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Human heat shock protein 27 overexpressing mice are protected against hepatic ischemia and reperfusion injury

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Abstract

Background—Hepatic ischemia reperfusion injury (IRI) is a major clinical problem during the perioperative period and occurs frequently after major hepatic resection or liver transplantation. Our laboratory previously demonstrated that exogenous A₁ adenosine receptor (AR) activation protects against renal IRI via upregulation and phosphorylation of heat shock protein 27 (HSP27).

Methods—In this study, we utilized mice overexpressing human HSP27 (huHSP27 OE) to determine whether these mice are protected against liver IRI.

Results—After hepatic IR, the huHSP27 OE mice had significant protection against liver injury (reduced alanine transferase) and necrosis (H&E staining) compared to the HSP27 WT mice. The huHSP27 OE mice also showed less induction of pro-inflammatory mRNA MIP-2, reduced neutrophil infiltration and decreased apoptosis (caspase 3 fragmentation and DNA laddering) compared to the HSP27 WT mice. Finally, the huHSP27 OE mice showed significantly less disruption of filamentous actin in hepatocytes as well as bile canaliculi of the ischemic lobes compared to the HSP27 WT mice. Depletion of Kupffer cells with gadolinium chloride provided significant protection against liver IRI in HSP27 WT mice but not in huHSP27 OE mice suggesting that the overexpression of huHSP27 in the Kupffer cells may be responsible for the hepatic protection observed in huHSP27 OE mice.

Conclusions—Our results show that the overexpression of huHSP27 in Kupffer cells of the liver may be responsible for the protection against hepatic IRI *in vivo* by reducing necrosis and apoptosis and by stabilizing F-actin with subsequent reductions in inflammation and pro-inflammatory neutrophil infiltration. Harnessing the mechanisms of cytoprotection with HSP27 may lead to new therapies for the management of perioperative hepatic IRI.

Keywords

apoptosis; inflammation; liver; necrosis; neutrophil

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Introduction

Hepatic ischemia reperfusion injury (IRI) is a major clinical problem during the perioperative period and frequently follows major hepatic resection or liver transplantation (1-3). Hepatic IRI not only causes liver dysfunction but frequently results in injury of extra-hepatic organs including the lung, kidney and heart (4-6). Therefore, a better understanding of the pathophysiology of hepatic IR and the identification of therapeutic methods to attenuate this injury could have significant clinical implications.

Our laboratory previously demonstrated that A₁ adenosine receptor (AR) activation protected against renal IRI in mice (7,8) as well as in rats (9,10). Mechanistically, we have previously shown that A₁AR activation phosphorylates and upregulates heat shock protein 27 (HSP27) in cultured renal proximal tubule cells and in mice (11,12). HSP27 is a member of family of chaperone proteins that are up-regulated in response to increases in temperature, as well as a wide range of cellular stresses including hypoxia, ischemia and exposure to toxic drugs (13-16). Increased expression of HSP27 serves to defend against cell injury or death by acting as chaperones facilitating proper polypeptide folding and aberrant protein removal (17-19). Furthermore, HSP27 is a potent anti-apoptotic protein and is a key stabilizer of the actin cytoskeleton; both of these cellular effects lead to increased resistance against cell death (20-22).

It is unknown whether overexpression of HSP27 would protect against warm hepatic IRI *in vivo*. Therefore, in this study, we tested the hypothesis that mice with global overexpression of human HSP27 (huHSP27) would show an increased resistance against liver IRI.

Materials and Methods

Mice

The generation and initial characterization of the huHSP27 OE and WT mice with a C57BL/10 and CBA/Ca background has been described previously (23). Heterozygous HSP27 transgenic mice were bred and resulting male littermates (huHSP27 OE or HSP27 WT mice) were used in the subsequent studies. We performed PCR on genomic DNA extracted from tails for and performed RTPCR for human HSP27 from total RNA extracted from every mouse studied using primers that distinguish human from mouse HSP27. In preliminary studies, we also confirmed human HSP27 protein overexpression by immunoblotting for the mouse and human forms of HSP27 (Santa Cruz Biotechnologies, CA) as described previously (11,24). We also purchased C57BL/6 mice (Harlan laboratories, Indianapolis, IN) to breed with huHSP27 OE and HSP27 WT mice.

