High-Mobility Group Box-1 and Liver Disease

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High-mobility group box-1 (HMGB1) is a ubiquitous protein. While initially thought to be simply an architectural protein due to its DNA-binding ability, evidence from the last decade suggests that HMGB1 is a key protein participating in the pathogenesis of acute liver injury and chronic liver disease. When it is passively released or actively secreted after injury, HMGB1 acts as a damage-associated molecular pattern that communicates injury and inflammation to neighboring cells by the receptor for advanced glycation end products or toll-like receptor 4, among others. In the setting of acute liver injury, HMGB1 participates in ischemia/reperfusion, sepsis, and drug-induced liver injury. In the context of chronic liver disease, it has been implicated in alcoholic liver disease, liver fibrosis, nonalcoholic steatohepatitis, and hepatocellular carcinoma. Recently, specific posttranslational modifications have been identified that could condition the effects of the protein in the liver. Here, we provide a detailed review of how HMGB1 signaling participates in acute liver injury and chronic liver disease. (*Hepatology Communications* 2018;2:1005-1020)

High-mobility group (HMG) proteins were isolated in the early 1960s and were later characterized by their chemical and physical properties, receiving their name due to their fast electrophoretic mobility on polyacrylamide gels.[1] They are among the most ubiquitous, abundant, and evolutionarily conserved proteins in eukaryotes. These and the other members of a larger HMG superfamily share a common structural HMG box (HMGB) motif that is a unique ~80 residue L-shaped domain that mediates DNA binding.[2]

Native HMGB proteins have molecular weights of approximately 22-25 kDa and are quite homologous within higher eukaryotic species (100% for human,

mouse, and rat HMGB1 or HMGB3 and 99% for HMGB2).[3] The expression of HMGB1 is ubiquitous, whereas HMGB2 is mainly expressed in lymphoid tissues, the gastrointestinal tract, and testis in adult animals. HMGB3 is present in embryos, lung, nasopharynx, bronchus, placenta, and hematopoietic stem cells, and HMGB4 is restricted to testis.[4–8] Among the four HMGB proteins, HMGB1 is the most abundant nonhistone nuclear protein and is also cytoplasmically expressed as it shuttles back and forth from the nucleus to the cytoplasm.[4,9]

HMGB1 has 215 residues and a predicted molecular mass of 24,894 Da in its native state. The protein is organized into two DNA-binding domains (box A

FIG. 1. HMGB1 protein structure. HMGB1 has 215 amino acids and two NLS (NLS1 spanning from histidine 27 to lysine 43 and NLS2 spanning from alanine 178 to lysine 184). Cysteines that can undergo oxidation are written in red (C23 and C45 form a disulfide bond), lysines that can undergo acetylation are written in blue, and a serine that can undergo phosphorylation is written in purple. These posttranslational modifications have been found in liver disease.

and box B, spanning nearly 75% of the protein) separated by a short linker sequence followed by a highly negatively charged C-terminal domain with 30 glutamic and aspartic acid residues (Fig. 1). Key features in the secondary structure are four β-strands and seven helical regions. The solution structure of the tandem HMGB domain has been resolved by nuclear magnetic resonance spectroscopy. Notably, the protein has three regions of interest in positions 80-96 (lipopolysaccharide [LPS] binding[10]), 89-108 (cytokine-stimulating activity[11]), and 150-183 (receptor for advanced glycation end products [RAGE] binding).

In addition, HMGB1 has two nuclear localization signals (NLSs) in positions 27-43 within box A (NLS1) and 178-184 within box B (NLS2) (Fig. 1).

