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ORIGINAL ARTICLE

High Mobility Group Box 1 Protein as a Marker of Hepatocellular Injury in Human Liver Transplantation

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High mobility group box 1 protein (HMGB1), a cytokine actively secreted by phagocytes and passively released from necrotic cells, is an inflammatory mediator in experimental hepatic ischemia/reperfusion injury. We characterized its expression in human liver transplantation. In 20 patients, in addition to systemic samples, blood was drawn from portal and hepatic veins during and after reperfusion to assess changes within the graft. Plasma HMGB1, tumor necrosis factor α (TNF-α), and interleukin-6 (IL-6) levels were measured, and HMGB1 immunohistochemistry was performed on biopsies taken before and after reperfusion. Plasma HMGB1 was undetectable before reperfusion, and levels in systemic circulation peaked after graft reperfusion. At portal declamping, HMGB1 levels were substantially higher in the caval effluent [188 (80-371) ng/mL] than in portal venous blood [0 (0-3) ng/mL, $P < 0.001$]. HMGB1 release from the graft continued thereafter. HMGB1 levels were not related to TNF-α or IL-6 levels. HMGB1 expression was up-regulated in biopsies taken after reperfusion ($P = 0.020$), with intense hepatocyte and weak neutrophil staining. HMGB1 levels in hepatic venous blood correlated with graft steatosis ($r = 0.497$, $P = 0.03$) and peak postoperative alanine aminotransferase levels ($r = 0.588$, $P = 0.008$). Our results indicate that HMGB1 originates from the graft and is a marker of hepatocellular injury in human liver transplantation. ***Liver Transpl 14: 1517-1525, 2008.*** © **2008 AASLD.**

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Liver transplantation is an established therapy for both acute liver failure and chronic liver disease. Although the results of liver transplantation have significantly improved during the past 15 years, primary graft dysfunction remains a problem. The etiology of primary graft dysfunction is multifactorial, with graft steatosis, graft preservation, and ischemia/reperfusion (I/R) injury among contributing factors.

Cold ischemia activates liver resident macrophages (Kupffer cells) and sinusoidal endothelial cells to produce cytokines and express adhesion molecules. Reperfusion is characterized by phagocyte activation upon contact with the activated sinusoidal endothelium. Once they have transmigrated, phagocytes elicit tissue destruction by producing oxygen free radicals, proteolytic enzymes, and proinflammatory cytokines. Activation of these inflammatory cascades involves the release of endogenous danger signals from damaged cells.

Abbreviations: A, systemic artery; ALT, alanine aminotransferase; CV, central veins; ELISA, enzyme-linked immunosorbent assay; H, hepatic vein (caval effluent during initial reperfusion); HMGB1, high mobility group box 1; IL, interleukin; INR, international normalized ratio; I/R, ischemia/reperfusion; P, portal vein; PA, portal areas; PBS, phosphate-buffered saline; TLR, toll-like receptor; TNF-α, tumor necrosis factor α; WB, Western blotting.

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These signals act in part through toll-like receptors (TLRs), pattern recognition molecules expressed in a variety of cells, including macrophages and endothelial cells.[1] TLR activation leads to the production of chemokines, cytokines, and complement proteins, thereby enhancing neutrophil activation and recruitment to injured tissue.[1] Liver I/R injury may be stimulated by endogenous TLR ligands.[1,2]

High mobility group box 1 protein (HMGB1; amphoterin), when nuclear, is involved in nucleosome stabilization and transcription regulation.[3] Extracellular HMGB1, secreted from activated phagocytes[4] and released passively from necrotic cells,[5] acts as an endogenous danger signal and inflammatory mediator.[6] HMGB1 exerts its effects through receptor for advanced glycation end products and/or TLR2 and TLR4,[3] leading to endothelial cell activation[7] and the release of multiple proinflammatory cytokines from phagocytes.[8] HMGB1 was initially identified as a late mediator in experimental endotoxemia and sepsis.[9,10] After local tissue injury, HMGB1 initiates systemic inflammation and injury at the end organ level.[11] In the clinical setting, elevated serum HMGB1 levels have been described in sepsis,[9,12,13] pneumonia,[14] and acute pancreatitis[15] and following cerebral and myocardial ischemia.[16]

Recently, HMGB1 was described as an early inflammatory mediator after acute local organ injury. In murine models of warm segmental hepatic ischemia, HMGB1 levels increase rapidly after reperfusion.[17,18] Furthermore, HMGB1 blockade protects against warm hepatic I/R injury. Anti-HMGB1 antibody decreases hepatic cytokine expression and hepatocellular damage through TLR4-dependent mechanisms[17] and improves survival.[18] HMGB1 given immediately after reperfusion worsens I/R injury,[17] whereas HMGB1 preconditioning before ischemia is protective.[19]

The role of HMGB1 in clinical liver transplantation is unknown. As HMGB1 is released from both damaged cells and activated phagocytes, it might be a useful marker of liver I/R injury in humans. We therefore characterized the cellular expression and bloodstream kinetics of HMGB1 during liver transplantation. Furthermore, we evaluated the relations between HMGB1 levels during surgery and subsequent graft function after liver transplantation.

