Published in final edited form as:

Am J Transplant. 2008 September ; 8(9): 1819–1828. doi:10.1111/j.1600-6143.2008.02336.x.

C-Jun N-Terminal Kinase 2 Promotes Graft Injury via the Mitochondrial Permeability Transition After Mouse Liver Transplantation

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Abstract

The c-Jun N-terminal kinase (JNK) pathway enhances graft injury after liver transplantation (LT). We hypothesized that the JNK2 isoform promotes graft injury via the mitochondrial permeability transition (MPT). Livers of C57BL/6J (wild-type, WT) and JNK2 knockout (KO) mice were transplanted into WT recipients after 30 h of cold storage in UW solution. Injury after implantation was assessed by serum ALT, histological necrosis, TUNEL, Caspase 3 activity, 30-day survival, and cytochrome *c* and 4-hydroxynonenal immunostaining. Multiphoton microscopy after LT monitored mitochondrial membrane potential *in vivo*. After LT, ALT increased three times more in WT compared to KO ($p < 0.05$). Necrosis and TUNEL were more than two times greater in WT than KO ($p < 0.05$). Immunostaining showed a >80% decrease of mitochondrial cytochrome *c* release in KO compared to WT ($p < 0.01$). Lipid peroxidation was similarly decreased. Every KO graft but one survived longer than all WT grafts ($p < 0.05$, Kaplan-Meier). After LT, depolarization of mitochondria occurred in 73% of WT hepatocytes, which decreased to 28% in KO ($p < 0.05$). In conclusion, donor JNK2 promotes injury after mouse LT via the MPT. MPT inhibition using specific JNK2 inhibitors may be useful in protecting grafts against adverse outcomes from ischemia/reperfusion injury.

Keywords

Apoptosis; c-Jun N-terminal kinase 2; ischemia/reperfusion injury; liver transplantation; mitochondrial permeability transition; necrosis; storage/reperfusion injury; survival

Introduction

After cold ischemic storage of livers for transplantation, reperfusion injury can occur that leads to poor early graft function and graft failure. Sinusoidal endothelial cells are particularly sensitive to ischemia/reperfusion (I/R) injury and undergo necrosis and apoptosis *in vivo* and *in vitro* (1–4). At later stages, hepatocytes undergo necrotic cell death and apoptosis (4–6). Apoptosis and necrosis share common pathways, including the mitochondrial permeability transition (MPT) (7).

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In liver and other organs, the MPT plays a prominent role in the pathogenesis of I/R injury (5,6). ATP depletion after MPT onset produces necrotic cell killing (oncosis), whereas swelling after the MPT leads to outer membrane rupture and release of proapoptotic proteins like cytochrome *c* (5,8). Whether necrosis or apoptosis occurs depends on the extent of ATP depletion. If ATP depletion is severe, necrosis occurs since caspase activation requires ATP (or dATP). Since necrosis requires severe ATP depletion, more moderate ATP depletion leads to apoptosis instead (5,6).

c-Jun N-terminal kinase (JNK) is a stress-activated protein kinase. JNK becomes activated in response to physical stresses, such as I/R, hypoxia, ultraviolet (UV) radiation and exposure to inflammatory mediators and pathogen-derived antigens (9,10). JNK-dependent phosphorylation of the transcription factor c-Jun/AP-1 is a central mechanism that promotes *de novo* gene expression leading to an enhanced immune response (10,11). In situations of both acute and chronic stress, JNK may also induce apoptosis via JNK-mediated phosphorylation of proapoptotic Bcl₂ family proteins, leading to permeabilization of mitochondria, release of cytochrome *c* and activation of Apaf-1/caspase 9 complexes (12). In contrast with wild-type cells, JNK-deficient cells are resistant to UV-induced apoptosis and do not release cytochrome *c* from mitochondria (13).

