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Plasma Biomarkers to Study Mechanisms of Liver Injury in Patients with Hypoxic Hepatitis

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Abstract

Background and Aims—Hypoxic hepatitis is a clinical condition precipitated by prolonged periods of oxygen deprivation to the liver. It can have several underlying causes. Despite its prevalence in critically ill patients, which can reach upwards of 10%, very little is known about the mechanisms of injury. Thus, we set out to measure previously identified circulating biomarkers in an attempt to describe mechanisms of injury following hypoxic hepatitis.

Methods—Plasma from patients diagnosed with hypoxic hepatitis was collected for this study. Biomarkers of hepatocellular injury, mitochondrial damage, and cell death were measured. These results were compared against results obtained from well characterized acetaminophen overdose patients.

Results—At peak injury, ALT measured 4082 ± 606 U/L and gradually decreased over 5 days, corresponding to the clinically observed pattern of hypoxic hepatitis. Levels of GDH showed a similar pattern, but neither ALT nor GDH were significantly higher in these patients than in acetaminophen patients. Plasma levels of DNA fragments mimicked hepatocellular injury as measured by ALT and miRNA-122. Interestingly, we found a significant increase in caspase-cleaved cytokeratin-18; however, the full-length form greatly exceeded the cleaved form at the time of maximum injury (45837 ± 12085 vs. 2528 ± 1074 U/L). We also found an increase in

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CONFLICT OF INTEREST DISCLOSURE:

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acHMGB1 at later time points indicating a possible role of inflammation, but cytokine levels at these times were actually decreased relative to early time points.

Conclusions—The mechanism of injury following hypoxic hepatitis involves mitochondrial damage and DNA fragmentation. Importantly, necrosis, rather than apoptosis, is the main mode of cell death.

Keywords

Hypoxic hepatitis; ischemic hepatitis; biomarkers; apoptosis; necrosis; miRNA-122; acetaminophen

INTRODUCTION

Hypoxic hepatitis (HH) is a condition resulting from prolonged periods of hypoxia to the liver. Hypoxic hepatitis is recognized clinically by an hypoxic insult accompanied by sharp increases in plasma transaminase activity to about >20 times normal, and lack of other confounding etiologies of liver disease, such as viral or drug-induced hepatitis [1,2]. Typically, the inciting cause for HH involves an episode of cardiogenic, circulatory, or respiratory failure leading to decreased oxygen delivery to the liver [2]. Hypoxic hepatitis represents a serious source of morbidity and mortality, with a prevalence of approximately 10% in intensive care patients [3]. HH resolves with treatment of underlying causes, but mortality has reached 30–60% within one month [1,3–5].

Despite the prevalence and mortality of HH, very few studies have examined the cellular mechanisms underlying liver injury, in part due to the lack of an animal model. The most relevant model employed experimentally is the hemorrhagic shock/resuscitation model in which rodents are hemorrhaged to a hypotensive state (low flow ischemia) for a period of time, followed by a resuscitation period, after which cellular mechanisms of liver injury can be assessed. A major problem with this model is that liver injury does not always approach the degree of injury seen during clinical cases of HH, during which ALT levels easily exceed 20 times normal [6–8], indicating that the model does not completely recapitulate what is occurring in a clinical setting. Additionally, HH can be precipitated by a number of causes other than hemorrhagic shock [2]. Furthermore, most of these studies are limited in that they evaluate a time point shortly after (<6hrs) resuscitation, rather than a prolonged time course. Therefore, a different approach to study the mechanisms of injury during HH is needed.