Murine model of hepatic IR

After Columbia University IACUC approval, male huHSP27 OE or HSP27 WT mice (25-30g) were anesthetized with intraperitoneal pentobarbital (50 mg/kg or to effect). Mice were placed under a heating lamp and on a 37°C heating pad. After a midline laparotomy and intraperitoneal application of 20 U heparin, left lateral and median lobes of the liver were subjected to ischemia with a microaneurysm clip occluding the hepatic triad above the bifurcation. This method of partial hepatic ischemia results in a segmental (~70%) hepatic ischemia but spares the right lobe of the liver and prevents mesenteric venous congestion by allowing portal decompression throughout the right and caudate lobes of the liver (25,26). The liver was then repositioned in the peritoneal cavity in its original location for 60 minutes. The liver was kept moist with gauze soaked in 0.9% normal saline. The body temperature was monitored by an infrared temperature sensor (Linear Laboratories, Fremont, CA) every 10 min. and maintained at 37°C using a heating lamp and a heating pad. After 60 minutes, the liver was reperfed and the wound

closed. Sham operated mice were subjected to laparotomy and identical liver manipulations without vascular occlusion. Two, 4 and 24 hr after reperfusion, plasma was collected for the measurement of alanine aminotransferase (ALT). The liver tissue subjected to IR was collected to measure percent liver necrosis (2, 4 and 24 hr after IR), neutrophil infiltration (with immunohistochemistry, 4 and 24 hr after IR), apoptosis (with terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling staining, caspase 3 immunoblotting and DNA laddering, 24 hr after IR), and inflammation by RT-PCR for pro-inflammatory mRNAs (4 hr after IR). In order to determine whether the Kupffer cells mediate the protective effects of huHSP27 overexpression, we pretreated a separate cohort of HSP27 WT and huHSP27 OE mice with gadolinium chloride (10 mg/kg i.v.) 24 hr prior to liver IRI (27).

Assessment of liver dysfunction

Plasma ALT was measured by using a Prep-Profile II kit and a VetScan VS2 Point-of-Care Analyzer (Union City, CA) and expressed as units/liter (U/L).

Histology and quantification of hepatic necrosis after IRI

For histological preparations, explanted murine livers were fixed in 10% formalin solution overnight. After automated dehydration through a graded alcohol series, transverse liver slices were embedded in paraffin, sectioned at 4 μ m, and stained with hematoxylin-eosin (H&E). To quantify the degree of hepatic necrosis, H&E stains were digitally photographed and the percent of necrotic area was quantified with NIH IMAGE (Image-J, 1.37v) software by a person who was blinded to the treatment each animal had received. Twenty random sections were investigated per slide to determine the percentage of necrotic area.

Assessment of hepatic inflammation

Liver inflammation after IR was determined by the detection of neutrophil infiltration using immunohistochemistry 24 hr after hepatic IR and by measuring mRNA encoding markers of inflammation, including keratinocyte derived cytokine (KC), intercellular adhesion molecule-1 (ICAM-1), monocyte chemoattractive protein-1 (MCP-1), macrophage inflammatory protein-2 (MIP-2), and tumor necrosis factor-alpha (TNF- α) 4 hr after liver IR with semiquantitative RT-PCR analysis as described previously (7,12,28).

To verify the results of our semiquantitative RT-PCR analysis, we also performed quantitative real-time RT-PCR analysis on the liver samples. The results were consistent with the semiquantitative analysis (data not shown). Q-RTPCR was performed with the MyiQ Real Time Detection System (Bio-Rad, Hercules, CA) using SYBR Green I Brilliant Mastermix (Stratagene, La Jolla, CA). The cDNA template was synthesized using Omniscript Reverse Transcriptase and oligo-dT primer (Qiagen, Valencia, CA). Specificity of the amplification was checked by melting curve analysis and by agarose gel electrophoresis. All reactions were performed in duplicate with appropriate negative controls. The Ct values were determined by using Mx3000P software. Values were normalized for GAPDH mRNA.

Detection of hepatic apoptosis

We utilized 2 independent assays to assess the degree of liver apoptosis after IR: DNA laddering and detection of caspase 3 fragmentation by immunoblotting. For DNA laddering, liver tissues were removed 24 hr after IR, apoptotic DNA fragments were extracted according to the methods of Herrmann et al. (29) and was electrophoresed at 70 V in a 2.0% agarose gel in Tris-acetate-EDTA buffer. This method of DNA extraction selectively isolates apoptotic, fragmented DNA and leaves behind the intact chromatin. The gel was stained with ethidium bromide and photographed under UV illumination. DNA ladder markers (100 bp) were added

to a lane of each gel as a reference for the analysis of internucleosomal DNA fragmentation. Caspase 3 immunoblotting was performed as described previously (11).