HMGB1 was originally discovered as a nuclear protein; however, when it is passively released or actively secreted after injury or cell stimulation, HMGB1 meets all the criteria of a damage-associated molecular pattern (DAMP) and works as a necrosis signal for the immune system through cell-surface receptors.[12-14] In this latter role, HMGB1 acts as a

proinflammatory cytokine that contributes to multiple injuries, including those from the liver, such as warm and cold ischemia/reperfusion (I/R) injury,[15-27] sepsis,[23,28-32] acetaminophen (APAP) intoxication,[23,33-40] alcoholic liver disease (ALD),[41-43] fibrosis,[44-49] nonalcoholic steatohepatitis (NASH),[50-52] and hepatocellular carcinoma (HCC).[53-60] By binding cell-surface receptors on immune cells, HMGB1 activates intracellular signaling pathways that regulate immune cell function, including chemotaxis and cytokine production.[37,61-63] When HMGB1 targets hepatic stellate cells (HSCs), it induces a fibrogenic response.[44,49] Currently, less is known on how it signals in hepatocytes.

Our understanding of the role of HMGB1 has increasingly advanced during the last decade. Recent findings in molecular, structural, and functional analyses of HMGB1 have revealed that specific posttranslational modifications (PTMs) determine the effects of this powerful protein. Here, we aimed to provide an abridged review focusing on how HMGB1 signaling participates in acute and chronic liver disease.

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Localization and Secretion

Under physiologic conditions, HMGB1 localizes to the nucleus due to its two NLSs; yet, its nuclear localization changes during development, aging, and injury.[64] In the nucleus, HMGB1 binds double- or single-stranded DNA and has affinity for four-way junctions, bent DNA, and DNA adducts. The binding occurs in the minor groove of the DNA in a sequence-independent manner that covers approximately 14 base pairs.[2,65] HMGB1 contributes to establishing the tridimensional structural framework and geometric requirements essential for the binding of some transactivators and therefore enables the formation of the pre-initiation complex by recruiting various transcription factors.[66–69] HMGB1 induces DNA bending into a helical structure,[70–72] facilitating gene expression or DNA replication by allowing the formation of large multiprotein complexes.[2] The nuclear role of HMGB1 appears critical for survival as *Hmgb1*^{−/−} mice die within 24 hours after birth due to a deficit in the glucocorticoid receptor gene[73]; however, conditional ablation of *Hmgb1* in specific cell subsets in the liver does not cause a deleterious phenotype.[41,74,75] Moreover, under basal conditions, HMGB1 is not present or significantly reduced in the nucleus of some liver cells[44,49] looking otherwise healthy, suggesting that compensatory mechanisms may possibly exist; to date they are unknown.

Although HMGB1 constantly shuttles bidirectionally between the nucleus and the cytoplasm due to the action of importin and exportin in the nuclear pore complex, it preferentially accumulates in the nucleus during normal cellular homeostasis. However, acetylation of critical residues located in NLS1 (lysines 28-30) and NLS2 (lysines 180, 182-185) precludes HMGB1 from re-entering the nucleus; as a result, it builds up in the cytoplasm and is eventually secreted to the extracellular space[9,76,77] (Fig. 2).

