Title
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Permalink
https://escholarship.org/uc/item/30m505zn

Journal
American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons, 18(5)

ISSN
1600-6135

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Publication Date
2018-05-01

DOI
10.1111/ajt.14586

Peer reviewed
Heme oxygenase-1 regulates sirtuin-1–autophagy pathway in liver transplantation: From mouse to human

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Funding information
Center for Scientific Review, Grant/Award Number: PO1 AI120944, R01 DK102110, R01 DK107533, R01 DK06

Liver ischemia–reperfusion injury (IRI) represents a major risk factor of early graft dysfunction and a key obstacle to expanding the donor pool in orthotopic liver transplantation (OLT). Although graft autophagy is essential for resistance against hepatic IRI, its significance in clinical OLT remains unknown. Despite recent data identifying heme oxygenase-1 (HO-1) as a putative autophagy inducer, its role in OLT and interactions with sirtuin-1 (SIRT1), a key autophagy regulator, have not been studied. We aimed to examine HO-1–mediated autophagy induction in human OLT and in a murine OLT model with extended (20 hours) cold storage, as well as to analyze the requirement for SIRT1 in autophagy regulation by HO-1. Fifty-one hepatic biopsy specimens from OLT patients were collected under an institutional review board protocol 2 hours after portal reperfusion, followed by Western blot analyses. High HO-1 levels correlated with well-preserved hepatocellular function and enhanced SIRT1/LC3B expression. In mice, HO-1 overexpression by genetically modified HO-1 macrophage therapy was accompanied by decreased OLT damage and increased SIRT1/LC3B expression, whereas adjunctive inhibition of SIRT1 signaling diminished HO-1–mediated hepatoprotection and autophagy induction. Our translational study confirms the clinical relevance of HO-1 cytoprotection and identifies SIRT1-mediated autophagy pathway as a new essential regulator of HO-1 function in IR-stressed OLT.

KEYWORDS
basic (laboratory) research/science, biopsy, immunobiology, liver disease: immune/inflammatory, liver transplantation/hepatology, organ perfusion and preservation, tissue injury and repair, translational research/science

1 | INTRODUCTION

Orthotopic liver transplantation (OLT) is the standard of care for patients with end-stage liver disease and those with hepatic malignancies, but liver graft shortage remains a major challenge. Liver ischemia–reperfusion injury (IRI), an innate immune-driven sterile inflammation response leading to hepatocellular death, is an inevitable consequence of multiple clinical conditions, including trauma, sepsis,
hepatic tumor resection, and liver transplantation. Indeed, hepatic IRI has been recognized as a major risk factor for delayed early graft function and acute and chronic rejection as well as a key obstacle to expanding the donor organ pool. However, despite obvious clinical importance, mechanisms that account for liver IRI are only partially understood and no effective therapy is available to prevent or treat this condition in humans.1

Autophagy is an evolutionarily conserved intracellular self-digesting pathway responsible for maintaining energy homeostasis and removing long-lived or damaged organelles and proteins.2 A growing body of evidence indicates a tissue-protective function for autophagy in various pathologic states, such as aging, diabetes, and neurodegenerative diseases,3 as well as in liver IRI.4-6 Although enhanced autophagy may represent a novel therapeutic target against IR damage, its efficacy and mechanism in OLT settings remain to be assessed, while clinical relevance of autophagy pathway in liver transplant patients has not been studied before.

Heme oxygenase-1 (HO-1; Hmox1), a rate-limiting enzyme that catalyzes the conversion of heme into biliverdin, carbon monoxide, and free iron, exerts potent antioxidative, anti-inflammatory, and cytoprotective functions.7 We and others have reported on the beneficial function of HO-1 in hepatic IRI murine models by using chemical HO-1 inducers,8-11 adenovirus (Ad) gene transfer,12,13 and genetically modified animals.13 Because macrophages are key mediators of innate immune-driven inflammation and the primary source of HO-1 in ischemia–reperfusion (IR)-stressed liver,10,14 we have established a novel molecular transfer approach by using ex vivo genetically modified HO-1–overexpressing macrophages. This regimen effectively transferred the target molecule into 40-50% of hepatic cells and successfully alleviated warm IRI in mice15,16; its putative efficacy in clinically relevant OLT models has not been tested. Although anti-inflammatory phenotype is central to HO-1 function in liver IRI, recent studies have identified HO-1 as a novel hepatic autophagy inducer18,19; its role in liver transplantation remains unknown, and putative regulatory mechanisms need to be studied.