Immunoblot analyses for phosphorylated and total HSP27 in liver tissue were determined as described previously (30). Primary mouse antibodies for phospho-HSP27 and total HSP27 were from Santa Cruz Biotechnologies (Santa Cruz, CA). The secondary antibody (goat anti-rabbit or anti-mouse IgG conjugated to horseradish peroxidase at 1:5000 dilution) was detected with enhanced chemiluminescence immunoblotting detection reagents (Amersham, Piscataway, NJ, USA), with subsequent exposure to a CCD camera coupled to a UVP Bio-imaging System (Upland, CA, USA) and a personal computer.

Filamentous (F)-actin staining of liver sections

As breakdown of F-actin occurs early after IR, we visualized the F-actin cytoskeleton by staining with phalloidin as an early index of liver injury (31,32). Twenty four hr after liver IR, liver tissues were embedded in Tissue-Tek oxytetracycline compound (Fisher Scientific, Pittsburgh, PA) and cut into 5µm sections. To reduce background staining, the sections were incubated in 1% FBS dissolved in PBS for 10 minutes at room temperature. The sections were then stained with Alexafluor 594 (Red)-labeled phalloidin (Invitrogen, Carlsbad, CA) for 30 min at 37°C in a humidified chamber in the dark. Sections were then washed twice in PBS and mounted with Vectashield (Vector Laboratories, Burlingame, CA). F-actin images were visualized with Olympus IX81 epifluorescence microscope (Tokyo, Japan) and captured and stored using SlideBook 4.2 software (Intelligent Imaging Innovations Inc., Denver, CO) on a personal computer. The mean fluorescent intensity after background correction was calculated from 5 random blinded sections with identical surface areas per slide to quantify F-actin degradation after liver IR. We also counted the number of intact bile canalicular membranes as well as their fluorescent intensities after F-actin staining (400× fields). To minimize the variations in fluorescent intensity, slides from sham-operated animals and animals subjected to liver were processed together.

Immunohistochemistry for HSP27 to determine co-localization with F-actin

Livers from HSP27 WT or huHSP27 OE mice were embedded in Tissue-Tek oxytetracycline compound and cut into 5µm sections. The sections were air dried and fixed in 4% paraformaldehyde in PBS and briefly washed in PBS. To reduce background staining, the sections were incubated in 1% FBS dissolved in PBS for 10 min. at room temperature. The sections were then stained with Alexafluor 488 (Green)-labeled phalloidin (Invitrogen, Carlsbad, CA) for 30 min. at 37°C in a humidified chamber in the dark. Excess phalloidin was removed by washing twice in PBS and the sections were incubated with anti-HSP27 antibody (Abcam, Cambridge, MA) antibody (recognizes both human and mouse HSP27) for 1 h at room temperature. After washing with PBS, the sections were incubated with a goat anti-rabbit Alexa Fluor 594 conjugated secondary antibody (red) for 1 h at room temperature in the dark and mounted with Vectashield (Vector Laboratories, Burlingame, CA). Co-localization (yellow) of F-actin (green) with HSP27 (red) was determined with Z sections taken with a step size of 0.25 µm (for total of Z-distance of 5µm) with an Olympus Spinning Disk Confocal System microscope and analyzed with the Slidebook software using Pearson's correlation.

Analysis of liver vascular permeability

Changes in liver vascular permeability were assessed by quantitating extravasations of Evans blue dye (EBD) into the tissue as described by Awad *et al.* (33) with some modifications. Briefly, 2% EBD (Sigma Biosciences, St. Louis, MO) was administered at a dose of 20 mg/kg iv 24 hr after liver injury. One hour later, mice were killed and perfused through the heart with PBS and EDTA with 10 cc of cold saline and heparin. Livers were then removed, allowed to dry overnight at 60°C, and the dry weights were determined. EBD was extracted in

formamide (20 ml/g dry tissue; Sigma Biosciences), homogenized, and incubated at 60°C overnight. Homogenized samples were centrifuged at 5,000 g for 30 min and the supernatants were measured at 620 and 740 nm in a spectrophotometer. The extravasated EBD concentration was calculated against a standard curve and the data expressed as micrograms of EBD per gram of dry tissue weight.

Protein Determination

Protein contents were determined with a bicinchoninic acid protein assay kit (Pierce Chemical Co., Rockford, IL), using BSA as a standard.