PATIENTS AND METHODS

Patients

The study was approved by the ethics committee of Helsinki University Central Hospital. After written informed consent, 20 consecutive patients participated in the study. Indications for liver transplantation are given in Table 1. Diagnoses in 15 patients transplanted for cirrhosis included cholestatic liver disease (n = 4), alcoholic cirrhosis (n = 4), cryptogenic cirrhosis (n = 3), nonalcoholic fatty liver disease (n = 2), and miscellaneous (n = 2). Histology revealed concurrent incidental hepatocellular carcinoma and cirrhosis in 4 explanted grafts.

TABLE 1. Patient and Graft Characteristics

Age (years)	50 (22–66)
Gender (male/female)	14/6
Diagnosis (ALF/cirrhosis/other)	3/15/2
Cold ischemic time (minutes)	305 (225–630)
Anhepatic time (minutes)	56 (35–69)
Graft steatosis (%)	0 (0–30)
Postoperative liver function	
ALT (IU/L)	267 (152–364)
INR	1.5 (1.4–1.6)
Bilirubin (μmol/L)	34 (20–101)

NOTE: Data are expressed as medians (range). Interquartile ranges are used for liver function tests.
Abbreviations: ALF, acute liver failure; ALT, alanine aminotransferase; INR, international normalized ratio.

Surgery

All grafts were retrieved from brain-dead donors. Grafts were preserved with University of Wisconsin solution at +4°C. The surgical techniques of transplantation and graft reperfusion were standardized. First, portal and suprahepatic caval anastomoses were completed. During initial reperfusion, with the suprahepatic caval vein clamped, the graft was first flushed with 1000 mL of Ringer's solution via the portal vein, and this was followed by 400 mL of portal venous blood (graft caval effluent). Subsequently, portal and suprahepatic caval veins were declamped, and this was followed by completion of the infrahepatic caval anastomosis and declamping. Finally, the hepatic arterial anastomosis was completed and the vessel was declamped approximately 46 minutes (median; range, 28-80 minutes) after portal declamping. Immunosuppression was achieved with calcineurin inhibitors (cyclosporin A, n = 7; tacrolimus n = 13) and steroids. As there was no difference in study parameters between immunosuppression groups (data not shown), the groups were combined.

Liver Biopsies

Two biopsies were obtained from each graft, the first during liver procurement immediately before graft perfusion and the second at the end of liver transplantation prior to skin closure.

Blood Samples

A single systemic blood sample was drawn from the organ donor before organ procurement. During liver transplantation, systemic arterial (A) blood samples were collected after induction of anesthesia but before surgery (A0), immediately before initial reperfusion of the graft with portal blood (A1), 10 minutes after portal vein declamping (A2), 10 minutes after hepatic artery declamping (A3), and 8, 24, and 48 hours after reperfusion. To assess changes across the graft, blood sam-

ples were obtained by puncture from both the portal vein (P) and the hepatic vein (H) at the following time points: during initial reperfusion [P1 and H1 (graft caval effluent)], 10 minutes after portal vein declamping (P2 and H2), and 10 minutes after hepatic artery declamping (P3 and H3). The volume of each sample was 10 mL. All samples were drawn into pyrogen-free syringes, transferred into sodium citrate anticoagulated tubes, and placed on melting ice at +0°C. Plasma was separated by centrifugation at 2000*g* for 10 minutes and stored at −80°C until the analyses were performed.

HMGB1 Western Blotting

Plasma HMGB1 levels were determined by combined heparin-Sepharose precipitation and Western blotting methods.[12] Briefly, plasma samples (0.2 mL) were centrifuged at 14,000 to 15,000*g* for 2 minutes, 120 μL of supernatant was collected, and 60 μL of 50% heparin-Sepharose slurry in phosphate-buffered saline (PBS) containing 1.2 M NaCl was added. Samples were mixed in rotation at +4°C for 1 to 2 hours, the supernatant was removed, and the gel was washed with PBS. Heparin-Sepharose–bound proteins were eluted with 30 μL of sodium dodecyl sulfate–polyacrylamide gel electrophoresis sample buffer by incubation at 99°C for 5 minutes in a shaker. Samples (15 μL) were electrophoresed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analyzed in a Western blot probed with chicken anti-HMGB1 immunoglobulin Y[20] and peroxidase-conjugated anti-chicken immunoglobulin Y (Zymed Laboratories, San Francisco, CA). Optical densities of immunoreactive 30-kDa bands were quantified as described.[12,20] Normal plasma samples containing known amounts of recombinant HMGB1 were analyzed equally in each gel, and a standard curve was generated to calculate sample HMGB1 levels.