Previous work from this laboratory showed that inhibition of JNK with the inhibitor CC-401 decreased graft injury, improved liver function and increased graft survival after liver transplantation (LT) (14). More recently, we showed that mitochondrial dysfunction via the MPT is a key event in liver graft injury and that specific inhibition of MPT decreases liver injury and improves graft survival (15). Mitochondrial dysfunction mediated through the MPT is also important in TNF α - and acetaminophen-dependent toxicity to hepatocytes (16,17). Recent work shows involvement of the JNK2 isoform in TNF- α and acetaminophen-induced liver toxicity, which implicates a role of JNK2 in mitochondrially mediated hepatic injury (18,19). Accordingly, we hypothesize that JNK2 activation after LT promotes MPT-dependent graft injury. Here, we test this hypothesis and show that transplantation of livers from JNK2 knockout mice decreases graft injury, improves hepatic function and increases graft survival. These improvements are associated with diminished mitochondrial dysfunction.

Materials and Methods

Mouse LT

All experiments were conducted using protocols approved by the Institutional Animal Care and Use Committee. Livers from male C57BL/6 (wild-type) and JNK2-deficient (B6.129S2-*Mapk9^{tm1Flv}/J* on a C57BL background) mice were transplanted into C57BL/6J mouse recipients after cold storage in University of Wisconsin (UW) solution, as described previously (20). Donor and recipient mice weighed 19–24 g. For donor operations under ether anesthesia, livers were freed from peritoneal attachments, the gall bladders were removed and the common bile ducts were cannulated with polytetrafluoroethylene (PTFE) tubing (0.2 mm inner diameter, Zeus, Orangeburg, SC) and divided. After portal infusion of ice-cold UW solution (3 mL), the livers were excised, cuffs were placed on the portal veins and subhepatic inferior vena cava using 20-gauge and 18-gauge i.v. catheters, respectively (Braun Medical, Bethlehem, PA). Excised livers were immersed in UW solution inside glass containers and stored in an ice-water bath for 30 h until implantation. Time for the donor procedure averaged 22 min.

In the recipient operation, livers of recipient mice were removed after dividing the bile duct at the hilum and clamping and dividing the suprahepatic inferior cava, portal vein and subhepatic inferior cava. Cold-stored donor livers were then implanted by anastomosing the suprahepatic vena cava with a running 10–0 nylon suture and connecting the portal veins and infrahepatic vena cava by cuff insertion. Bile ducts were anastomosed over PTFE stents. The recipient

operation averaged 45 min, and the portal vein clamp time averaged 15 min. For sham operations under ether anesthesia, wild-type and JNK2-deficient mice were laparotomized. After 45 min, the abdomen was closed.

Alanine aminotransferase

Blood samples to measure alanine aminotransferase (ALT) were collected from the inferior vena cava 8 h after transplantation for analysis by standard methods.

Histology

Histology was evaluated 8 h after LT. Liver tissues were fixed by immersion in 4% buffered paraformaldehyde and embedded in paraffin. Sections (4 μ m) were stained with hematoxylin and eosin (H&E). Ten random fields were assessed for necrosis by standard morphologic criteria (*e.g.* loss of architecture, vacuolization, karyolysis, increased eosinophilia). Images were captured on a microscope (Zeiss Axiovert 100 microscope, Thornwood, NY), and the area percentage of necrosis was quantified using a computer program (AxioQuant, BD Bioimaging Systems, San Jose, CA).

Immunohistochemistry

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) was performed on paraffin sections using an *in situ* cell death detection kit (Roche Diagnostics, Penzberg, Germany). TUNEL-positive cells were counted by light microscopy in 10 random high-power fields (HPF). To assess cytochrome *c* and lipid peroxidation in graft tissue, immunocytochemistry with mouse cytochrome *c* antibody (BD Pharmingen, Franklin Lakes, NJ) and rabbit 4-hydroxy-2-nonenal (HNE) antibody against HNE-adducts (Alpha Diagnostic International, San Antonio, TX) was performed. Visualization of cytochrome *c* and HNE adducts was done with horse radish peroxidase (HRP) and diaminobenzidine (DAB) chromogen with hematoxylin counterstaining according to the manufacturer's instructions (DAKO, Carpinteria, CA). Parenchymal cells with punctate cytochrome *c* staining were counted by light microscopy in 10 random HPF as a percentage of total cells.