Statistical Analyses

The data were analyzed with Student's *t*-test when comparing means between two groups or with one-way analysis of variance plus Tukey's *post hoc* multiple comparison test to compare mean values across multiple treatment groups. In all cases, a probability statistic of <0.05 was taken to indicate significance. All data are expressed throughout the text as mean $\pm$ S.E.

Reagents

Unless otherwise specified, all reagents were purchased from Sigma (St. Louis, MO).

Results

huHSP27 OE mice showed reduced hepatic injury after IRI

Because of the concerns for the potential genetic variability associated with non-congenic strain of mice, we bred our huHSP27 OE and HSP27 WT mice with C57BL/6 mice for 4 generations. Sham-operated HSP27 WT and huHSP27 OE mice had normal plasma ALT at 24 hr after surgery (ALT=81 $\pm$ 14 U/L, N=8 and ALT=71 $\pm$ 13 U/L, N=8, respectively). The HSP27 WT mice subjected to liver IR developed severe liver dysfunction at 24 hr after hepatic ischemic injury with significantly higher plasma ALT levels (24949 $\pm$ 3244 U/L, N=11, p <0.0001 compared to sham-operated mice). However, the huHSP27 OE mice showed significantly less elevations in plasma ALT (6083 $\pm$ 891 U/L, N=12) compared to the HSP27 WT mice 24 hr after liver IRI. Surprisingly, the degree of liver injury did not statistically differ between the HSP27 WT mice and huHSP27 OE mice at 2 hr (8205 $\pm$ 2005 U/L, N=4 vs. 6067 $\pm$ 1367 U/L, N=4, respectively, P =0.3) and 4 hr (15454 $\pm$ 1328 U/L, N=10 vs. 11712 $\pm$ 2753 U/L, N=8, respectively, P =0.2) after liver IR (although the huHSP27 OE mice showed reduced % necrosis at these time points, see below).

Depletion of Kupffer cells with gadolinium chloride provided significant protection against liver IRI only for the HSP27 WT mice (ALT=3577 $\pm$ 700 U/L, N=5, p <0.001 vs. HSP27 WT mice not treated with gadolinium chloride) and but not for the huHSP27 OE mice (ALT=5727 $\pm$ 1489, N=7, p =0.62 vs. huHSP27 OE mice not treated with gadolinium chloride) 24 hr after liver IR. Therefore, overexpression of huHSP27 in the Kupffer cells may be responsible for the hepatic protection in huHSP27 OE mice.

huHSP27 OE mice show reduced hepatic necrosis after IRI

Sixty min. of partial hepatic IRI produced time-dependent increases in the necrotic areas of livers after reperfusion (Fig. 1). Correlating with significantly improved function, reduced necrosis was observed in huHSP27 OE mice subjected to hepatic IRI compared to the HSP27 WT mice (Fig. 1). Representative histological slides from liver tissues from HSP27 WT mice and huHSP27 OE mice subjected to 60 min. ischemia and 24 hr reperfusion or to sham-operation are shown in Fig. 1A. The quantifications of percent necrotic area are shown in Fig. 1B (N=4-6). We failed to detect necrosis in liver sections from sham-operated mice. The HSP27

WT mice subjected to hepatic IR resulted in severe necrosis 2, 4 and 24 hr after hepatic IR whereas the huHSP27 OE mice had significantly less necrosis ($p < 0.05$ vs. HSP27 WT mice).

huHSP27 OE mice showed reduced MIP-2 mRNA expression in the liver after IRI

We measured the expression of pro-inflammatory cytokine mRNAs in the livers 4 hr after IR with RT-PCR. Hepatic IRI was associated with significantly increased pro-inflammatory mRNA expression (ICAM-1, TNF- α , KC, MCP-1 and MIP-2) in HSP27 WT mice (Figure 2A). However, the huHSP27 OE mice showed significantly reduced increases in MIP-2 after liver IRI compared to the HSP27 WT mice (Figure 2B). There were no significant differences in induction of other pro-inflammatory mRNAs. Both HSP27 WT and huHSP27 OE mice showed similar induction of HSP27 mRNA after liver IR (Figure 2A)