HMGB1 Enzyme-Linked Immunosorbent Assay (ELISA)

In 4 patients, in addition to HMGB1 Western blotting, HMGB1 ELISA was performed with HMGB1 ELISA Kit II (Shino-Test Corp., Kanagawa, Japan). All donor samples were analyzed with ELISA only. The sensitivity of the assay was 1.0 ng/mL.

HMGB1 Immunohistochemistry

As tissue HMGB1 occurs mainly in an oxidized form,[21,22] a monoclonal antibody recognizing the oxidized form was used. Liver biopsies were formalin-fixed and paraffin-embedded. Sections of 4 μm were cut, deparaffinized in xylene, and rehydrated in a series of graded alcohols. The endogenous peroxidase activity was inhibited with 0.1% H_2O_2 in PBS for 20 minutes at room temperature, and sections were blocked for ≥2 hours with 5% milk powder and 0.3% Triton X-100 in PBS. Sections were then incubated with anti-HMGB1 (clone KS1, MBL International, Woburn, MA) in a blocking solution overnight and washed 3 times with PBS. Horseradish peroxidase–conjugated anti-mouse IgG (GE Healthcare, Little Chalfont, United Kingdom), diluted 1/400 in a blocking buffer, was added to sections and incubated overnight. Sections were washed 2 times with PBS and once with 50 mM Tris-HCl buffer (pH 7.6). Bound antibodies were detected with 0.1 mg/mL 3,3′-diaminobenzidine tetrahydrochloride hydrate (Sigma-Aldrich, St. Louis, MO) in a 0.0003% H_2O_2 50 mM Tris-HCl buffer, and after color development, sections were washed once with PBS and once with H_2O. Counterstaining was done with toluidine blue.

HMGB1 immunostaining in liver sections was evaluated with a semiquantitative scoring system, with grade 0 representing completely negative staining, grade 1 occasional positive staining, grade 2 intermediate staining, and grade 3 intense positive staining, respectively. Results were scored independently by a pathologist and 2 members of the research group.

Tumor Necrosis Factor α (TNF-α)

The plasma TNF-α concentration was measured with WEHI-13VAR cells.[23,24] Briefly, 40,000 WEHI-13VAR cells per microwell were cultured overnight on a 96-well plate. The medium was changed to 100 μL of an Actinomycin D (2 μg/mL; Sigma-Aldrich) containing medium, and cells were cultured for 1 hour. Diluted plasma samples (100 μL; 25% and 50% plasma in PBS) or recombinant TNF-α standards (0-1000 pg/mL in 1% bovine serum albumin–PBS, Pierce-Endogen, Rockford, IL) were added to wells, and cells were cultured overnight. Thirty microliters of 5 mg/mL methylthiazolyldiphenyl-tetrazolium bromide (Sigma-Aldrich) was added to wells, and cells were cultured for 2 hours. The medium was replaced with 150 μL of dimethyl sulfoxide (Riedel-de Haen, Seelze, Germany) and mixed for 10 minutes, and the absorbance at 595 nm was measured. A standard curve derived from TNF-α standards was generated, and plasma TNF-α concentrations were calculated. All samples were measured twice on separate assays, and the mean was calculated.

Interleukin-6 (IL-6)

Plasma IL-6 levels were determined with a commercial ELISA assay (Human IL-6 Instant ELISA, Bender MedSystems, Vienna, Austria). The sensitivity of the assay was 0.92 ng/mL.

Clinical Assessment

Measurements of the serum alanine aminotransferase (ALT), plasma international normalized ratio (INR), and serum bilirubin were performed daily as a part of the routine follow-up. For ALT and INR, values measured within 72 hours of reperfusion (3 postoperative days) were included in the data analysis. The serum bilirubin level was recorded at postoperative day 7. The outcome within 3 months was recorded.

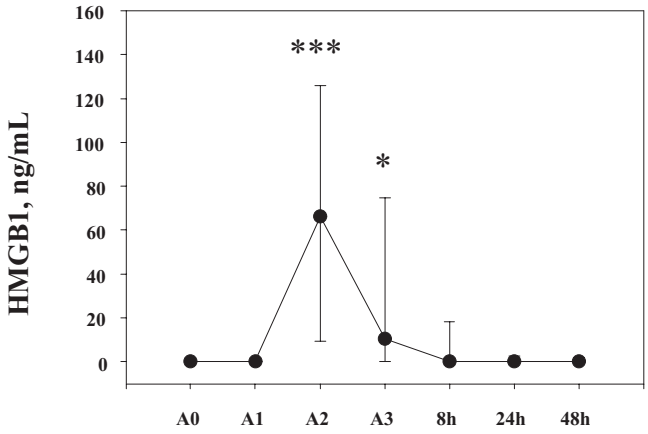

Figure 1. Plasma HMGB1 levels in systemic circulation during and after transplantation. Time points: before surgery (A0), immediately before initial reperfusion (A1), 10 minutes after portal vein declamping (A2), 10 minutes after hepatic artery declamping (A3), and 8, 24, and 48 hours after reperfusion. Data are expressed as medians (interquartile range); that is, whiskers denote 25th and 75th percentiles, respectively. Friedman's test revealed significant change over time ($P < 0.001$). *$P < 0.05$ and ***$P < 0.001$ versus before surgery, Wilcoxon signed rank test with Holm's correction for multiple comparisons. Abbreviation: HMGB1, high mobility group box 1.