One such approach is the use of mechanistic biomarkers of liver injury, such as mitochondrial DNA, caspase activities, microRNA-122 (miRNA-122), total and acetylated HMGB-1 (acHMGB1), and the ratio of caspase-cleaved to full-length cytokeratin-18. Our laboratories and others have previously used this approach to characterize both modes of cell death (apoptosis vs. necrosis) and cellular mechanisms of liver injury in ischemia/reperfusion injury in mice, as well as acetaminophen toxicity and cholestasis in both mice and humans [9–13]. In addition, a previous study has demonstrated that miRNA-122 may be a more sensitive marker of liver injury than ALT during HH in a porcine model of cardiogenic shock [14]. However, to date, no studies have employed the use of these biomarkers to characterize the mechanisms of liver injury following HH in humans.

Therefore, the aim of the current study was to assess the mechanisms of liver injury following HH using circulating plasma biomarkers in order to better understand cellular mechanisms leading to injury in these patients.

PATIENTS AND METHODS

Patient Characteristics and Study Design

Subjects with HH were selected from 266 patients who presented to Banner-University Medical Center Phoenix with initially suspected acetaminophen (APAP) toxicity. However, only those patients with peak plasma ALT > 1,000 IU/L whose hepatic necrosis was not caused by APAP (as determined by plasma APAP, APAP-protein adduct levels and medical history demonstrating a definitive history or strong likelihood of hypotension/shock) and who were subsequently diagnosed with HH were selected (total of 14 patients). A total of 15 age- and gender-matched subjects with APAP toxicity were selected among inpatients at the University of Kansas Medical Center or Banner-University Medical Center Phoenix as comparison. Patients with comorbidities contributing to liver injury (such as alcoholism or viral hepatitis) were excluded. Blood was obtained upon admission after informed consent, and then approximately every 24 hours. Because duration of hospitalization differed for each patient, for the purposes of this study, the day of peak injury (as assessed by clinically measured ALT levels) was considered “day 0”. Following blood collection, blood tubes were centrifuged, plasma collected, frozen, and sent to the University of Kansas Medical Center or the University of Liverpool for analysis. All patient information is summarized in Table 1. All biochemical parameters reported are averages of maximum values, not necessarily values at the time of peak ALT. All patient samples were procured with approval by, and in accordance with, both the Institutional Review Board at the University of Kansas Medical Center and Banner University Medical Center.

Biochemical measurements

All analytical methods for measurements of plasma biomarkers are described in the supplemental material.

RESULTS

Hypoxic hepatitis causes profound liver injury

We first measured the time course of injury in these patients to ensure they fit the clinical profile of HH. We found that plasma ALT activities rapidly rose to peak injury (4082 ± 606 U/L; Figure 1A) and steadily decreased over the next 5 days. When compared to healthy volunteers (HV), the difference in ALT levels was significantly higher at peak injury (4082 ± 606 vs. 23.8 ± 3.1 U/L) but not significantly different from patients with liver injury from APAP overdose (4082 ± 606 vs. 5744 ± 588 U/L; Figure 1B). Because ALT is not specific to the liver, and HH affects all organs simultaneously, we measured levels of the liver-specific microRNA-122 to ensure the majority of the ALT was from liver injury. Again, in HH patients, we found a dramatic increase to peak injury followed by a gradual but steady decline (Figure 1C). When compared to HV, levels of miRNA-122 in HH patients were significantly higher than controls (13.2 ± 3.1 vs. 0.52 ± 0.26 , respectively; Figure 1D). To

confirm liver injury was not caused by APAP, plasma levels of APAP-protein adducts were measured in both APAP and HH patients (Figure S1). Plasma levels of APAP-CYS in HH patients were $0.46 \pm 12 \mu\text{M}$, well below the toxic threshold of $1 \mu\text{M}$ (15). In contrast, the adduct levels for the APAP patients ($3.0 \pm 0.7 \mu\text{M}$) were 3-times above the threshold (Figure S1).