huHSP27 OE mice showed reduced hepatic neutrophil infiltration 24 hr after IRI

Sixty min. of hepatic ischemia resulted in recruitment of neutrophils into the liver (Fig. 3). Neutrophil infiltration 24 hr after hepatic ischemia was significantly less in huHSP27 OE mice compared to the HSP27 WT mice. Figure 3 shows representative images of neutrophil immunohistochemistry of liver sections from HSP27 WT or huHSP27 OE mice subjected to sham-operation or to 60 min. of liver ischemia and 24 hr reperfusion. In sham-operated HSP27 WT or huHSP27 OE mice, we were unable to detect any neutrophils. Twenty four hr after liver IR, we detected 31 ± 7 neutrophils/field ($200\times$ magnification, $N=7$) in HSP27 WT mice. The huHSP27 OE mice had significantly reduced neutrophil infiltration 24 hr after liver IR (11 ± 4 neutrophils/field ($200\times$ magnification, $N=7$, $p < 0.05$). Neutrophil infiltration at 4 hr of reperfusion did not differ between the 2 groups of mice (26 ± 7 neutrophils/field, $N=6$ for HSP27 WT mice and 23 ± 5 neutrophils/field, $N=7$ for huHSP27 OE mice, $200\times$ magnification)

huHSP27 OE mice show decreased liver apoptosis after IRI

We utilized 2 separate indices to detect apoptosis: DNA laddering and caspase 3 fragmentation. Twenty four hr after hepatic IR, huHSP27 OE mice showed significantly reduced apoptosis of the liver compared to the HSP27 WT mice with reduced DNA laddering (Fig. 4A) and caspase 3 fragmentation (Fig. 4B).

huHSP27 OE mice show decreased degradation of F-actin after IRI

Liver staining in sham-operated mice show localization of F-actin at the hepatocyte basolateral membranes and around the bile canaliculi membranes (Figure 5A). As expected, 60 min. of liver ischemia and 24 hr of reperfusion resulted in severe disruption of liver parenchymal F-actin compared to the sham-operated mice (Figure 5A, representative of 6 experiments) in HSP27 WT mice as the staining of basolateral hepatocyte membranes as well as bile canaliculi membranes is strongly decreased and disorganized. However, huHSP27 OE mice show significantly better preserved F-actin structure after liver IR as the staining is quite similar to that of sham-operated mice. Mean fluorescent F-actin intensity showed reduced F-actin degradation in huHSP27 OE mice after liver IR (Figure 5B). We also show that the huHSP27 OE mice demonstrate improved preservation of bile canaliculi membrane F-actin number as well as fluorescent intensity (Fig. 5C and 5D).

huHSP27 OE mice show increased phosphorylated and total HSP27 protein expression and enhanced colocalization of F-actin with HSP27

We determined that huHSP27 OE mice show increased expression of both phosphorylated and total HSP27 protein compared to the HSP27 WT mice (data not shown). We also observed increased HSP27 protein expression (red fluorescence) detected with immunocytochemistry (utilizing antibody that recognized both human and mouse form of HSP27) in huHSP27 OE

mice (Figure 6). Furthermore, we saw increased co-localization of HSP27 (red fluorescence) and F-actin (green fluorescence) in huHSP27 OE mice (Figure 6).

huHSP27 OE mice show decreased vascular permeability after IRI

We measured liver vascular permeability after liver IR with EBD injection. EBD binds to plasma proteins and its appearance in extravascular tissues reflects an increase in vascular permeability. Analysis of EBD extravasations in HSP27 WT and huHSP27 OE mice subjected to sham-operation and liver IRI is shown in Figure 7. Liver IR increased the EBD content in the liver for both groups, however, the increase in EBD content was significantly higher for the HSP27 WT mice (363 ± 33 μ g EBD/g dry liver, N=6) compared to the huHSP27 OE mice (231 ± 21 μ g EBD/g dry liver, N=7, $p < 0.001$).

Discussion

The major findings of this study are that mice overexpressing human HSP27 (huHSP27 OE mice) demonstrate reduced liver injury after hepatic IRI. Moreover, the huHSP27 OE mice had less liver necrosis, apoptosis and reduced neutrophil infiltration coupled with less induction of MIP-2 cytokine. Furthermore, we demonstrate that the huHSP27 OE mice showed reduced F-actin breakdown and improved preservation of vascular integrity after hepatic IR. Finally, we demonstrate that huHSP27 overexpression in Kupffer cells may be responsible for the liver protection observed in huHSP27 OE mice as depletion of Kupffer cells with gadolinium chloride abolished the protective benefit of HSP27 overexpression.

Hepatic IR induced acute liver dysfunction is a very common clinical problem and frequently complicates several major surgical procedures including major liver resection, procedures requiring prolonged portal vein occlusion and orthotopic liver transplantation. Unfortunately, neither effective prevention nor therapy exists for hepatic IRI induced liver dysfunction and the current management remains largely supportive (3).