Caspase 3

Liver tissue (~50 mg) was homogenized (Polytron PT-MR2100, Kinematica, Luzern, Switzerland) in 1 mL of lysis buffer containing 0.1% CHAPS, 2 mM EDTA, 5 mM DTT, 1 mM pepabloc, 10 ng/mL pepstatin A, 10 ng/mL aprotinin, 20 μ g/mL leupeptin and 10 mM HEPES buffer, pH 7.4. The lysate was centrifuged at 15 000 rpm for 30 min. Activity of caspase 3 in the supernatant was determined using a Caspase 3 Colorimetric Assay Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Activity was normalized to protein concentration of each sample and expressed as fold increase compared to sham.

Survival study

Livers of wild-type and JNK2-deficient mice were transplanted into wild-type recipients in a randomized, prospective fashion by allocation to shuffled preplanned dates of surgery. The recipients were followed 30 days after surgery. Mice dying or requiring euthanasia for presumed surgical complications within 4 h of transplantation were excluded.

Intravital microscopy

At 4 h after transplantation, mice were anesthetized with pentobarbital (50 mg/kg) and connected to a small animal ventilator via a tracheostomy and respiratory tube (20-gauge catheter), as described previously (20). A catheter (0.4 mm inner diameter, Zeus, Orangeburg, SC) was inserted into the right carotid artery. Using a syringe pump, the membrane potential-

indicating fluorophore rhodamine 123 (2 μ mol/mouse) was infused via the catheter over 10 min. During this time a laparotomy was performed using the previous incision line. After prone positioning of the mouse, the liver was gently withdrawn from the abdominal cavity and placed over a glass coverslip on the stage of a Zeiss LSM 510 NLO inverted laser scanning confocal microscope. Multiphoton images were collected with a 63X 1.3 NA water-immersion objective lens using 820-nm excitation light from a Chameleon Ultra Ti-Sapphire pulsed laser (Coherent, Santa Clara, CA), as described previously (15). Green rhodamine 123 fluorescence was collected through a 525 ± 25 nm band pass filter. During image acquisition, the respirator was turned off for ~ 5 s to eliminate breathing movement artifacts. In 20 fields per liver, parenchymal cells were scored for bright punctate rhodamine 123 fluorescence representing cells with polarized mitochondria or a dimmer diffuse cytosolic fluorescence representing cells with depolarized mitochondria. Image analysis was performed in a blinded fashion.

Statistical analysis

Group sizes are indicated in the figure legends and were typically 5 to 6 mice per experimental condition to adequately power the experiments, as determined in previous studies from our laboratory (20,21). Data are presented as means $\pm$ SE, unless otherwise noted. Statistical analysis was performed by Student's *t* test, ANOVA plus Student-Newman-Keuls test or Kaplan-Meier test, as appropriate, using $p < 0.05$ as the criterion of significance. Slides, images and tissue samples were given numerical codes and all analyses were performed in a blinded fashion.

Results

Decreased ALT release and graft necrosis after transplantation of JNK2-deficient livers

Livers from wild-type and JNK2-deficient mice were stored for 30 h and transplanted, or mice were subjected to sham operation. Sham-operated wild-type and JNK2 knockout mice had serum ALT averaging 121 ± 17 U/L at 8 h postoperatively (Figure 1). When wild-type livers were stored for 30 h and transplanted into wild-type mice, recipient ALT increased to $30\,001 \pm 4899$ U/L. By comparison, transplantation of JNK2-deficient livers into wild-type recipients resulted in ALT levels of $12\,219 \pm 3460$ U/L ($p < 0.05$ vs. wild-type graft; Figure 1).

Liver histology was normal and indistinguishable in unoperated and sham-operated wild-type and JNK2-deficient mice (Figure 2A and data not shown). By contrast at 8 h after transplantation of wild-type liver grafts, large areas of necrosis developed with a predominantly pericentral and midzonal distribution (Figure 2B). Hepatic necrosis decreased from $39 \pm 4.2\%$ in wild-type grafts to $14.7 \pm 2.9\%$ in JNK2-deficient liver grafts ($p < 0.05$, Figure 2C, D). Thus, transplantation of JNK2-deficient livers decreased hepatic necrosis by more than 60%.

