaffinized, dehydrated, and exposed to 3% hydrogen peroxide for inactivation of endogenous peroxidase activity. Sections were stained for apoptotic cells with a TUNEL *in situ* staining kit from R&D Systems, Inc and apoptotic cells were stained in brown with 3,3-diaminobenzidine color development, and then were counted in each section for 10-high power fields, and expressed as cells/10 high fields [8].

DNA binding activity by electrophoretic mobility shift assay

For determining the activation of NF-κB, electromobility shift assay (EMSA) was performed using a nonradioactive detection method. All the reagents and kits needed for this assay were obtained from Pierce Biochemicals, Rockford, IL, USA. Nuclear fractions from snap-frozen liver

tissue were isolated as previously [9], and DNA binding activity was determined with the 3′-end biotin labeled NF-κB oligos, followed by separation in 6% polyacrylamide DNA retardation gels, and detected with an EMSA detection kit as described by us earlier [10].

Statistical analysis

All data are shown in mean values ± SD. Data were analyzed by ANOVA test plus Newman–Keuls test for multiple comparisons between two given groups. $P < 0.05$ was considered statistically significant.

Results

We performed both whole liver transplantation and SSGLT in rats. As shown in Fig. 1, 3 weeks after the whole liver transplantation, 88% of recipient rats survived, whereas, only 37% of the recipient rats with small size liver grafts (45% of the standard liver volume) survived ($P < 0.01$). The survival rates in groups of SSGLT plus PIM and SSGLT plus NF-κB ODN treatment were significantly improved compared with the SSGLT (75 and 56 vs. 37%, $P < 0.01$ and 0.05). Thus, both PIM and the treatment with phosphorothioate-modified ODN antisense against NF-κB p65 subunit during the implantation of liver grafts were beneficial for the improvement of recipient animal survival.

Consistent with the improvement of survival rates in recipient rats with SSGLT by either PIM or the treatment of NF-κB ODN, serum ALT and AST levels were significantly lower in these two groups than SSGLT alone at all time points during 3-weeks follow-up (Fig. 2). Moreover, enhanced DNA binding activity in SSGLT was markedly attenuated by either PIM or NF-κB ODN antisense treatment (Fig. 3a). In addition, serum TNF-α levels which were markedly elevated in SSGLT group were decreased significantly in the recipient rats with SSGLT plus preischemic intervention or NF-κB ODN treatment (Fig. 3b). Hence, it is evident that both PIM and the treatment of NF-κB ODN antisense during a window of donor harvest and implantation of small size liver grafts improved the graft function, and reduced NF-κB activation and the release of toxic cytokines, such as TNF-α. These two factors are responsible for the inflammatory and apoptotic processes in the grafts after the transplantation.

To investigate the apoptotic process during the SSGLT, we performed TUNEL assay staining in tissue sections of

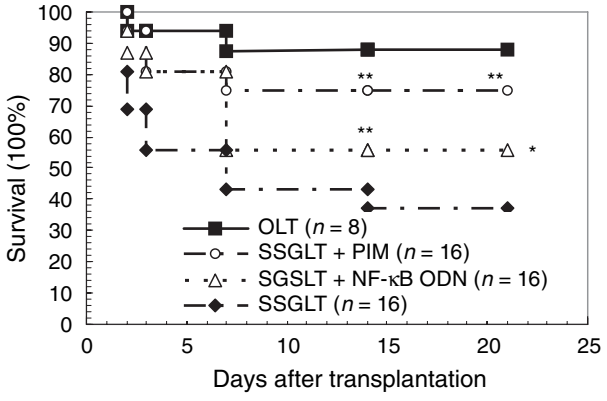

Figure 1 The survival rates of animals that received whole liver or small size liver graft transplantation. WLT = whole liver transplantation ($n = 8$); SSGLT = small size graft transplantation ($n = 16$); PIM = preischemic manipulation; NF-κB ODN = nuclear factor-κB oligodeoxynucleotide antisense treatment ($n = 16$). *, **$P < 0.05$ and 0.01 compared with corresponding time points in SSGLT group ($n = 16$).