Sirtuin 1 (SIRT1), an NAD+–dependent type III histone/protein deacetylase involved in cellular senescence, inflammation, and stress resistance,20 plays a key role in autophagy induction.21,22 In addition to an anti-inflammatory role in the pathogenesis of liver IRI,23 recent studies have demonstrated the significance of SIRT1-induced autophagy in hepatocyte resistance against IR stress.24,25 Molecular communication between SIRT1 and HO-1 in OLT has not been studied, and the mechanism by which SIRT1 may regulate the HO-1 autophagy pathway remains to be elucidated.

To gain further insight into HO-1–mediated autophagy in liver transplantation, we used an ex vivo genetically modified HO-1 macrophage adoptive transfer approach in a clinically relevant murine OLT model and analyzed human OLT samples (N = 51) in parallel. Our translational study confirms the clinical relevance of HO-1 hepatoprotection and identifies SIRT1-dependent autophagy as a novel and essential regulator of HO-1 function in OLT under IR stress.

2 | MATERIALS AND METHODS

2.1 | Clinical liver transplant study

Fifty-six consecutive adult primary OLT recipients were recruited under an institutional review board protocol (13-000143; May 2013-August 2015). Routine standard of care and immunosuppressive therapy were administered as specified by University of California, Los Angeles (UCLA) liver transplant protocols. Study data were collected and managed by using REDCap electronic data capture tools.26 Donor organs, procured according to standardized techniques, were perfused with and stored in cold University of Wisconsin (UW) solution (ViaSpan; Bristol-Myers Squibb Pharma, New York, NY). Cold ischemia time was defined as the time between the perfusion of the donor liver with UW solution and its removal from cold storage. Recipient venous blood was collected within the hour before transplantation and on postoperative days (PODs) 1-14. Hepatocellular injury was evaluated based on serum alanine aminotransferase (sALT) levels. Posttransplantation biopsy (Bx) specimens were obtained from the left liver lobe by using a Tru-Cut biopsy needle (Carefusion, Medline Industries, Northfield, IL) approximately 2 hours after portal reperfusion (before abdominal closure) and then snap-frozen. Of 56 cases, 51 were examined; 4 were excluded because the Bx samples were too small for Western blot analyses, and 1 was excluded due to unavailability of clinical data. Early allograft dysfunction (EAD) was defined by the presence of ≥1 of the following: total bilirubin ≥10 mg/dL (171 μmol/L) on POD 7, international normalized ratio ≥1.6 on POD 7, and alanine aminotransferase/aspartate aminotransferase >2000 IU/L within the first 7 PODs.27,28 Posttransplantation rejection was diagnosed by follow-up Bx performed per clinical standard of care.

2.2 | Animals

C57BL/6 mice aged 6-8 weeks were used (Jackson Laboratory, Bar Harbor, ME). Animals were housed in the UCLA animal facility under specific pathogen-free conditions and received humane care according to the criteria outlined in the “Guide for the Care and Use of Laboratory Animals” (National Institutes of Health publication 86-23 revised 1985). All studies were reviewed and approved by the UCLA Animal Research Committee.

2.3 | Generation of genetically modified bone marrow–derived macrophages

L929 cells (ATCC, Rockville, MD) were cultured in RPMI-1640 medium supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 10% fetal calf serum. The conditioned medium was collected from cells grown for 7 days. The bone marrow–derived macrophages (BMMs) were generated according to standard procedures.15 In brief, bone marrow cells were removed from the femurs and tibias of C57BL/6 mice and cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mmol/L L-glutamine, with
the addition of 15% L929-conditioned medium. Cells were cultured at 5 × 10^6 cells/well for 7 days with 5% CO₂-95% air at 37°C and used for in vitro transfection.