Decreased graft apoptosis and mitochondrial cytochrome c release after transplantation of JNK2-deficient livers

TUNEL was performed on tissue sections to assess double-stranded DNA breaks that are characteristic of apoptosis. TUNEL-positive cells were rare after sham operation in both wild-type and JNK2-deficient grafts, averaging less than one cell/HPF (Figure 3A, G). At 8 h after transplantation of wild-type livers, TUNEL in nonnecrotic areas increased to 16.8 ± 2.6 cells/HPF (Figure 3B, G). By contrast in JNK2-deficient liver grafts, TUNEL was 60% less at 6.8 ± 1.8 cells/HPF ($p < 0.05$, Figure 3C, G). As a percentage of all cells, the percentage of TUNEL was $3.2 \pm 1.2\%$ after transplantation of wild-type livers vs. $0.6 \pm 0.2\%$ after transplantation of JNK2-deficient livers, respectively ($p < 0.05$).

As another measure of apoptosis, caspase 3 activity was measured in liver extracts at 8 h after transplantation of wild-type and JNK2-deficient livers in comparison to sham-operated livers

(Figure 3H). After sham operation, caspase activity was nearly undetectable. After transplantation of wild-type livers, caspase 3 activity increased significantly by 18-fold, which decreased to 9-fold in JNK2-deficient grafts, a difference that approached statistical significance ($p = 0.08$, Figure 3H). Unfortunately, because of insufficient liver tissue, caspase 3 activity could only be measured in about half the liver grafts, which may account for the lack of significance despite the large difference between means.

Cytochrome *c* release was also assessed in tissue sections at 8 h after transplantation. After sham operation, brown immunolabeling for cytochrome *c* was punctate in all hepatocytes, indicating mitochondrial localization (Figure 3D). After transplantation of wild-type livers, hepatocellular cytochrome *c* staining became diffuse, indicating mitochondrial release of cytochrome *c* into the cytosol (Figure 3E). In JNK2-deficient grafts, however, most cells retained punctate cytochrome *c* staining, indicating that cytochrome *c* was not released from mitochondria (Figure 3F). Overall, $12.8 \pm 2.6\%$ of parenchymal cells in wild-type grafts had punctate cytochrome *c* labeling compared to $97.8 \pm 0.7\%$ for sham livers ($p < 0.01$, Figure 3J) and $73.1 \pm 6.6\%$ for JNK2-deficient liver grafts ($p < 0.01$, Figure 3J). Thus, punctate cytochrome *c* staining was more than 5-fold greater in JNK2-deficient liver grafts than wild-type grafts ($p < 0.01$, Figure 3J).

Delayed death after transplantation of JNK2-deficient livers

Graft survival was assessed out to 30 days. After transplantation of wild-type livers, six of six mice died within 36 h, and 30-day survival was 0% (Figure 4). After transplantation of JNK2-deficient livers, four of the six rats survived 48 h or more after surgery, and one mouse survived for 30 days ($p < 0.05$, Figure 4). Overall, survival was prolonged in all but one JNK2-deficient grafts compared to all wild-type grafts ($p < 0.05$, Kaplan-Meier).

Improved mitochondrial function in vivo after transplantation of JNK2-deficient livers

At 4 h after sham operation, intravital multiphoton microscopy revealed bright fluorescence of rhodamine 123 in hepatocytes whose punctate pattern denoted polarization of individual mitochondria. Cytosolic and nuclear areas had little rhodamine 123 fluorescence, and no differences were observed between livers of wild-type and JNK2-deficient mice (Figure 5A). We also imaged rhodamine 123 fluorescence at 4 h after LT, since we previously found in rat liver grafts destined to fail that mitochondrial depolarization but not yet cell death occurred at this time point (15). In agreement with our earlier study in rats, rhodamine 123 staining became diffuse and dim in many hepatocytes at 4 h after transplantation of wild-type mouse livers (Figure 5B, white arrows).

After transplantation of JNK2-deficient livers, mitochondria depolarized in fewer hepatocytes than in wild-type grafts (Figure 5C, white arrow). In JNK2-deficient liver grafts, most hepatocytes exhibited bright, punctate staining by rhodamine 123, indicative of polarized mitochondria. We also assessed rhodamine 123 fluorescence in JNK2-deficient grafts at 36 h after transplantation, a timepoint at which most recipients of wild-type grafts had already died, but recipients of JNK2-deficient grafts were still living. At 36 h postoperatively, mitochondria of many more hepatocytes became depolarized compared to the 4 h time point (Figure 5D, white arrows). Thus, JNK2 deficiency delayed rather than prevented mitochondrial dysfunction after LT.