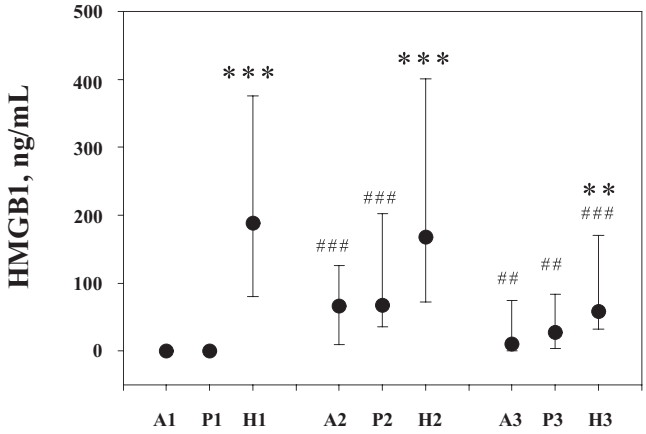

Figure 2. Plasma HMGB1 levels during reperfusion. Data are expressed as medians (interquartile range), that is, whiskers denote 25th and 75th percentiles, respectively. **$P < 0.01$ and ***$P < 0.001$ for P versus H, Wilcoxon signed rank test. ##$P < 0.01$ and ###$P < 0.001$ versus previous time point (for example, A2 versus A1), Wilcoxon signed rank test. Abbreviations: A, systemic artery; H, hepatic vein (caval effluent during initial reperfusion); HMGB1, high mobility group box 1; P, portal vein.

Statistical Analysis

As the data distribution was skewed by the Kolmogorov-Smirnov test, nonparametric tests were used. Time-dependent changes were evaluated with Friedman's test for repeated measurements followed by Wilcoxon signed rank test with Holm's correction for multiple comparisons. For the transhepatic gradient, the portal vein value was subtracted from the hepatic vein value. Pairwise comparisons were made with the Wilcoxon signed rank test. Bivariate correlations were tested with Spearman's rank correlation. The α level was 0.05 for all statistical tests. Data are expressed as medians with interquartile ranges or ranges when separately indicated.

RESULTS

Clinical Outcome

Patient and graft characteristics are shown in Table 1. After transplantation, the degree of hepatocellular damage was mild (Table 1). During the 3-month follow-up period, acute rejection occurred in 8 (40%) patients. Graft survival and patient survival were both 100%.

Plasma HMGB1 Levels

In the donors, low HMGB1 levels were detected before surgery [4 (2-10) ng/mL in ELISA analysis]. In the recipients, apart from 3 patients, HMGB1 was undetectable in systemic circulation before surgery and reperfusion (Fig. 1). After reperfusion, HMGB1 was released into systemic circulation, peaking 10 minutes after portal vein declamping [66 (9-126) ng/mL, $P < 0.001$ versus preoperatively; Fig. 1]. Peak HMGB1 levels in individual patients were as high as 611 ng/mL. HMGB1 levels declined rapidly (Fig. 1), and postoperatively, HMGB1 was still detectable in only 6 patients.

Throughout surgery, HMGB1 levels remained equal in systemic arterial and portal venous blood (Fig. 2). In contrast, HMGB1 levels were significantly higher in hepatic venous blood than in portal blood (Fig. 2). During initial reperfusion, HMGB1 levels in both systemic arterial and portal venous blood were low or undetectable [artery, 0 (0-0) ng/mL; portal vein, 0 (0-3) ng/mL], whereas HMGB1 levels were very high in the caval effluent [188 (80-371) ng/mL; transhepatic gradient, 187 (80-311) ng/mL; $P < 0.001$, caval effluent versus portal vein; Fig. 2]. Peak HMGB1 levels in individual patients were as high as 795 ng/mL. Although HMGB1 levels in both portal and hepatic venous blood decreased toward the end of surgery, HMGB1 release from the graft continued at 10 minutes after both portal vein and hepatic artery declamping [transhepatic gradient, 63 (46-185) ng/mL, $P < 0.001$, and 16 (0-91) ng/mL, $P < 0.01$, respectively; Fig. 2].

In 4 randomly selected patients, we measured HMGB1 levels with both Western blotting and a commercially available ELISA kit (Table 2; preoperative and postoperative data are not shown). Overall, HMGB1 levels measured with Western blotting were 5 times greater (median) than those measured with ELISA, although the results from the 2 methods correlated well ($r = 0.737$, $P < 0.001$). When recombinant HMGB1 was mixed with plasma to a concentration of 30 ng/mL, ELISA yielded values of 14.5 $\pm$0.3 ng/mL. Thus, ELISA seems to underestimate the HMGB1 plasma levels.