Replication-defective recombinant Ad encoding HO-1 (AdHO-1) was generated as described. BMMs (5 × 10^6/well) were incubated for 1 hour with AdHO-1 or Adj-gal (at a multiplicity of infection of 10). After medium change, cells were cultured for additional 48 hours before in vivo adoptive transfer. By 48 hours of transfection, the X-Gal staining was >90%, compared with control BMMs.15

2.4 | Mouse liver cold ischemia and transplantation model

We used a well-established mouse model of ex vivo hepatic cold storage and orthotopic liver transplantation (OLT) as described. Donor livers were stored in UW solution at 4°C for 20 hours before transplantation into syngeneic mice. BMMs (5 × 10^6) transfected with AdHO-1/Adj-gal (2.5 × 10^9 pfu) or untreated (5 × 10^9) were injected via portal vein before liver reperfusion at the completion of transplant surgery. In some experiments, SIRT1 inhibitor (EX527; Sigma Aldrich, St. Louis, MO) was administered (10 mg/kg i.p.) at 30 minutes before OLT surgery.15 Liver and serum samples were collected 6 hours after reperfusion, the peak of hepatocellular damage in this model. The sham group underwent the same procedures except for OLT.

2.5 | Serum biochemistry

sALT levels, an indicator of hepatocellular injury, were measured at IDEXX Laboratories (Westbrook, ME).

2.6 | Liver histology and IRI grading

Formalin-fixed paraffin-embedded OLT sections (5 µm) were stained with hematoxylin and eosin (HE). The severity of hepatic IRI was graded by using the Suzuki’s criteria.31

2.7 | Quantitative RT-PCR analysis

RNA was extracted from liver tissue samples using the RNase Mini Kit (Qiagen, Germantown, MD). A total of 5.0 µg of RNA was reverse-transcribed into cDNA. Quantitative PCR was performed using DNA Engine with Chromo 4 Detector (MJ Research, Waltham, MA). The primer sequences are listed (Table S1). The expression of the target gene was normalized to the housekeeping gene HPRT.

2.8 | Western blot assay

Proteins were extracted from liver tissues, and their concentrations were measured (BCA Protein Assay Kit, Thermo Scientific). Equal amounts of protein were electrophoresed, blotted, and incubated with primary Abs and secondary HRP-conjugated Abs and developed. Primary Abs detecting HO-1 (Enzo Life Sciences, Farmingdale, NY), SIRT1, LC3B, and β-actin (Cell Signaling Technology, Danvers, MA) were used. To compare target protein expression in multiple human OLT samples, densitometry quantification was conducted by using a reference sample and normalization with β-actin as reported.23

2.9 | Immunohistochemistry

OLT-infiltrating neutrophils were detected by using monoclonal rat anti-Ly6G Abs (BD Biosciences, San Jose, CA). Immunostaining signals were visualized with a labeled polymer in the EnVision+ Kit (HPR; Dako, Carpinteria, CA). Positively stained cells were counted blindly (10 HPF/section). LC3B expression in OLT was detected by using rabbit anti-LC3B Abs, and signals were visualized with secondary Abs: Alexa Fluor 488 anti-rabbit IgG.

2.10 | TdT-mediated dUTP nick end labeling (TUNEL) assay

Cell death in formalin-fixed paraffin-embedded liver sections (5 µm) was detected by use of the ApopTag Plus Peroxidase In Situ Apoptosis Kit (Millipore, Temecula, CA). Results were scored semiquantitatively by blindly counting the number of positive cells in 10 HPF/section.

2.11 | Statistical analysis

In experiments in mice, group comparisons were performed by using a Student t-test. For human data, continuous values were analyzed by using a Mann-Whitney U test, and categorical variables were analyzed by using the Fisher exact test. The Spearman correlation coefficient (r) was used to evaluate the strength of linear relationship between variables. The cumulative survival rate was analyzed by using the Kaplan-Meier method, and differences between groups were compared by using a log-rank test. JMP for Windows 8.0 (SAS Institute, Cary, NC) was used for statistical analyses. A P-value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | AdHO-1–transfected BMMs alleviate IRI in OLT