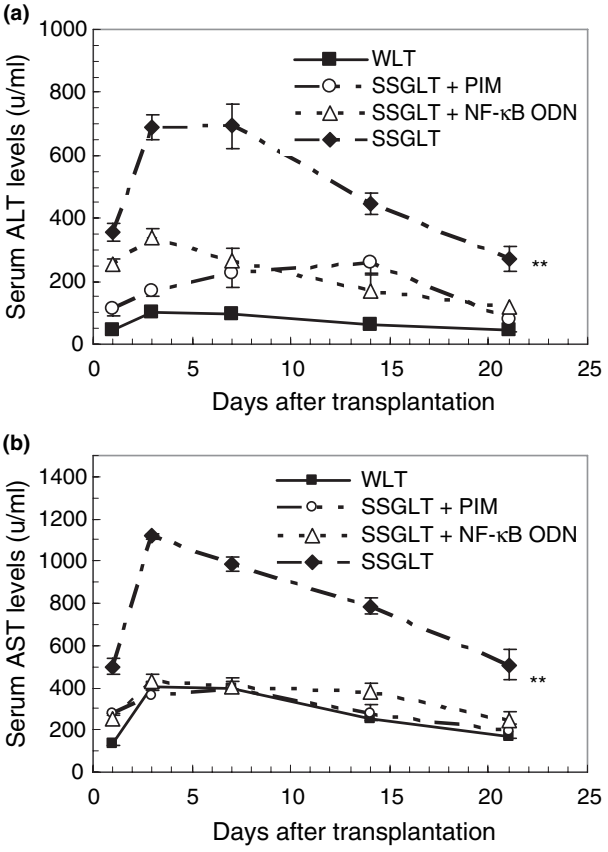

Figure 2 Serum alanine aminotransferase (a) and aspartate aminotransferase (b) levels in recipient rats after whole liver or small size graft liver transplantation (SSGLT). The number of animals in each group is the same as Fig. 1. WLT = whole liver transplantation; SSGLT = small size graft liver transplantation; PIM = preischemic manipulation; NF-κB ODN = nuclear factor-κB oligodeoxynucleotide antisense treatment. **$P < 0.01$ compared to the SSGLT group at all corresponding time points.

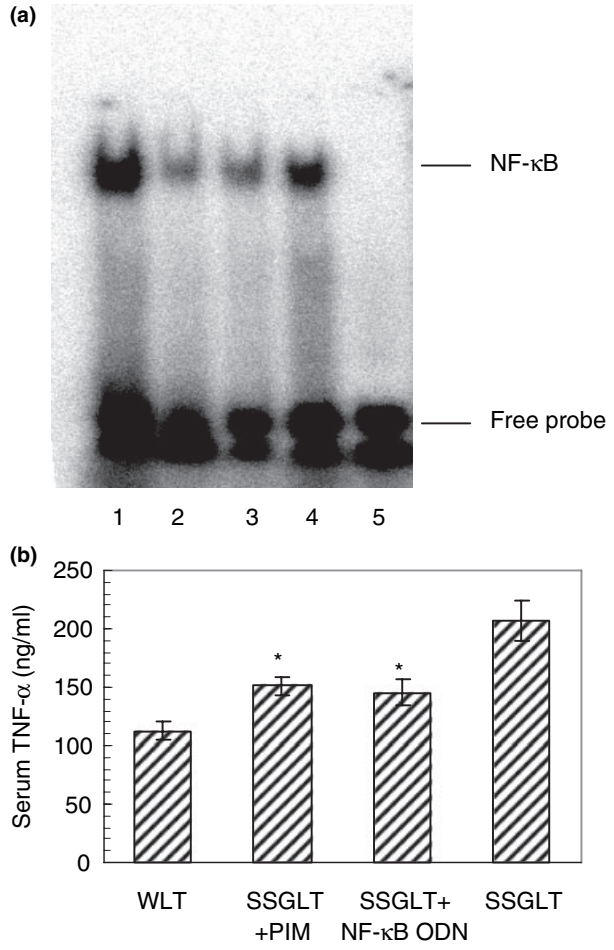

Figure 3 Representative of DNA binding activity of nuclear factor-κB (NF-κB) in small size graft liver transplantation (SSGLT) (a) and serum TNF-α levels. The DNA binding activity of NF-κB was determined 2 h after the completion of transplant by electromobility shift assay as detailed in the Materials and Methods section. The tissue was obtained from the right lobe of the grafts in all groups. Lane 1 = SSGLT; lane 2 = SSGLT + NF-κB ODN; lane 3 = SSGLT + preischemic manipulation; lane 4 = whole liver transplantation; lane 5 = sense control. (b) The serum TNF-α was determined 2 h after the completion of the whole or small size graft liver transplantation with an ELISA kit from R & D Systems Ins.