After transplantation and sham operation, hepatocytes were scored for rhodamine 123 staining (Figure 6). In sham-operated livers, 0.05 ± 0.002 hepatocytes/HPF contained depolarized mitochondria. At 4 h after transplantation with wild-type livers, 7.6 ± 1.1 hepatocytes/HPF contained depolarized mitochondria (Figure 6). By contrast, at 4 h after transplantation of JNK2-deficient livers, hepatocytes with depolarized mitochondria decreased by nearly half to

4 ± 0.5 hepatocytes/HPF ($p < 0.05$ vs. vehicle, Figure 6). At 36 h after transplantation, however, the number of depolarized hepatocytes in JNK2-deficient grafts increased to 6.9 ± 3.2 hepatocytes/HPF, which was not significantly different to mitochondrial depolarization occurring in wild-type grafts after 4 h. Thus, JNK2-deficient livers conferred initial protection against mitochondrial depolarization after transplantation.

Decreased oxidative stress after transplantation of JNK2-deficient livers

We used 4-HNE immunohistochemistry to evaluate oxidative stress in liver grafts 8 h postoperatively. HNE is a product of lipid peroxidation that forms protein adducts that are recognized by anti-HNE antibodies. After sham operation, the brown reaction product of HNE immunohistochemistry was virtually undetectable (Figure 7A). By contrast at 8 h after transplantation of wild-type livers, wide confluent areas of HNE immunoreactivity developed in pericentral and midzonal areas with relative sparing the periportal regions (Figure 7B, areas noted by stars). After transplantation of JNK2-deficient livers, HNE immunoreactivity was substantially decreased and confined mostly to pericentral regions (Figure 7C).

Discussion

Decreased graft injury after transplantation of JNK2-deficient livers

Damage to liver caused by ischemia and reperfusion can be a limiting factor in clinical settings of liver surgery and transplantation. The aim of this study was to evaluate the hypothesis that cold liver ischemia and reperfusion induce mitochondrial dysfunction through the JNK signaling pathway. Specifically, we evaluated whether the JNK isoform JNK2, previously shown to promote hepatic injury after various nonsurgical liver stresses (18,19), increases graft injury after LT. In liver, mitochondrial dysfunction via the MPT precedes hepatocellular killing in *in vitro* and *in vivo* models of I/R (5,8,15,22,23). Our findings showed that JNK2-deficient liver grafts had decreased necrosis and apoptosis after transplantation. In addition, in comparison to wild-type liver grafts, failure of JNK2-deficient grafts was delayed. Protection in JNK2-deficient grafts was associated with the prevention of mitochondrial depolarization, as assessed by intravital multiphoton microscopy and decreased oxidative stress.

Reperfusion injury after transplantation induces necrosis and apoptosis through JNK2 signaling

The goal of this study was to investigate the specific role of the JNK2 isoform in graft injury. Our previous study showed that pan-JNK inhibition with the JNK-inhibitor CC-401 decreases graft necrosis and apoptosis and improves survival after orthotopic rat LT (14). Similarly, the JNK inhibitor, leflunomide, protects against acetaminophen-induced hepatotoxicity (24). However, existing JNK inhibitors are nonspecific and inhibit both JNK isoforms, JNK1 and JNK2, that are present in liver, and which play specific roles in either protection or propagation of liver injury (25). Investigations of deoxycholic acid-induced hepatocyte apoptosis suggest that JNK2 signaling is cytoprotective (26). Furthermore, TNF α -dependent apoptosis increases in JNK2-deficient mouse fibroblasts, whereas apoptosis decreases in JNK1-deficient cells (27). Other studies, however, indicate that JNK2 promotes cell death. In PC12 cells, JNK2 translocates to mitochondria and mediates cytochrome *c* release and apoptosis in response to 6-hydroxydopamine (28). In liver particularly, TNF α - and acetaminophen-induced hepatotoxicity is decreased in JNK2-deficient mice (18,19).