We first aimed to validate the efficacy of HO-1 induction after the adoptive transfer of AdHO-1 BMMs in a clinically relevant mouse IRI-OLT model. Cold-stored liver grafts were infused with BMMs via portal vein immediately before reperfusion. At 6 hours after reperfusion, AdHO-1–transfected BMM–treated OLT showed increased HO-1 expression compared with naive or Adj-gal–transfected BMM–treated controls (Figure 1A). AdHO-1 BMM–conditioned OLT displayed attenuated sinusoidal congestion, edema/vacuolization, and hepatocellular necrosis compared with control OLT infused with untreated or Adj-gal–transfected cells (Figure 1B). These correlated with depressed Suzuki’s grading of histologic liver damage (OLT: + AdHO-1 BMMs = 1.7 ± 0.6 vs OLT: + BMMs = 3.7 ± 1.2 or OLT: +Adj-gal BMMs = 3.3 ± 0.6; n = 4-6/group, P < .05, Figure 1C) and
preserved liver function, evidenced by sALT levels (OLT: + AdHO-1 BMMs = 3900 ± 2642 vs. OLT: + BMMs = 7997 ± 2068 or OLT: +Adβ-gal BMMs = 7726 ± 2129 IU/L; mean ± SD, n = 4-6/group, P < .05, Figure 1C) in AdHO-1 BMM–treated OLT. Thus, infusion of AdHO-1 BMMs into the graft enhanced HO-1 expression and minimized the adverse effects of hepatic IRI in OLT.

3.2 | AdHO-1 gene transfer promotes anti-inflammatory signature in OLT

Because anti-inflammatory function is one of the key HO-1 biological features, we examined whether AdHO-1 gene transfer alters the anti-inflammatory/proinflammatory gene expression profile in IR-stressed OLT. Indeed, AdHO-1 BMMs infused directly into the graft increased mRNA coding for anti-inflammatory interleukin (IL)-10 and transforming growth factor (TGF)β, while decreasing proinflammatory TNFα, MCP1, and CXCL10 compared with naïve/Adβ-gal BMM–treated controls (Figure 2A, B). This confirms that HO-1 overexpression exerts an anti-inflammatory function and inhibits inflammatory cytokine-induced OLT injury. We also tested whether AdHO-1 gene transfer may affect cytokine-induced neutrophil OLT influx. As expected, HO-1 overexpression reduced neutrophil (Ly6G) numbers in IR-stressed OLT infused with AdHO-1 BMMs (Figure 3A, B), indicating HO-1 gene transfer enhanced the anti-inflammatory phenotype and diminished cytokine-induced inflammation.

3.3 | AdHO-1 gene transfer attenuates hepatocellular death while enhancing SIRT1/LC3B expression in OLT

We next aimed to determine whether AdHO-1 gene transfer affects cell death and autophagic activity in IR-stressed OLT. As shown in Figure 3A, B, a reduced frequency of TUNEL-positive apoptotic/necrotic cell death was observed in AdHO-1 BMM–treated OLT compared with naïve BMM or Adβ-gal BMM controls. Moreover, we found that AdHO-1 BMM therapy not only upregulated hepatic SIRT1
3.4 | HO-1 expression correlates with IR liver damage and SIRT1/LC3B signaling in human OLT

Having demonstrated AdHO-1 BMM–facilitated hepatoprotection accompanied by increased SIRT1/LC3B levels in a murine OLT model, we next aimed to validate the relevance of these findings in human liver transplantation. Liver postreperfusion Bx specimens from 51 human OLTs were screened by Western blots for HO-1, SIRT1, and LC3B expression (Figure 5A). Hepatic HO-1 expression profile exhibited weak negative correlation with sALT levels at POD1 (r = −0.3306, P = .0178, Figure 5B), indicating higher levels of HO-1 were associated with milder IR-liver injury. In addition, graft HO-1 expression showed positive correlation with SIRT1 (r = 0.4229, P = .0020, Figure 5C) and weak positive correlation with LC3B (r = 0.3594, P = .0096, Figure 5D).

To evaluate the impact of hepatic HO-1 on clinical outcomes, 51 OLT samples were split evenly into low (n = 26) and high (n = 25) HO-1 expression groups (Figure 6A). The patients’ demographic data and clinical parameters are shown (Table S2). There was no correlation between HO-1 expression and donor background, including age, gender, weight, body mass index (BMI), or preprocurement sALT level. We also found no correlation between HO-1 expression and recipient factors, including age, gender, weight, BMI, race, disease etiology, presence of hepatocellular carcinoma, ABO compatibility, Model for End-Stage Liver Disease score, pretransplantation sALT, or dialysis. Furthermore, there was no correlation between HO-1 expression and cold ischemic time or intraoperative blood loss.