TABLE 2. Actual High Mobility Group Box 1 Protein Concentrations (ng/mL) by Western Blotting and ELISA

		Initial Reperfusion			10-Minute Portal Vein Declamping			10-Minute Hepatic Artery Declamping		
		A	P	H	A	P	H	A	P	H
Patient 1	WB	0	67	378	75	261	341	14	243	214
	ELISA	3	4	127	32	37	86	19	26	41
Patient 2	WB	0	0	235	70	64	140	0	4	106
	ELISA	1	1	101	40	39	44	17	14	18
Patient 3	WB	16	15	658	197	257	421	7	23	34
	ELISA	1	2	68	29	29	34	2	4	3
Patient 4	WB	0	0	23	99	34	632	17	4	171
	ELISA	2	5	113	21	2	26	8	15	8

Abbreviations: A, systemic artery; ELISA, enzyme-linked immunosorbent assay; H, hepatic vein (caval effluent at initial reperfusion); P, portal vein; WB, Western blotting.

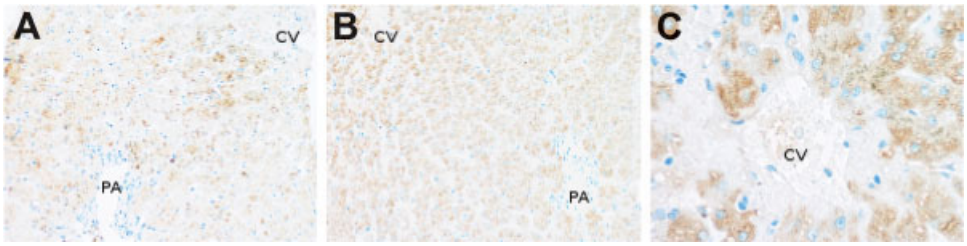

Figure 3. Liver HMGB1 staining (A) before and (B,C) after reperfusion (20× magnification and 60× magnification, respectively). HMGB1 is expressed predominantly in hepatocytes located close to CV with only occasional staining next to PA. Abbreviations: CV, central veins; HMGB1, high mobility group box 1; PA, portal areas.

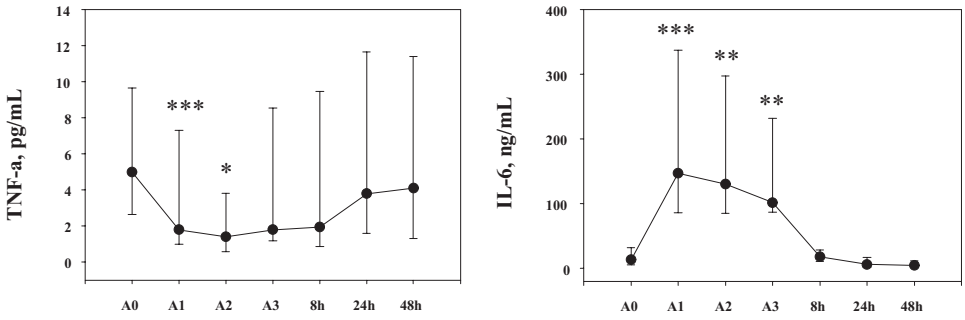

Figure 4. Plasma TNF-α and IL-6 levels in systemic circulation during and after transplantation (for time points and detailed explanations, see Fig. 1). *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$ versus before surgery. Abbreviations: IL-6, interleukin-6; TNF-α, tumor necrosis factor α.

HMGB1 Immunohistochemistry

Immunohistochemistry revealed weak HMGB1 positivity in biopsies taken before graft perfusion during procurement [grade 1 (1-3)]. HMGB1 staining was more intense in biopsies taken after reperfusion [grade 2 (0-3), $P = 0.020$, before reperfusion versus after reperfusion]. HMGB1 was expressed predominantly in the cytoplasm of hepatocytes located close to the central veins, with only occasional staining in portal regions (Fig. 3). Neutrophils exhibited only occasional HMGB1 staining (grade 1). The sinusoidal endothelium and portal structures, including bile ducts, were HMGB1-negative (grade 0). The intensity of HMGB1 staining in biopsies taken both before ($r = 0.619$, $P = 0.009$) and after reperfusion ($r = 0.579$, $P = 0.015$) correlated with HMGB1 levels in the caval effluent.

Plasma TNF-α Levels

During surgery, TNF-α levels in systemic circulation decreased slightly, returning postoperatively close to the preoperative levels (Fig. 4). There was no difference in TNF-α levels across the hepatic circulation at any point during reperfusion (Table 3).

Plasma IL-6 Levels

IL-6 levels in systemic circulation increased already before reperfusion (Fig. 4). IL-6 levels remained high throughout surgery but declined postoperatively (Fig. 4). During initial reperfusion, IL-6 uptake into the graft occurred, as IL-6 levels were higher in portal venous blood than in the caval effluent ($P = 0.038$, caval effluent versus portal vein; Table 3). At 10 minutes after portal vein declamping, slight IL-6 release from the

graft was evident ($P = 0.044$, hepatic vein versus portal vein; Table 3).