In other models of liver injury, such as cold storage/reperfusion injury, the specific role of different JNK isoforms is unknown. Hence, we investigated a possible role of JNK2 in liver graft injury by comparing transplanted wild-type and JNK2-deficient mouse liver grafts. JNK2 deficiency decreased both necrosis and apoptosis in liver grafts. Necrosis assessed by ALT and histology and apoptosis by TUNEL and caspase 3 activity were both decreased by about 60%

in JNK2-deficient liver grafts compared to wild-type (Figures 1–3). Necrosis was the predominant mode of cell death in our studies. After transplantation of wild-type livers, necrosis involved 39% of the parenchyma, whereas TUNEL occurred in only 3.2% of cells. These findings support earlier assessments that apoptosis contributes much less to graft storage/reperfusion injury than necrosis (6,15,29). However, both modes of cell death can occur through a mitochondrial pathway involving the MPT, a phenomenon of necroptosis (5). Since the absence of JNK2 protected against both necrosis and apoptosis, graft injury and cell death after storage/reperfusion would appear to be largely a necroptotic phenomenon.

JNK2 involvement in the formation of reactive oxygen species (ROS) after LT

Generation of ROS in reperfused livers is a major factor contributing to warm I/R injury (30–32). ROS formation also contributes to cold storage/reperfusion injury in livers preserved for transplantation surgery (33–35). A consequence of ROS formation is peroxidation of polyunsaturated fatty acids like linoleic and arachidonic acids, which leads to HNE generation and formation of HNE-protein adducts. After transplantation of wild-type livers, immunocytochemistry showed a marked increase of HNE adducts in the liver parenchyma and more specifically in hepatocytes (Figure 7). After transplantation of JNK2-deficient livers, HNE formation was greatly decreased (Figure 7). Thus, JNK2 signaling may also upregulate ROS generation after cold storage/reperfusion. ROS directly damage proteins, lipids and DNA and also potentially induce the MPT in mitochondria. Hence, JNK2-dependent ROS formation may contribute to the MPT and mitochondrial dysfunction in transplanted liver grafts either directly or secondarily due to damage to proteins and other cellular constituents.

JNK2 signaling after cold storage and reperfusion induces mitochondrial depolarization and promotes graft failure

To test the hypothesis that JNK2 promotes the MPT and mitochondrial dysfunction, we used intravital multiphoton microscopy to assess mitochondrial polarization, as described previously (15). This technique directly assesses mitochondrial polarization in the livers of living animals. After transplantation of wild-type livers, widespread mitochondrial depolarization occurred in graft hepatocytes within 4 h. Mitochondrial depolarization occurred prior to cell death and thus was not simply the consequence of onset of necrosis. After transplantation of JNK2-deficient livers, mitochondrial depolarization was substantially attenuated (Figures 5 and 6). Previously, we showed that mitochondrial depolarization in liver grafts after transplantation is prevented by NIM811 and minocycline, MPT inhibitors that have no direct effect on mitochondrial respiration, respiratory control or oxidative phosphorylation (15). Hence, mitochondrial polarization visualized by intravital microscopy as utilized in this study represents a reliable tool to monitor the occurrence of the MPT, which was attenuated in JNK2-deficient mice.

Several studies also indicate involvement of the MPT in acetaminophen hepatotoxicity (17, 36,37). After acetaminophen, activated JNK translocates to mitochondria and promotes MPT onset, which is prevented by JNK inhibition (37). Thus, protection against depolarization in JNK2-deficient liver grafts implies that JNK2 is directly involved in promoting the MPT in wild-type grafts after cold I/R stress. After the MPT, mitochondria swell and release cytochrome *c*, which can promote apoptosis. In accordance, cytochrome *c* immunohistochemistry revealed release of cytochrome *c* in wild-type liver grafts that was attenuated more than 80% in JNK2-deficient hepatocytes (Figure 3D–F, J). However, other factors are likely promoting the MPT, since mitochondrial depolarization was delayed rather than prevented in JNK2-deficient liver grafts and still occurred at 36 h after transplantation (Figures 5 and 6). Future studies will be needed to characterize how other mitochondrial and nonmitochondrial changes contribute causally to graft injury and failure from storage/reperfusion injury and promote the MPT.