Consistent with Figure 5C,D data, the high–HO-1 group showed increased SIRT1 (low HO-1: 0.83 ± 0.10 vs high HO-1: 1.25 ± 0.10, mean ± SEM, P < .05, Figure 6B, left) and LC3B (low HO-1: 0.87 ± 0.08 vs high HO-1: 1.13 ± 0.10, mean ± SEM, P < .05, Figure 6B, right) expression. Representative Western blots are shown (Figure 6C). In addition, the high–HO-1 group exhibited significantly lower sALT levels at POD1 (low HO-1: 809 ± 367 vs high HO-1: 255 ± 34 IU/L, mean ± SEM, P < .05, Figure 6D). To examine the relationship between HO-1 expression and OLT outcomes, we analyzed cumulative posttransplantation survival,
with the median follow-up of 740 days (range, 4-1432 days). None of the patients underwent secondary liver transplantation. Despite consistent trends, the improved survival in the high–HO-1 expression group failed to reach statistical significance compared with the low–HO-1 expression group (P = .1963, Figure 6E). The high–HO-1 expression group was characterized by lower frequency of early graft dysfunction (3.8% vs 8.0%) and post-OLT rejection episodes (3.8% vs 16.0%). However, these differences failed to reach statistical significance (P = .5150 and P = .1871, respectively). Representative TUNEL and LC3B staining in low- versus high–HO-1 Bx groups are shown (Figure 6F).

3.5 SIRT1 inhibition abrogates hepatoprotection and depresses autophagy despite HO-1 induction in mouse OLT

Based on the aforementioned preclinical and clinical data, preservation of hepatocellular function (sALT) and enhanced autophagy (LC3B) in HO-1–overexpressing OLT was accompanied by increased SIRT1 signaling, a putative regulator of autophagy and stress resistance against hepatic IR damage.25 Hence, we hypothesized SIRT1 might be essential in cytoprotection after HO-1 induction in IR-stressed OLT. As shown in Figure 7A, adjunctive SIRT1 inhibition before transplantation (EX527, 10 mg/kg i.p.) recreated cardinal features of hepatic IRI in otherwise well-functioning AdHO-1 BMM–treated OLT, evidenced by increased sALT levels and Suzuki's histologic grading scores (Figure 7B). Moreover, SIRT1 inhibition depressed LC3B expression in AdHO-1 BMM–treated liver grafts. These data indicate the SIRT1–autophagy pathway is required for HO-1–mediated hepatoprotection against IRI in OLT.

4 DISCUSSION

This study is the first to document HO-1–mediated autophagy enhancement in human liver transplant settings and in a clinically relevant mouse OLT model. Although anti-inflammatory signaling is
critical for HO-1 function against IR stress, our findings document autophagy regulation as a novel mechanistic component of its biological effects. Our clinical study showed positive correlation between HO-1 and SIRT1 levels in human OLT (Figures 5C,D and 6B,C), while SIRT1 inhibition in parallel mouse studies diminished autophagy phenotype seen otherwise after HO-1 induction in IR-resistant OLT (Figures 4A,B 7C,D). These data are consistent with the notion that HO-1 regulates hepatic autophagy in an SIRT1-dependent manner. Although previous studies suggested the role of p38 MAPK in HO-1–induced liver autophagy, it remains unknown as to how HO-1 may regulate hepatic p38 signaling. In the context of our present data, a recent finding of liver-specific SIRT1 increasing p38 translocation/phosphorylation in a YAP/MKK3-dependent manner implies SIRT1-p38 signaling may underlie the autophagy regulation by HO-1. These complex molecular mechanisms require further in-depth analyses.