Hypoxic hepatitis causes hepatocellular necrosis

To differentiate whether liver injury is caused by necrosis or apoptosis, we measured plasma levels of full-length and caspase-cleaved cytokeratin-18. We observed a rapid increase in both forms of cytokeratin-18 at the time of peak ALT, followed by a gradual and steady decline over the next 5 days (Figures 2A,B). However, the magnitude of increase in the full-length form was approximately 18-fold higher than the caspase-cleaved form (45837 ± 12085 vs 2528 ± 1074) at the time of peak ALT. Furthermore, levels of full-length cytokeratin-18 were significantly higher than the caspase-cleaved form at each time point except for day 5, demonstrating that necrosis predominates during HH (Figure 2C). However, because there was an increase in caspase-cleaved cytokeratin-18, we measured caspase-3 activity in the plasma but found no measureable caspase activity in these samples (Figure 2D). Plasma from galactosamine/endotoxin (Gal/ET)-treated animals served as controls for parenchymal cell apoptosis [16]. The readily detectable caspase-3 activity in these animals suggested that if significant caspase-dependent apoptotic cell death occurs, plasma caspase-3 activity can be measured.

Mitochondrial injury occurs in HH

Numerous *in vitro* studies suggest mitochondria are targets of ischemia-reperfusion injury [17]. To test this hypothesis in these patients, we measured levels of the mitochondria-specific biomarkers GDH and mitochondrial DNA (mtDNA). Because these macromolecules are located within the mitochondrial matrix, only mitochondrial damage would be expected to lead to their release in plasma [10]. Accordingly, we found an increase in GDH which closely mimicked the increase in ALT – that is, a rapid increase at the time of peak ALT (1381 ± 229) followed by a gradual but steady decline (Figures 3A and S2). Similarly, plasma levels of mtDNA as measured by RT-PCR of the cytochrome c oxidase (CytC) gene showed a similar downward trend following peak injury (Figure 3C). Interestingly, neither levels of GDH nor CytC mtDNA in plasma of HH patients were significantly different compared to APAP overdose patients (Figures 3B,D).

Nuclear DNA fragmentation in HH

Because mitochondrial injury during necrosis has been shown to release endonucleases leading to nuclear DNA fragmentation and cell death [18], we measured plasma levels of nuclear DNA fragments. Our results show an increasing concentration of nuclear DNA fragments in plasma of HH patients which peaks at day zero ($1444 \pm 182\%$; Figure 4A). As with GDH, this time course of injury closely mimics that of ALT (Figure S3F). We then compared the levels of nuclear DNA fragments to those observed in APAP patients. As with GDH and mtDNA, there was no significant difference between HH and APAP patients (1444 ± 182 vs. $2531 \pm 552\%$, respectively; Figure 4B).

The inflammatory response does not exacerbate HH in later stages

HMGB1 is a nuclear protein that sits in the minor groove of DNA and modulates transcription of numerous genes. HMGB1 can be either passively released during necrosis or actively secreted in its acetylated form by inflammatory cells. Therefore, the two forms of HMGB1 can provide information on the mode of cell death as well as possible inflammatory cell activation during injury. To confirm necrosis as the mode of cell death, we measured total levels of HMGB1 and found a similar pattern of change over the course of injury as all other parameters measured thus far (Figure 4C). These data are consistent with our cytokeratin-18 measurements and provide further evidence for necrosis as the primary mode of cell death. To assess whether or not there is an immune component, we also measured levels of acetylated HMGB1 and found a reverse pattern – an increase in acHMGB1 at the later time points. When comparing acHMGB1 to total HMGB1, there is a time-dependent increase in the percent of acHMGB1 from less than 5% at the peak of injury to 20–30% during the recovery phase (Figure 4D). To determine whether the elevated acHMGB1 ratio we observed was an indication of a robust inflammatory response during the later stages of HH, we measured multiple cytokines. For most cytokines and chemokines, the highest levels were observed at the early injury with a downward trend at the later stages (Figures S5A – D).