liver grafts. As shown in Fig. 4, liver sections from the small size graft (Fig. 4b) in recipient rats displayed much more cells with positive brown nuclear staining than the whole liver grafts (Fig. 4a). The PIM or the treatment of NF-κB ODN antisense minimized cells with positive TUNEL staining (Fig. 4c and d), which was verified by the apoptotic cell counts in these sections (Fig. 4e). Thus, it is clear that enhanced apoptosis in SSGLT accounts for the poor graft function and recipient survival, and that both PIM and the treatment of NF-κB ODN antisense attenuated the apoptotic process in SSGLT, and improved the recipient survival and graft function.

Discussion

In the present study, we employed SSGLT in rats as a model to assess the protective effects of PIM and the treatment of ODN antisense against NF-κB. We found that recipient rats with 45% graft volume had a much lower survival rate than those with orthotopic (whole) liver transplantation (OLT), as well as poorer graft function, and increased serum TNF-α, and enhanced DNA binding activity. These changes were partially corrected by either PIM before the donor organ harvest, or administration of ODN antisense against NF-κB p65 subunits after establishing the portal vein circulation. These two approaches were undertaken during the window of donor organ harvest from a recipient and the implantation of the liver graft in a recipient; thus, they did not add extra procedures to either the donor or the recipient, and are clinically acceptable when biosafety issues are fully validated.

The shortage of donor organs drives to use marginal grafts whenever available, and to undertake LDLT, both of which are alternatives for shortening the duration on a waiting list for those with a failing liver or occurrence of metastasis of liver cancer. However, either marginal grafts or grafts from living donors are small in size, and may accompany with other conditions, such as steatosis or fibrosis. These are the critical factors affecting the graft function and survival after the transplantation. Hence, developing novel strategies to improve the donor organ quality, reduce graft damage as a result of I/R, and improve graft survival is one of the major tasks in achieving an ideal outcome and improving prognosis of liver transplant recipients. The approaches we employed are to meet these needs; and they were evaluated in a preclinical model of SSGLT, which mimics LDLT and provides a tool for assessing any potential therapeutic strategies.

Ischemia-reperfusion-associated donor organ damage is inevitable in almost every transplant depending on the duration of cold ischemia preservation, so is in the SSGLT. To reduce the damage, a various approaches or antioxidant therapeutics have been explored to target specific factors or pathways which lead to the damage during the I/R procedure [10,11]. One of these approaches, the ischemic precondition, has been shown to be effective in improving graft function after OLT in both animal experiments and clinical practice [12,13] by improving overall energy status, minimizing inflammatory infiltration. We employed this approach before the donor liver was harvested, and the results showed that the PIM significantly improved animal survival in small size graft recipients. At the same time, the graft function was much