It is well documented that hepatocyte autophagy is essential in liver stress resistance. Indeed, the loss of Atg4B increased sensitivity of aged mouse livers to warm IRI, whereas rapamycin increased autophagy and protected against IR stress. In contrast, recent studies in myeloid-specific Atg5 knockout mice have shed new light on macrophage autophagy regulation. While macrophage Atg5 deficiency was shown to limit acute toxic liver injury by downregulating IL-1β, the impaired macrophage Atg5 promoted proinflammatory macrophage polarization in obese mice. Although macrophages are pivotal in innate immune-driven IR damage, the significance of macrophage autophagy in liver IRI, using myeloid autophagy-deficient animals, awaits future research.

Despite numerous preclinical reports identifying HO-1 as a major cytoprotective molecule, Geuken and coworkers reported high "pre-transplant" HO-1 expression in human donor livers paradoxically correlated with augmented posttransplantation injury. As "post-transplant" HO-1 features specific to clinical liver transplantation have not been well defined, we have recently reported that high HO-1 expression in human OLT (n = 21) at the time of reperfusion are
associated with suppressed IR liver damage.\textsuperscript{41} In agreement with the latter, our present data from human OLT recipients (n = 51) consistently demonstrated negative correlation between HO-1 levels and posttransplantation sALT levels (Figures 5B and 6D), implying therapeutic benefit of HO-1 induction in clinical liver transplantation. Unlike hepatocytes under basal conditions, liver macrophages (infiltrating and tissue resident) are the major HO-1 producers, in both human OLT and IR-stressed murine livers.\textsuperscript{10,41}

Although aging and obesity are poor prognostic factors influencing HO-1 expression, we found no correlation between posttransplantation HO-1 levels and donor–recipient demographic parameters or surgical factors (Table S2). Because HO-1 is not only a cytoprotective molecule but also stress-inducible heat shock protein 32 (hsp32), its expression increases with the severity of IR stress. Paradoxically, high HO-1 levels were associated with more attenuated liver damage in the clinical arm of our study (Figure 6D). We speculate that the divergent HO-1 expression in OLT might be caused by varying individual inducitivity of HO-1 in response to IR stress, resulting in varying susceptibility against IRI. Indeed, short guanine-thymine nucleotide repeats polymorphism in the promotor region on chromosome 22q13.1 of human HO-1 was shown to enhance HO-1 expression in response to oxidative stress,\textsuperscript{42} whereas a more recent study associated HO-1 polymorphism with post-OLT hepatic cellular damage.\textsuperscript{43} As others failed to correlate HO-1 polymorphism with its expression profile,\textsuperscript{42} more studies on putative factors influencing human HO-1 responses are warranted.

We have also recently reported on the anti-inflammatory and hepatoprotective role of SIRT1 activation in a warm liver IRI mouse model alongside negative correlation between SIRT1 and T-bet/IL-1β levels in 21 clinical OLT cases.\textsuperscript{23} The positive SIRT1 regulation by HO-1 seen in a mouse IRI model was then confirmed in a 21-OLT patient cohort.\textsuperscript{41} The current study documents the functional significance of the HO-1–SIRT1 axis in a clinically relevant mouse hepatic cold ischemia/OLT model (Figure 4A,B) and in 51 human OLT cases (Figures 5C and 6B). Although in our previous clinical study (n = 21), post-OLT “high” HO-1 expression was accompanied by a statistically significant improvement in OLT survival,\textsuperscript{41} we failed to detect a similar statistically significant survival difference in the current (n = 51) liver transplant patient cohort (Figure 6E, P = .1963). As discussed earlier,\textsuperscript{41} the link between graft and recipient long-term outcomes and IRI severity remains controversial. The presence of early lesions on the time-zero Bx in older grafts (n = 16) did not translate into early graft dysfunction or graft loss,\textsuperscript{44} while histologic IRI grading in postreperfusion liver Bxs and peak transaminase levels (n = 55) did not show any correlation with clinical outcomes in the first posttransplantation month.\textsuperscript{45} Likewise, despite preferable trends, our present study (n = 51) failed to detect significant differences in EAD, post-OLT rejection, or overall survival. In marked contrast, severe IRI on the time-zero Bx and peak of posttransplantation ALT in 476 OLT cases were significantly associated with a 1-year graft survival in an earlier study.\textsuperscript{46} The correlation between graft/recipient outcomes and IRI severity may thus require considerably larger number of study subjects.