The long cold storage time of 30 h used in this study caused 100% recipient mortality within 36 h after transplantation of wild-type grafts. After transplantation of JNK2-deficient livers, recipient mortality was delayed, and one of the recipients survived to 30 days. Overall, every JNK2-deficient graft but one survived longer than all WT grafts, and Kaplan-Meier survival improved significantly. Nonetheless, the majority of JNK2 grafts failed at a later time (Figure 4). This delay of graft failure was consistent with the delayed onset of mitochondrial depolarization (Figures 5 and 6).

In clinical LT, storage/reperfusion injury is strongly dependent on the time of cold ischemic storage (38,39). Current clinical practice is to limit storage time to less than 20 h so that initial graft dysfunction and failure are relatively rare. Similarly, in rodent LT, most grafts are successful after such shorter times of storage, and a more prolonged storage time is required to cause substantial graft injury and failure. Accordingly, we chose a cold storage time of 30 h, since our previous work showed that 18 h of storage did not cause enough injury to produce graft failure (20). Moreover, initial experiments showed that pronounced mitochondrial depolarization that occurred in liver grafts after 30 h of storage did not occur after 18 h of storage (data not shown).

Previous work shows improved survival in JNK2-deficient mice after treatment with the hepatotoxicants, galactosamine/lipopolysaccharide and acetaminophen (18,19). In these studies, extrahepatic JNK2 deficiency cannot be ruled out as contributing to protection. The present work utilizing JNK2-deficient and wild-type liver grafts implies, however, that hepatic cytoprotection by JNK2 deficiency is largely attributable to the intrahepatic rather than the systemic effects of JNK2 deficiency. More generally, comparison of hepatotoxicity in transplanted wild-type and JNK2-deficient liver graft recipients could provide a way to evaluate the specific roles of intrahepatic versus extrahepatic JNK2 deficiency on liver injury and mortality.

Our findings suggest that JNK2 could provide a unique therapeutic target in the treatment and prevention of cold storage/reperfusion injury after LT. At present, CC-401 and other existing JNK inhibitors are nonspecific and inhibit all JNK isoforms, JNK1, JNK2 and JNK3 (25). JNK2 in our model of LT plays a detrimental role, but JNK1 and/or JNK3 may have beneficial effects in livers and other tissues, especially since JNK1/JNK2 double knockout mice are not viable. Thus, a specific JNK2 inhibitor might provide a more specific benefit after LT and decrease the potential of toxicity mediated by JNK1 and JNK3 inhibition. Marginal donor livers have increased susceptibility to storage/reperfusion injury (40,41). Thus, treatment of marginal livers with a specific JNK2 inhibitor has the potential for allowing more frequent and successful use of marginal livers in clinical transplantation.

Acknowledgements

This work was supported, in part, by Grants DK37034, DK73336, DK70844 and C06 RR015455 from the National Institutes of Health and Grant DFG TH1328/1-1 from the Deutsche Forschungsgemeinschaft. Dr. Theruvath is the recipient of an American Liver Foundation Postdoctoral Research Fellowship Award. Portions of this work were presented at the American Transplant Congress, San Francisco, CA, May 5-9, 2007.

Abbreviations

ALT	alanine aminotransferase
ATP	adenosine triphosphate
CHAPS	

	3[(3-cholamidopropyl)dimethylammonio]-propanesulfonic acid
DAB	3,3'-diaminobenzidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H&E	hematoxylin and eosin
HNE	hydroxyl-2-nonenal
HPF	high power field
HRP	horse radish peroxidase
JNK	c-Jun N-terminal kinase
LT	liver transplantation
MPT	mitochondrial permeability transition
ROS	reactive oxygen species
TUNEL	terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
UW	University of Wisconsin cold storage solution

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Figure 1. ALT release is decreased after transplantation of JNK2-deficient livers

Serum ALT was assessed 8 h after sham operation or mouse LT. Transplants were performed with either wild-type (JNK2 ^{+/+}) or JNK2-deficient (JNK2 ^{-/-}) liver donors into wild-type recipients. Sham groups had normal and comparable values and only the JNK2 ^{-/-} sham group is shown. Group sizes were: sham, 3; JNK2 ^{+/+} LT, 5; JNK2 ^{-/-} LT, 6. *, $p < 0.05$ versus JNK2 ^{+/+}.