TABLE 3. TNF-α and IL-6 Levels During Reperfusion

	Initial Reperfusion			10-Minute Portal Vein Declamping			10-Minute Hepatic Artery Declamping		
	A	P	H	A	P	H	A	P	H
TNF-α, pg/mL	1.7 (1.0–4.8)	1.9 (0.7–4.9)	1.7 (0.6–3.2)	1.3 (0.5–3.3)	1.2 (0.5–2.1)*	1.3 (0.2–4.5)	1.8 (1.0–6.9)	2.1 (0.7–7.3)	2.5 (1.2–6.9)
IL-6, ng/mL	147 (86–337)	153 (81–361)	135 (60–248)†	124 (85–184)	122 (77–264)	121 (67–222)†	96 (86–224)	113 (80–242)	97 (58–222)

NOTE: Data are expressed as medians (interquartile range).
Abbreviations: A, systemic artery; H, hepatic vein (caval effluent at initial reperfusion); IL-6, interleukin-6; P, portal vein; TNF-α, tumor necrosis factor α.
*$P < 0.05$, A versus P.
†$P < 0.05$, P versus H.

HMGB1 and Other Inflammatory Markers

Plasma HMGB1 levels were not related to TNF-α or IL-6 levels at any time point or blood sampling location (data not shown). Plasma TNF-α and IL-6 levels correlated in systemic arterial blood but not in samples drawn across the hepatic circulation (data not shown).

HMGB1 and Clinical Outcome

The cold ischemic time had no influence on HMGB1 levels. Graft macrovesicular steatosis and peak postoperative ALT levels correlated with higher plasma HMGB1 levels in hepatic venous blood 10 minutes after hepatic artery declamping ($r = 0.497$, $P = 0.03$, and $r = 0.588$, $P = 0.008$, respectively; Fig. 5). Likewise, ALT levels correlated with HMGB1 levels in systemic arterial blood 10 minutes after hepatic artery declamping ($r = 0.496$, $P = 0.026$; Fig. 5). Of note, peak postoperative ALT levels correlated with neither the cold ischemic time nor graft steatosis. Neither postoperative INR nor bilirubin levels correlated with HMGB1 levels.

DISCUSSION

In this study, we describe extensive HMGB1 release from the graft during reperfusion in human liver transplantation. HMGB1 levels did not correlate with those of TNF-α and IL-6, and liver sequestered neutrophils exhibited only weak HMGB1 expression. Instead, hepatocytes expressed HMGB1, and reperfusion was associated with HMGB1 outflow from the graft. This HMGB1 release was associated with both graft steatosis and peak postoperative ALT levels. These results suggest that HMGB1 is a marker of hepatocellular injury in human liver transplantation.

Several findings in this study point to the liver graft and hepatocytes in particular as the primary source of HMGB1 during reperfusion. First, graft preservation and reperfusion induced up-regulation in hepatocyte HMGB1. Second, in systemic circulation, HMGB1 levels were undetectable before graft reperfusion and increased only after graft reperfusion. Third, there was no difference between systemic arterial and portal venous HMGB1 levels, and this suggested that HMGB1 was not released from the splanchnic area. Finally, during initial reperfusion, significant hepatic HMGB1 efflux was evident. HMGB1 levels were low or undetectable in portal venous blood, in contrast to considerably high levels in the caval effluent. After reperfusion, HMGB1 levels remained significantly higher in hepatic venous than in portal blood, indicating ongoing HMGB1 release from the graft.

Although hepatic HMGB1 outflow continued, both the absolute HMGB1 levels in hepatic venous blood and the calculated transhepatic gradient decreased after initial reperfusion. HMGB1 efflux appears to reflect washout of HMGB1 released into extracellular space