A chemical HO-1 inducer (cobalt protoporphyrin [COPP]) was recently shown to increase SIRT1 expression in hepatocytes, while adjunctive competitive HO-1 inhibitor (tin mesoporphyrin) abrogated SIRT1 upregulation, indicating HO-1–dependent SIRT1 induction by exogenous COPP.\textsuperscript{47,48} Likewise, in our current study, transfer of genetically modified HO-1-overexpressing BMMs consistently enhanced liver graft SIRT1 expression levels (Figure 4A,B). The mechanisms accounting for HO-1–mediated SIRT1 upregulation remain largely unknown. Because oxidative stress suppresses hepatic SIRT1,\textsuperscript{23,49}
antioxidant properties may be the key for HO-1 to regulate SIRT1 expression.

Ad is an efficient vector for in vivo gene therapy due to its ability to mediate transgene expression in various cell types. However, serious off-target effects, including activation of innate immune responses, thrombocytopenia, coagulopathy, and liver damage, restrict systemic use of Ad vector in clinical practice. In our present and previous preclinical studies, we used replication-defective recombinant Ad to generate HO-1-overexpressing BMMs. Indeed, the ex vivo gene-modified local BMM infusion upregulated hepatic HO-1/LC3B expression and alleviated IRI in a mouse OLT model. This regimen makes Ad-related adverse effects negligible, while direct BMM infusion into the liver improves macrophage transfer efficacy as a cellular delivery system for HO-1 trafficking into the disease site. The present study using ex vivo gene-modified HO-1-overexpressing BMM in a clinically relevant mouse model is likely to validate and support future experiments in translational and clinical settings.

In our current study, the injection of BMMs (Adβ-gal) to OLT increased hepatic HO-1 expression compared with untreated BMM (no virus)–treated graft (Figure 1A). This result is consistent with our previous findings in a mouse model of liver warm IRI. As a foreign pathogen, virus infection itself can cause cellular stress to activate antioxidant system, accompanied by increased antioxidant gene expression, such as manganese superoxide dismutase, HO-1, indoleamine-2,3-dioxygenase, and glutathione peroxidase. We speculate that increased HO-1 expression following Adβ-gal BMM injection was induced by virus-related BMM stress, which was accompanied by increased SIRT1 and slightly higher LC3B levels (Figure 4A). However, Adβ-gal BMMs failed to ameliorate liver damage in IR-stressed OLT (Figure 1B,C) as "pure" viral-mediated HO-1 induction may not be

![FIGURE 6](image-url)
sufficiently proficient to protect the liver graft. In contrast, unlike the Adβ-gal BMM controls, infusion of Ad HO-1 BMMs markedly enhanced local HO-1 (Figure 1A) and SIRT1/LC3B (Figure 4A) levels, leading to attenuated hepatocellular injury in IR-stressed OLT (Figure 1B,C).

In conclusion, our study with genetically modified HO-1-overexpressing BMM transfer in a mouse OLT model and parallel clinical examination of 51 human liver transplants not only confirmed the clinical relevance of HO-1 hepatoprotection but also identified a novel regulatory role of SIRT1-mediated autophagy as a part of cytoprotective function in transplant recipients. The present data, in agreement with our recent reports, validate a novel investigative tool in which host macrophages can be transplanted ex vivo with cytoprotective HO-1 and then infused, if needed, into prospective recipients to mitigate IR-mediated inflammation during liver transplantation, resection, or trauma. As decreasing donor organ quality represents one of the most challenging problems, our findings provide the rationale for a new and clinically attractive gene-targeted strategy to "rejuvenate" extended criteria donor livers and improve their function via an HO-1-SIRT1-autophagy pathway in transplant recipients.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health (NIH) grants R01 DK102110; R01 DK107533; and R01 DK062357 (JWKW); NIH PO1 AI120944 and Keck Foundation Award 986722 (JWKW and EFR); and Ruth L. Kirschstein National Research Service Award T32CA009120 (RAS). The authors declare that no conflicts of interest exist. We thank Ko Takanashi at UCLA-TPCL for immunohistochemical assistance.

DISCLOSURE

The authors of this manuscript have no conflicts of interest to disclose as described by the American Journal of Transplantation.


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