We demonstrated previously that A₁ adenosine receptors protected against renal IRI via phosphorylation and upregulation of HSP27 *in vitro* as well as *in vivo* (11,12). However, the role of HSP27 in protecting the liver against IR has never been investigated. Therefore, in this study we tested the hypothesis that upregulation of human HSP27 would lead to hepatic protection after IR. HSP27 is a molecular chaperone protein with diverse cytoprotective effects (34) and is known to attenuate apoptosis, stabilize cytoskeletal architecture and decrease necrotic cell death (19,22,35). It is well established that HSP27 phosphorylation or up-regulation improves cell survival against stress or injury (14,35). Phosphorylation of HSP27 enhances resistance of actin to breakdown and maintains cytoskeletal architecture after ATP depletion and IR injury (22,36,37). This may be due in part to preservation of cytoskeletal actin in a filamentous state over a globular state. Furthermore, overexpression of HSP27 has been shown to confer significant cytoresistance against heat shock, simulated ischemia, pro-apoptotic agents (*i.e.*, TNF- α), oxidant stress and a number of cytotoxic drugs (38). HSP27 can protect by reducing oxidative stress mediated injury, molecular chaperoning by preventing unfolded proteins from irreversible aggregation (35,39). These principles may explain reduced liver injury and less apoptosis and necrosis after IR in huHSP27 OE mice.

In this study, we demonstrate that overexpression of human HSP27 strongly reduced necrosis of the liver parenchyma after IR. Necrosis after IR is a key component of organ failure (1, 40). Necrotic cell death occurs directly by total breakdown of cellular homeostatic machinery due to massive depletion of ATP during and after the ischemic period or indirectly during reperfusion where uncontrolled delivery of free radicals as well as pro-inflammatory hematopoietic cells cause further cellular derangements. Our study is the first to demonstrate reduced necrosis of the liver with HSP27 overexpression after IRI.

We also demonstrate that the huHSP27 OE mice not only had reduced necrosis after IR, they also showed significantly less apoptosis in the liver (reduced DNA laddering and caspase 3 fragmentation). Apoptosis is an important contributor in the development of liver failure after IRI (41,42). Apoptotic cell death represents the execution of an ATP-dependent death program often initiated by death ligand/death receptor interactions, such as Fas ligand with Fas, which leads to a caspase 3 activation and cleavage of nuclear materials such as DNA and PARP (11,43). Recent reports suggest that HSP27 may also enhance the biological activity of Akt by acting as a scaffolding protein of Akt, a well known anti-apoptotic kinase (44-46). Overexpression of phosphorylation deficient HSP27 has also been shown to confer significant cytoresistance against pro-apoptotic agents (*i.e.*, TNF- α) by counteracting apoptosis via interacting with caspases and cytochrome c (35,39). Our data further extends these previous *in vitro* findings in that *in vivo* overexpression of human HSP27 in mice protects against liver IR induced apoptosis.

Ischemia reperfusion injury *in vivo* results in degradation of filamentous (F)-actin which contributes significantly to the development of acute organ injury (47-49). Moreover, F-actin disruption promotes apoptosis in several cell lines (50). We saw severe disruption of F-actin after liver IR in HSP27 WT mice. Specifically, we saw severe disruptions in both hepatocyte and bile canalicular F-actin cytoskeleton after liver IR. We demonstrated in this study that the huHSP27 OE mice had increased expression of both phosphorylated and total HSP27 compared to the HSP27 WT mice. We also observed increased HSP27 protein expression is correlated with increased co-localization of HSP27 and F-actin in huHSP27 OE mice. Our data clearly demonstrate significantly better preserved F-actin cytoskeleton in livers of huHSP27 OE mice after IRI which may have resulted in reduced necrosis as well as apoptosis after liver IRI. Both basolateral hepatocyte membrane F-actin as well as bile canalicular membrane F-actin was better preserved in huHSP27 OE mice. The bile canalicular F-actin filaments play an important role in bile flow via regulating the contraction of bile canaliculi (47).