In order to highlight the strong correlation between each of these plasma parameters with ALT, the time course of ALT versus miR-122, full-length cytokeratin-18, caspase-cleaved cytokeratin-18, mtDNA, nuclear DNA fragments, and total HMGB1 is shown in the Supplemental Figure 3 (S3). All parameters show a similar time-dependent increase and subsequent decrease in plasma as ALT (Figure S3A–G). This is further supported by linear regression analysis, which demonstrated a significant correlation of each parameter with ALT on each day (Figure S4A–G). The only exception is plasma levels of acetylated HMGB1, an indicator of inflammatory cell activation, which does neither show a temporary correlation (Figure S3H) nor a significance with linear regression (Figure S4H).

DISCUSSION

In the present study, we set out to investigate the mechanisms of injury during HH in patients. To accomplish this, we investigated a series of circulating plasma biomarkers, which have previously been shown to characterize mode and mechanisms of cell death in experimental models and other human liver diseases [9–13]. Because APAP hepatotoxicity is well- characterized in both animal models and humans [19], we compared the findings in patients with HH to those in patients with APAP toxicity.

Liver injury during HH

We found liver injury in our patients fits the clinical profile of HH, mostly characterized by a sharp rise in ALT greater than 20 times normal. Although not liver specific, ALT is clinically used for assessment of liver injury. Multiple studies showed that miRNA-122 is a more sensitive and specific indicator of ongoing liver injury, rising earlier and to a much greater degree than ALT [13,20,21]. Because we detected a dramatic increase of the liver-specific miRNA-122 in HH patients similar to that previously observed in APAP hepatotoxicity, we

conclude that the majority of ALT release in these patients was derived from hepatocytes – an important distinction in the setting of HH, since hypoxia is not limited to the liver. Our results are consistent with previously reported values of miRNA-122 in a porcine model of cardiogenic shock [14]. Interestingly, our cohort of HH patients were found to have low levels of APAP adducts in plasma (Figure S1), however, such low levels of adducts are well under the level which would be expected if APAP caused a significant amount of liver injury [15]. More likely, these individuals consumed a therapeutic dose of APAP and adducts were passively released as a result of the hypoxic event leading to HH [22].

Mode of cell death during HH

To differentiate whether or not hepatocellular injury was occurring as a result of apoptosis or necrosis, we measured plasma levels of cytokeratin-18, an intermediate filament protein which composes part of the cytoskeleton. During apoptosis, active caspases cleave cytokeratin creating a neo-epitope, which can be recognized by the M30 antibody. In contrast, during necrosis, caspases are not active and mainly full-length cytokeratin-18 is released. Thus, the ratio of cleaved/full-length cytokeratin-18 can be used to determine whether cells undergo apoptosis or necrosis. Given that at any time during the observation period >95% of the detectable cytokeratin-18 levels in blood of these patients was the full-length form, our data strongly suggest that necrosis was the dominant form of liver cell death during HH. Even still, we saw significant elevations in the caspase-cleaved form of cytokeratin-18 at various time points. However, no detectable caspase activity could be found in plasma of HH patients despite the fact that plasma caspase-3 activities are readily measurable in experimental models of apoptosis [10,16]. In light of elevated levels of caspase-cleaved cytokeratin-18, however, these results should be interpreted with caution, since little is known about the stability or half-life of active caspases in plasma. It is possible that caspases are rapidly degraded or eliminated from plasma before blood samples are obtained. Therefore, caspase-3 activities, especially at later time points during the recovery phase could be lower than in the liver. Regardless, when compared quantitatively with the full-length form of cytokeratin-18 (Figure 2C), it becomes clear that the vast majority of cell death is caused by necrosis, and that apoptosis, as indicated by cleaved cytokeratin-18, contributes overall relatively little to liver injury in this setting.