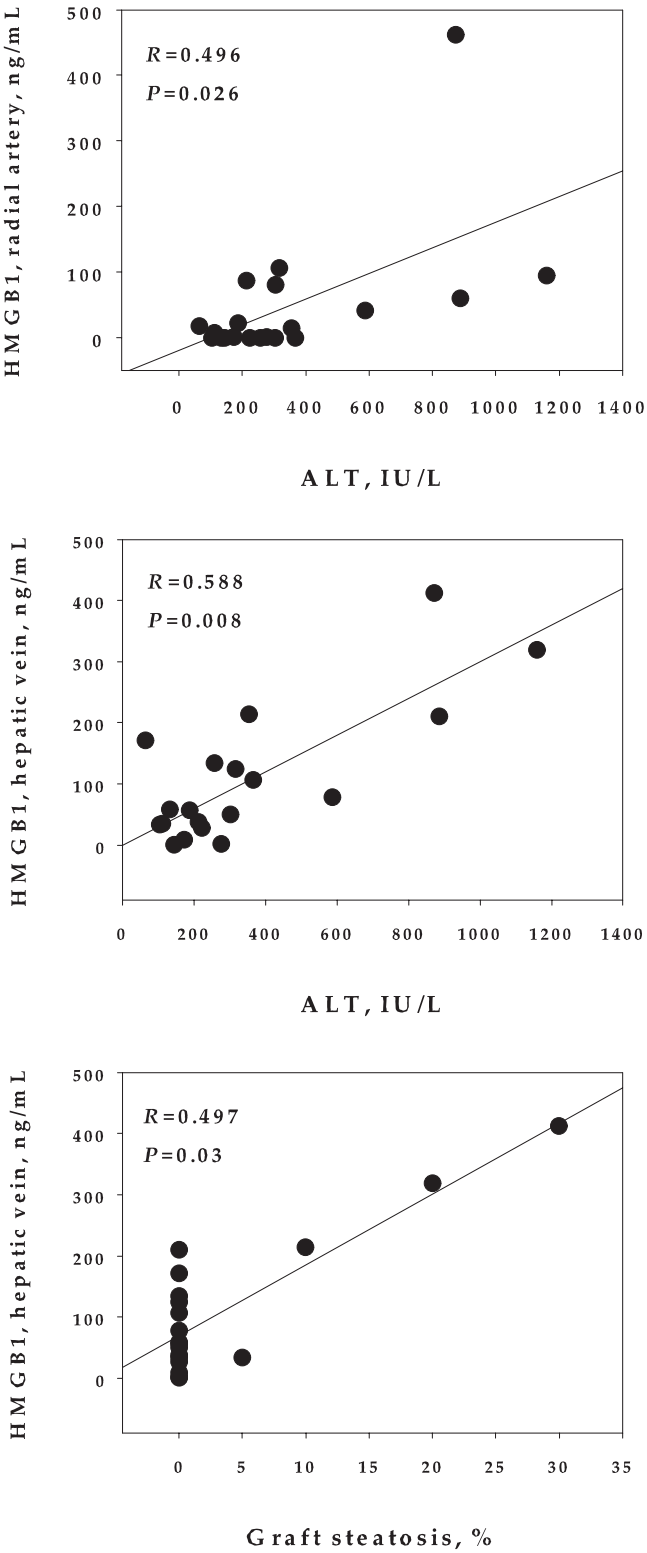

Figure 5. Correlations between HMGB1 levels, ALT, and graft steatosis. For HMGB1, levels represent those measured from radial arterial and hepatic venous blood at 10 minutes after hepatic artery declamping (H3). For ALT, values represent peak postoperative levels within 72 hours from reperfusion. Abbreviations: ALT, alanine aminotransferase; HMGB1, high mobility group box 1.

already during graft preservation and immediately after reperfusion. HMGB1 may be released both through active secretion from various cells, including activated monocytes/macrophages,[9] neutrophils,[25] and endothelial cells,[26] and passively from necrotic cells.[5] Both mechanisms likely contribute to the described HMGB1 release from the graft during liver transplantation.

HMGB1 is released as a danger signal from damaged cells.[17] Through the TLR4 system, HMGB1 produces an early inflammatory response,[17] leading to amplification of HMGB1 secretion.[27] Active HMGB1 secretion from phagocytes displays delayed kinetics.[3,9,20] In experimental models of endotoxemia, HMGB1 levels increase 12 to 18 hours after peak TNF-α levels.[10] This delayed active HMGB1 release may partly explain some of our findings. Plasma HMGB1 levels were slightly elevated in organ donors already before graft procurement, reflecting a systemic proinflammatory response induced by brain death.[28-30] Furthermore, surgical manipulation of the liver during procurement activates Kupffer cells, leading to hepatic expression of proinflammatory mediators.[30,31] Indeed, weak hepatocyte HMGB1 staining was already evident in biopsies taken immediately before graft perfusion during procurement. Inflammatory responses related to both brain death and surgical stress likely enhanced hepatic HMGB1 expression. Moreover, liver nonparenchymal cells, including tissue macrophages (Kupffer cells) and the sinusoidal endothelium, are sensitive to cold ischemia.[2,32] Although the cold ischemic time was short (median, 5 hours), active HMGB1 secretion during graft procurement and preservation may partly account for hepatic HMGB1 outflow during reperfusion.

Although hepatocytes are relatively resistant to cold ischemia, preservation ultimately damages cell structures. Cell destruction is exacerbated during reperfusion by activated and adherent neutrophils producing oxygen free radicals and proteolytic enzymes.[2,33] Necrotic cells readily release HMGB1. Even when cellular integrity is maintained, hepatocyte HMGB1 expression increases quickly after noxious stimuli.[17,34] In this study, immunohistochemistry revealed HMGB1 expression predominantly in hepatocytes located close to the central veins, that is, in areas most susceptible to ischemic injury. Furthermore, hepatocyte HMGB1 expression correlated with HMGB1 levels in the caval effluent. The observed hepatic HMGB1 efflux appears to originate mainly from hepatocytes through either active secretion from ischemic cells or passive release from necrotic ones.