The importance of neutrophils in the development of IRI in the liver is well established (1,2, 51). Activated neutrophils release substances to produce further tissue injury such as products of arachidonic acid metabolism, oxygen free radicals and neutrophil elastase (51). In this study, we demonstrate significant recruitment of PMNs to the liver of HSP27 WT mice after IR. Neutrophils are activated during and after liver ischemia and activated neutrophils attach to and then transverse the hepatic capillary endothelium into the subendothelial space, where they release enzymes and cytokines, causing direct hepatocyte injury and the recruitment of other injurious cells, such as monocytes and macrophages (52,53). In this study, we showed that the huHSP27 OE mice showed reduced EBD infiltration into the liver parenchyma indicating improved vascular endothelial integrity with huHSP27 overexpression. Weakened vascular defenses after liver IR may potentiate the PMN infiltration across the endothelial barrier and conversely, increasing the survival of endothelial cells after liver IR with HSP27 overexpression can limit the PMN infiltration into the liver and improve hepatic function.

The production of several pro-inflammatory cytokines and adhesion molecules after hepatic IR is critically important in the pathophysiology of liver IRI (2,5). By RTPCR, we examined whether the hepatic pro-inflammatory mRNAs (TNF- α , KC, MCP-1, MIP-2 and ICAM-1) expression is upregulated after IR and whether overexpression of human HSP27 modulates the pro-inflammatory mRNA expression. We demonstrate that as expected, all of the pro-inflammatory mRNAs examined show enhanced expression after hepatic IR. However, we only saw reductions in MIP-2 mRNA after liver IR in huHSP27 mice. MIP-2 is a member of the C-X-C chemokine family with inflammatory and immunoregulatory activities. MIP-2 is secreted by monocytes and macrophages and is chemotactic for polymorphonuclear leukocytes and hematopoietic stem cells (5). We demonstrate a novel finding that huHSP27 OE mice express selectively reduced MIP-2 expression after liver IR. This is consistent with our finding

that neutrophil infiltration in huHSP27 OE mice was significantly attenuated after liver IR (Figure 3).

Since the huHSP27 OE mice have huHSP27 overexpressed throughout the body, it is important to determine the cell type(s) responsible for the liver protection against IRI. In order to elucidate the cell type(s) involved in the protective effects of huHSP27 overexpression, we pretreated mice with gadolinium chloride to deplete the Kupffer cells in both HSP27 WT and huHSP27 OE mice. Kupffer cells are the resident macrophages of the liver and these cells produce several powerful pro-inflammatory mediators including (TNF- α , IL-1 and IL-6) and further activates/attracts other pro-inflammatory leukocytes (lymphocytes, NK cells) to the liver after IR (54). Kupffer cell activation is implicated in mediating the pathogenesis of liver IRI (1,54). Our findings support the pathogenic role of Kupffer cells in liver IRI as gadolinium chloride-mediated depletion provided significant protection against liver IRI in HSP27 WT mice. However, huHSP27 OE mice were not protected further against liver IRI after Kupffer cell depletion – both HSP27 WT and huHSP27 OE mice show similar degrees of liver IR injury after Kupffer cell depletion. Therefore, one interpretation of our findings is that hepatic protection against ischemia reperfusion injury with huHSP27 overexpression is Kupffer cell dependent. It is interesting that Kupffer cell depletion failed to provide further liver protection in huHSP27 OE mice after IR. We hypothesize that overexpression of huHSP27 in Kupffer cells negate the deleterious effects of Kupffer cells in producing liver injury after IR; therefore, depletion of Kupffer cells failed to provide further liver protection in huHSP27 OE mice.

Global overexpression of HSP27 may not always lead to organ protection against IRI. We were recently surprised to discover that systemic overexpression of HSP27 increased renal injury after IRI *in vivo* (30). Our findings in the liver contrast our previous finds in the kidney where huHSL27 OE mice demonstrated increased renal inflammation and neutrophil infiltration after IR (30). The reason for this discrepancy remains to be elucidated.

In summary, we demonstrate in this study that mice overexpressing human HSP27 are protected against liver necrosis, apoptosis and inflammation after IRI via protective effects of huHSP27 overexpression in Kupffer cells of the liver. The mechanisms of protection after liver IRI in huHSP27 OE mice involve reduced necrosis and apoptosis of liver parenchyma, better preservation of vascular barrier function, reduced neutrophil infiltration and significantly better preserved hepatocyte F-actin after liver IR. Given the protective benefit of HSP27 against hepatic IR and that hepatic IR is common in patients after liver surgery, liver transplantation or sepsis, our findings may have important future therapeutic implications.

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Figure 1B

Figure 1.