Mitochondrial damage as a hallmark of cell death in HH

In other causes of liver injury, such as APAP toxicity, in which necrosis is the predominant form of cell death, mitochondria play a major role in the pathophysiology of injury [23]. In the experimental model and in APAP overdose patients, mitochondrial rupture leads to release of matrix macromolecules which can be measured in plasma [10]. Furthermore, endonuclease G released from damaged mitochondria translocates to the nucleus where it causes DNA fragmentation and cell death [18,23]. Our results show that the increase in mitochondrial injury as measured by GDH and mitochondrial DNA mirror the pattern of injury as measured by ALT. Similarly, levels of nuclear DNA fragments follow this trend. These results provide strong evidence for mitochondrial damage as a key event in the mechanism of liver cell death in HH patients. Interestingly, our *in vivo* results in humans correlate with many experimental studies which demonstrate a critical role of mitochondria in reperfusion injury [17,24,25]. It has been shown that during hypoxia intracellular pH

decreases, which actually exerts a protective effect against hypoxic injury [26]. However, upon re-oxygenation, intracellular pH returns to normal, precipitating formation of the mitochondrial membrane permeability transition pore (MPTP) and mitochondrial rupture [26,27]. Mechanisms behind pH-induced mitochondrial injury are thought to involve mitochondrial calcium uptake [27]. Collectively, a clinical picture begins to emerge: during a period of hypoxia, ATP levels fall leading to a decreased ability to sequester calcium within mitochondria [28,29]. Return of oxygen allows for oxidative phosphorylation and elevated cytosolic calcium levels, leading to mitochondrial calcium sequestration. This incites formation of the MPTP, which triggers matrix swelling, rupture and necrotic cell death. Based on our previous data, it is possible that endonuclease G released from ruptured mitochondria produces DNA fragmentation, but further studies are needed for verification.

Role of inflammation in HH

Interestingly, we found an increase in acetylated HMGB1 (acHMGB1) protein in plasma of HH patients at later time points. Since macrophages actively secrete acHMGB1 upon activation [30], our data suggest the activation of inflammatory cells during the recovery phase. These findings are consistent with activation of macrophages and other phagocytes for removal of cell debris and regeneration of the damaged tissue. However, the majority of cytokines were slightly elevated earlier. Since the gastrointestinal tract is very susceptible to microcirculatory changes during ischemic injury [31], it is possible that intestinal injury allows for bacterial translocation and macrophage activation before liver injury occurs. Thus, the initial inflammatory response is on the decline at the point when these blood samples were drawn and the increased acHMGB1 levels, reflecting a sterile inflammatory response, could be the result of macrophage activation in an attempt to clear debris rather than exacerbate injury [32]. This has also been shown in APAP overdose patients, where neutrophil and monocyte activation and hepatic infiltration are crucial for liver repair and regeneration [33,34]. This seems very likely given that injury is decreased at later time points despite an apparent activation of inflammatory cells. This is in stark contrast to models where inflammation is involved in propagation of injury, such as no-flow ischemia, in which sterile inflammation leads to exacerbation of injury well past the point of reperfusion [35].

Overall, our data demonstrated that HH, similar to APAP hepatotoxicity, is characterized by extensive mitochondrial damage and necrotic cell death. Although these mechanistic biomarkers gave novel insight into the mechanism of these disease states in humans, there was no relevant difference between the parameters in APAP and HH that would allow a more accurate diagnosis. However, our previous studies looking at the miRNA profile between these patient groups indicated that similar miRNAs with different levels and unique miRNAs can be detected in plasma of these patients suggesting that the miRNA profile may be used to differentiate between APAP hepatotoxicity and HH [36]. Thus, plasma biomarkers are useful in both better understanding the mechanisms of the disease in humans and can be used to aid in the differential diagnosis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

ALT	alanine aminotransferase
GDH	glutamate dehydrogenase
miRNA-122	microRNA-122
HMGB1	high mobility group box 1
acHMGB1	acetylated HMGB1
HH	hypoxic hepatitis
APAP	acetaminophen
HV	healthy volunteers
Gal/ET	galactosamine/endotoxin
mtDNA	mitochondrial DNA
CytC	cytochrome c oxidase

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KEY POINTS

- During hypoxic hepatitis, cell death occurs primarily by oncotic necrosis.
- Mitochondrial injury appears to be a prominent feature of injury as a result of hypoxic hepatitis.
- The pattern of changes in most plasma biomarkers of injury in hypoxic hepatitis patients closely mimics the changes observed in acetaminophen overdose patients.
- Circulating plasma biomarkers represent a promising new approach to the study of hypoxic hepatitis.