Figure 2. Necrosis is decreased after transplantation of JNK2-deficient livers

Sham operation or LT was performed, as described in Figure 1. At 8 h postoperatively, necrosis was assessed by H&E histology after sham-operation (A) and after transplantation of wild-type (JNK2 +/+) (B) and JNK2-deficient (JNK2 -/-) (C) livers. Panel (D) shows necrosis as percentage of area in liver sections averaged from five livers per group. Necrosis in sham-operated wild-type and JNK2 -/- livers was absent. Group size was five per transplant group. Bar is 100 μ m. *, $p < 0.05$ versus JNK2 +/+.

Figure 3. Apoptosis and cytochrome *c* release are decreased after transplantation of JNK2-deficient livers

Sham operation or LT was performed, as described in Figure 1. At 8 h postoperatively, TUNEL was assessed after sham operation (A) and after transplantation of wild-type (JNK2 +/+) (B) and JNK2-deficient (JNK2 -/-) (C) livers. Cytochrome *c* immunostaining was also performed after sham operation (D) and after transplantation of wild-type (JNK2 +/+) (E) and JNK2-deficient (JNK2 -/-) (F) livers. Panel (G) quantifies the TUNEL positive cells per high power field (HPF). Panel (H) shows Caspase 3 activity in liver homogenates at 8 h after transplantation. Panel (J) quantifies liver cells with punctate mitochondrial cytochrome *c*, expressed as percentage of total cells. For both TUNEL and cytochrome *c* staining, wild-type and JNK2 -/- sham groups were comparable and only data for JNK2 -/- sham operations are shown. Bar is 50 μm for A–C and 20 μm for D–F. *, $p < 0.05$ versus JNK2 +/+; **, $p < 0.01$ versus sham. #, $p = 0.08$ versus JNK2 +/+. Group sizes were five per group except for Caspase 3 and sham groups in which group sizes were three.

Figure 4. Graft survival is improved after transplantation of JNK2-deficient livers

Thirty-day survival was assessed in mice transplanted with either wild-type (JNK2 +/+) or JNK2-deficient (JNK2 -/-) livers. Size of individual groups was 6. *, $p < 0.05$ compared to JNK2 +/+.

Figure 5. JNK2-deficient liver grafts have decreased mitochondrial depolarization

Livers from wild-type (JNK2 +/+) or JNK2-deficient (JNK2 -/-) mice were transplanted, as described in METHODS. At 4 h and 36 h postoperatively, liver grafts were visualized by intravital multiphoton microscopy of rhodamine 123 fluorescence. Shown are representative images of green rhodamine 123 fluorescence collected 4 h after sham operation (A) and after transplantation of wild-type (JNK2 +/+) (B) and JNK2-deficient (JNK2 -/-) (C) livers. Panel (D) shows a representative image of a JNK2-deficient graft at 36 h after reperfusion. Punctate staining of rhodamine 123 denoted polarization of individual mitochondria, whereas dim diffuse cellular staining indicated mitochondrial depolarization, as examples at white arrows illustrate. Depolarization in wild-type and JNK2 -/- sham livers was comparable and virtually zero. Only a JNK2 -/- sham liver is shown. Individual group size was five. Bar is 30 μ m.

Figure 6. Quantification of mitochondrial depolarization after LT

The number of hepatocytes with depolarized mitochondria (dim and diffuse rhodamine 123 staining) at 4 h and 36 h after transplantation from intravital multiphoton microscopy was counted as percentage of hepatocytes. Depolarization in wild-type and JNK2 ^{-/-} sham groups was comparable and virtually zero. Only the JNK2 ^{-/-} sham group is plotted. Individual group size was five. *, $p < 0.05$.

Figure 7. JNK2-deficient liver grafts have decreased oxidative stress

At 8 h after reperfusion, immunohistochemical staining was performed for 4-hydroxynonenal-modified proteins (4-HNE, *brown*) after sham operation (A) and after transplantation of wild-type (JNK2 +/+) (B) and JNK2-deficient (JNK2 -/-) (C) livers. Note greater brown staining (asterisks) in wild-type liver grafts (B) compared to JNK2-deficient grafts (C). 4-HNE immunoreactivity in wild-type and JNK2 -/- sham livers was comparable and virtually zero. Only a JNK2 -/- sham liver is shown. Individual group size was 3–4. Bar is 50 μ m.