In contrast to intense hepatocyte HMGB1 expression, mostly weak HMGB1 staining was evident in neutrophils sequestered within the graft. This does not, however, exclude neutrophils as a potential source of HMGB1 in liver transplantation. Because of the substantial delay in HMGB1 secretion from activated phagocytes,[3,9,20] it is possible that neutrophils sequestered into the graft during reperfusion may not have expressed HMGB1 at the time of liver biopsy, a few hours after reperfusion. Although we could not obtain blood samples across the hepatic circulation postoper-

atively to investigate hepatic HMGB1 release, HMGB1 levels in systemic circulation declined rapidly after reperfusion, and postoperatively, HMGB1 was undetectable in most patients. This suggests that, despite inducing intense momentary hepatic phagocyte activation and sequestration,[33,35] reperfusion does not lead to extensive delayed hepatic HMGB1 release into systemic circulation.

Our results are partly different from those for liver I/R injury in an experimental setting. In comparison with cold ischemia in liver transplantation, the 60- to 75-minute warm ischemia time used in experimental studies[17,18] is very robust. After warm liver I/R injury, both liver tissue[17,18] and bloodstream[18] HMGB1 levels increase in a time-dependent manner up to 24 hours, reflecting ongoing cellular damage and escalating inflammation. In contrast, we have reported that HMGB1 levels begin to decrease progressively within 1 to 2 hours after reperfusion, indicating that both ischemic damage and inflammatory response are limited after cold ischemia. Although HMGB1 kinetics are quite different after warm and cold hepatic ischemia, hepatocytes seem to be the main source for HMGB1, regardless of the type of ischemia.[17]

Cold ischemia time, a known risk factor for primary graft dysfunction, correlated with neither perioperative HMGB1 nor postoperative ALT levels. Furthermore, steatosis and peak ALT levels were not related. Hepatic venous HMGB1 levels measured at 10 minutes after hepatic artery declamping (median, 45 minutes after initial reperfusion) correlated with both graft steatosis and peak postoperative ALT. The correlation between HMGB1 release and graft steatosis suggests that even mild steatosis predisposes the graft to pronounced injury during preservation and reperfusion. Furthermore, graft HMGB1 outflow was associated with higher peak postoperative ALT levels, and this suggests that HMGB1 is an indicator of hepatocellular injury. Some limitations regarding the clinical implications of the results exist. The patient sample was relatively small with good postoperative liver function, and only a few steatotic grafts were transplanted during the study period. Moreover, blood sampling from the hepatic vein and HMGB1 detection with Western blotting are impractical in routine clinical use. However, we also demonstrated that HMGB1 levels in systemic arterial blood correlated with peak postoperative ALT levels. In addition, HMGB1 levels determined by Western blotting and ELISA correlated strongly, although ELISA yielded somewhat lower concentrations. Therefore, HMGB1 determination from arterial blood with commercially available ELISA seems feasible in the clinical setting. Our findings call for future studies with sufficient sample size to explore whether HMGB1 is a useful marker in identifying grafts developing primary graft dysfunction.

Recently, cytokine properties of HMGB1 have gained attention. HMGB1 is secreted by, and activates proinflammatory responses in, phagocytes and endothelial cells.[3] During liver transplantation, HMGB1 kinetics were distinct from those of TNF-α and IL-6. Surgery itself induced a significant elevation in IL-6 levels, with a concomitant decrease in TNF-α levels without significant changes during reperfusion. In contrast, HMGB1 levels increased only after reperfusion. We could not demonstrate any correlation between HMGB1 and TNF-α or IL-6 levels. This is in line with previous work on sepsis patients failing to find an association between HMGB1 and various cytokines (IL-6, IL-8, IL-10, and TNF-α)[12] and reflects the complexity of inflammatory response in the clinical setting. In experimental models, HMGB1 was initially identified as a late acting inflammatory mediator.[9,10] In clinical sepsis, elevated HMGB1 levels persist for several days, indicating ongoing inflammation.[12-14] The rapidly declining HMGB1 levels described in this study argue against prolonged systemic inflammatory response after liver transplantation. Corroborating this, plasma IL-6 declined to presurgery levels quickly after transplantation. High plasma HMGB1 levels have been suggested to be lethal.[9] In this study, high systemic HMGB1 levels (up to 600 ng/mL) were not lethal, perhaps because of the short exposure time.

In conclusion, reperfusion in human liver transplantation was associated with extensive HMGB1 efflux from the graft. Hepatocytes expressed HMGB1, and its release correlated with peak postoperative ALT levels. These results suggest that HMGB1 is primarily a marker of hepatocellular injury in human liver transplantation. Our findings call for future studies to delineate the possible prognostic value of HMGB1 in I/R injury in human liver transplantation.

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