Figure 1. Time course of injury following hypoxic hepatitis

(A) Plasma levels of ALT in both HH and APAP patients from -1 to +5 days after peak injury. (B) Dot histogram comparing plasma levels of ALT between HH patients and healthy volunteers (HV) or patients with APAP toxicity at the time of peak ALT. (C) miRNA-122 for patients with HH are shown from -1 to +5 days after peak injury. (D) Dot histogram comparing plasma levels of miRNA between HH patients and HV at the time of peak ALT. Line graph data are presented as mean $\pm$ SE. Box plots show the 25th and 75th percentiles. Whiskers show 5th and 95th percentiles. * $p < 0.05$.

Figure 2. Necrotic cell death predominates during hypoxic hepatitis

Plasma levels of (A) full-length cytokeratin-18 (M65) and (B) caspase-cleaved cytokeratin-18 (M30) from -1 to +5 days after peak injury. (C) Total cytokeratin-18 levels during the same time course demonstrating the relative contributions of each form of cytokeratin. (D) Caspase activity in plasma at various time points following peak injury. As positive control for apoptosis, plasma samples were obtained from mice treated with galactosamine/endotoxin for 6 h. Data are represented as mean $\pm$ SE. * p <0.05 (compared to M30).

Figure 3. Mitochondrial injury occurs in hypoxic hepatitis

Plasma levels of (A) GDH and (C) cytochrome c oxidase (CytC) mtDNA for patients with HH or APAP overdose shown from -1 to +5 days after peak injury. Data are presented as mean $\pm$ SE. Dot histograms comparing plasma levels of (B) GDH and (D) CytC mtDNA between HH patients and healthy volunteers (HV) or patients with APAP toxicity at the time of peak ALT. Box plots show the 25th and 75th percentiles. Whiskers show 5th and 95th percentiles. * $p < 0.05$.

Figure 4. Cell death involves DNA fragmentation and release of HMGB1

(A) Plasma levels of DNA fragments in HH and APAP patients from -1 to +5 days after peak injury. (B) Dot histogram comparing plasma levels of DNA fragments between HH patients and healthy volunteers (HV) or patients with APAP toxicity at the time of peak injury. (C) Total HMGB1 in HH patients measured from -1 day to +5 days after peak injury. (D) Percent of hyperacetylated HMGB1 of total HMGB1 in the plasma of HH patients from -1 to +5 days after peak injury. Line graph data are presented as mean $\pm$ SE. Box plots show the 25th and 75th percentiles. Whiskers show 5th and 95th percentiles. * $p < 0.05$.

Table 1

Patient characteristics

Parameter	Hypoxic Hepatitis	Acetaminophen
% M:F	56:44	33:67
Median Age (Range)	39 (18–62)	35 (19–65)
Survival (%)	92.9 (13/14)	93.3 (14/15)
Median Hospital Stay (Range)	10.8 (2–30.2)	8 (3–19)
AST (U/L)	8574 ± 1696	9324 ± 1251
ALT (U/L)	4082 ± 606	5744 ± 588
Creatinine (mg/dL)	5.3 ± 0.9	3.1 ± 0.7
Bilirubin (mg/dL)	1.8 ± 0.4	9.7 ± 2.4 *

Clinical parameters and other characteristics of patients diagnosed with HH and acetaminophen toxicity. Peak values for AST, ALT, creatinine and bilirubin are reported as average ± SEM of peak value, not necessarily at the time of peak ALT.

NR = Not Reported.

*
p<0.05.