A. Representative hematoxylin and eosin staining photomicrographs in liver sections for HSP27 WT and huHSP27 OE mice subjected to sham-operation or to liver IRI (magnification 40×). Necrotic hepatic tissue appears as light pink. B. Quantification of percent necrosis area from sham-operated HSP27 WT and huHSP27 OE mice (HSP27 WT Sham, N=4; huHSP27 Sham, N=4), HSP27 WT or huHSP27 OE mice subjected to hepatic ischemia and 2, 4 or 24 hr of reperfusion (HSP27 WT IR, N=6; huHSP27 OE IR, N=6).

Figure 2A

Figure 2B

Figure 2.

A. Representative gel images of semi-quantitative RT-PCR of the pro-inflammatory markers ICAM-1, KC, MCP-1, MIP-2 and TNF- α and mouse and human HSP27 from liver tissue of HSP27 WT or huHSP27 OE mice subjected to sham-operation or to liver IR (60 min. ischemia and 4 hr reperfusion). B. Densitometric quantifications of relative band intensities from RT-PCR reactions for MIP-2 mRNA (normalized to GAPDH). Data in bar graphs are means $\pm$ SEM. * $p < 0.05$ vs. HSP27 WT Sham group. # $p < 0.01$ vs. HSP27 WT IR group.

Figure 3A HSP27 WT mice

Figure 3B huHSP27 OE mice

Sham Surgery

Ischemia and Reperfusion

Figure 3.

A: Representative photomicrographs of neutrophil accumulation (dark brown punctate stain) in liver sections (magnification $\times 200$). Few scattered neutrophils are present in sham operated mice. HSP27 WT mice (A) subjected to IR injury exhibited large increases in neutrophil accumulation whereas huHSP27 OE mice (B) showed significantly less neutrophil infiltration (representative of 7 experiments). Inserts show higher magnification of image demonstrating PMN infiltration.

Figure 4A

Figure 4B

Figure 4.

huHSP27 OE mice show reduced apoptosis after liver IR compared to HSP27 WT mice. We utilized 2 separate indices to detect apoptosis after sham-operation or liver IR in HSP27 WT or huHSP27 OE mice. A. Representative DNA ladder (of 4 experiments). Apoptotic DNA fragments were separated from intact chromatin and loaded into the agarose gel. B. (top) Representative caspase 3 fragmentation (from 4 experiments). (bottom) Densitometric quantifications of unfragmented caspase 3 (pro-caspase 3) from HSP27 WT and huHSP27 OE mice subjected to sham-operation or liver IR (N=4 for each group). * $p < 0.05$ vs. HSP27 WT Sham group.

Figure 5B

Figure 5C

Figure 5.

A. Representative (of 5 experiments) fluorescent photomicrographs of FITC-phalloidin labeling to visualize F-actin in the liver from HSP27 WT or huHSP27 OE mice subjected to sham-operation or to 60 min. liver ischemia and 24 hr reperfusion (magnification $\times 400$). F-actin is localized in basolateral membranes (arrow) and bile canaliculi membranes. Transversal sections of bile canaliculi are seen as typical dots (circle). After liver IR in HSP27 WT mice, the basolateral as well as bile canaliculi membrane F-actin stains were reduced and disorganized.

B-D. Quantification of mean fluorescent total hepatocyte F-actin intensity (B, $N=5$), number of intact bile canaliculi membranes (C, $N=4$) and mean fluorescent intensities of bile canaliculi membranes (D, $N=4$) as measures of F-actin preservation in liver sections.

* $p < 0.05$ vs. HSP27 WT Sham group. # $p < 0.01$ vs. HSP27 WT IR group.

Figure 6.

Representative fluorescent photomicrographs of phalloidin labeling (green, A and D) to visualize F-actin and HSP27 protein immunocytochemistry (red, B and E; antibody recognized both human and mouse form of HSP27) in the livers from HSP27 WT (A, B, C) or huHSP27 OE mice (D, E, F). Note that increased HSP27 expression (red) in huHSP27 OE mice. Moreover, HSP27 and F-actin co-localize together (yellow, C and F) demonstrated with 0.25 μm thickness Z-sections. Representative of 5 independent experiments.

Figure 7.

Evans blue dye extravasation as an index of vascular permeability. HSP27 WT or huHSP27 OE mice were subjected to sham-operation (N=4) or to 60 min. liver ischemia and 24 hr reperfusion (N=6 for HSP27 WT mice and N=7 for huHSP27 OE mice). EBD was extracted in formamide and the amount of extravasated EBD concentration in the liver was calculated against a standard curve. * $p < 0.05$ vs. HSP27 WT Sham group. # $p < 0.01$ vs. HSP27 WT IR group.