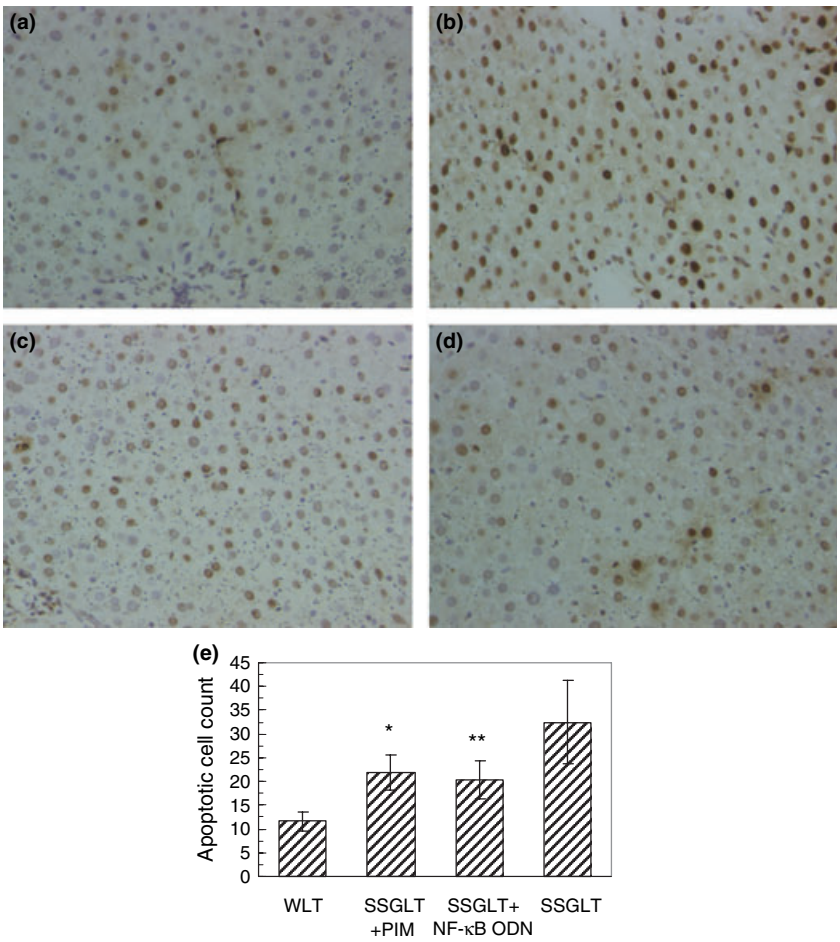

Figure 4 Apoptotic cells stained by an *in situ* TUNEL detection method. Representative micrographs of TUNEL staining of apoptotic cells. (a) Whole liver transplantation. (b) Small size graft liver transplantation (SSGLT). (c) SSGLT plus preischemic manipulation. (d) SSGLT plus NF-κB ODN. (e) Apoptotic cell counts in the liver grafts one day after the transplantation. Apoptosis-positive cells were stained with an *in situ* TUNEL method. WLT = whole liver transplantation; NF-κB ODN = nuclear factor-κB oligodeoxynucleotide antisense treatment; PIM = preischemic manipulation; *, **$P < 0.05$ and 0.01 compared to the small size graft liver transplantation group.

better than those without the manipulation as indicated by decreased serum aminotransferase and TNF-α levels, as well as less liver apoptotic staining. Therefore, the PIM protects the grafts from the subsequent I/R-associated damage not only in orthotopic liver transplantation as reported previously [14], but also in small size graft transplants. The protection against the I/R-induced damage is associated with reduced toxic cytokine TNF-α levels, decreased DNA binding activity of NF-κB as indicated in the present study, as well as the induction of heat shock protein-70 and hem-oxygenase-1, both of which are beneficial in the subsequent I/R procedure [15]. It seems that TNF-α production mainly originated from Kupffer cells because the use of a Kupffer cell depleting agent, gadolinium chloride, or transplant in TNF-α receptor-1 null mice improved the survival rates in 30% graft volume-transplanted mice [16].

It has been evident that I/R-induced injury in liver grafts is associated with enhanced nuclear translocation and DNA binding activity of NF-κB [10,17]. Another approach we employed in this study to minimize the I/R-induced damage in SSGLT was to use ODN antisense against NF-κB p65 subunit. As shown in our EMSA experiment, DNA binding activity was much more enhanced in recipient animals with SSGLT than the whole liver transplantation group, and enhanced DNA binding activity of NF-κB was associated with toxic cytokine release, such as TNF-α, and oxidant stress-associated inflammation. Thus, administering a solution containing antisense against NF-κB would inhibit NF-κB expression, and its down-stream effects in the mediation of inflammation and apoptosis. Our results showed that the administering ODN antisense against NF-κB during the completion of graft implantation is a practical and effective strategy in improving the recipient animal survival, correcting graft function, and attenuating apoptosis of hepatocytes, as a result of reduced serum TNF-α levels and reduced proinflammatory activity of NF-κB. This result is consistent with a report by Banafsche *et al.* [18] in orthotopic liver transplantation in rats. Our approach in modulating NF-κB activity at the transcriptional levels confers a proof of the principle and the effectiveness of

this innovative molecular therapy in liver transplant during the window of transplant surgery, and goes along with other donor cytoprotective or immunomodulating approaches as summarized in an extensive review [19].

In conclusion, the findings in the present study suggest that enhanced NF-κB activation and production of TNF-α may be involved in the I/R-associated small size graft injury, and that PIM and ODN antisense against NF-κB are effective in the attenuation of the small size graft injury and improvement of recipient animal survival. These innovative approaches would be beneficial in improving the graft function and survival especially when marginal liver grafts are used in liver transplantation.

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Authorship

J-MQ designed the study, collected and analyzed data. HZ, X-FW, G-QL performed all animal experiments. X-PC critical review and comments. JW validated the data, wrote the paper.

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