HMGB1 can be cleaved by thrombin and thrombomodulin between arginine 10 and glycine 11 and by caspase-1 between aspartate 67 and lysine 68. It is unknown, however, if cleavage by caspase-1 in this region localized within NLS1 also prevents the protein from re-entering the nucleus or if the cleavage contributes to protein instability and degradation.
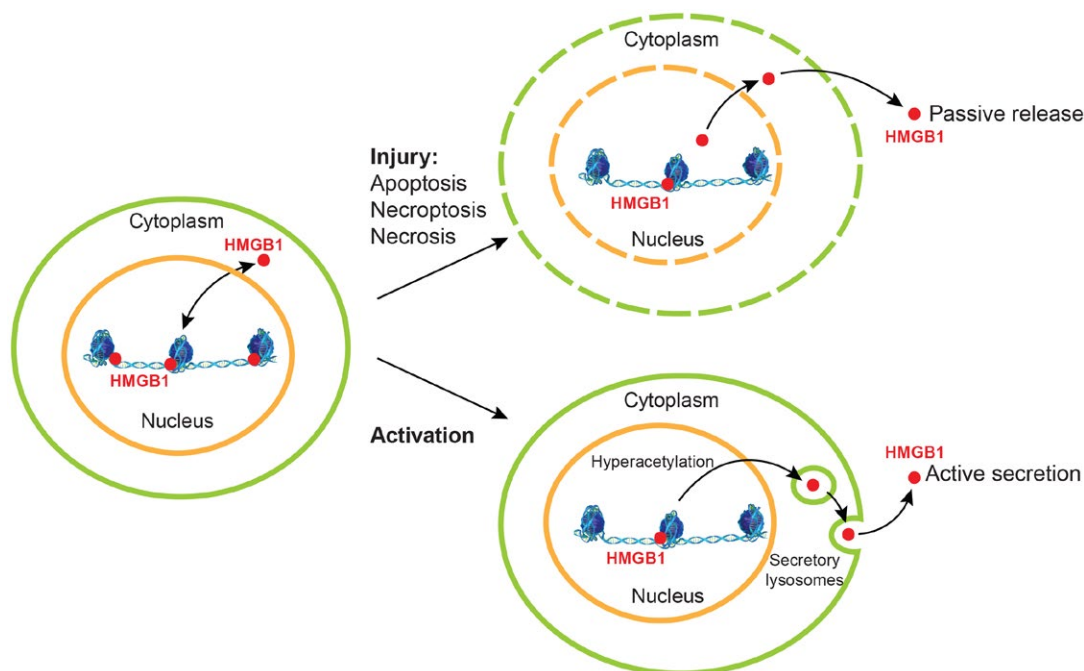
FIG. 2. HMGB1 localization and secretion. During normal cellular homeostasis, HMGB1 preferentially accumulates in the nucleus due to its two NLSs; however, it can shuttle bidirectionally between the nucleus and the cytoplasm by the nuclear pore complex. Passive release of HMGB1 typically occurs in injured, apoptotic, necroptotic, or necrotic cells, where damage to the plasma and nuclear membranes occurs. Because HMGB1 lacks a hydrophobic secretion signal peptide, it is actively secreted by secretory lysosomes. Acetylation of critical residues located in both NLSs precludes HMGB1 from re-entering the nucleus; as a result, it builds up in the cytoplasm and is eventually secreted to the extracellular space.
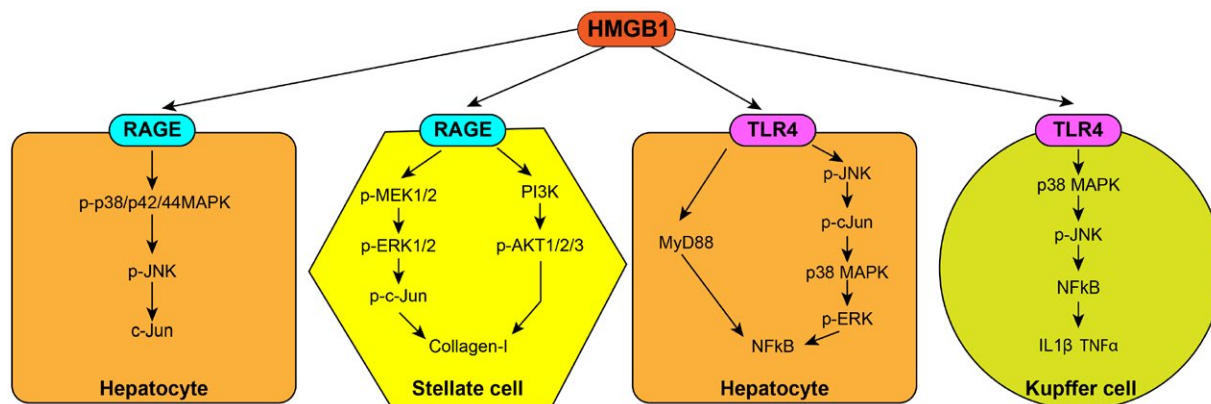
FIG. 3. HMGB1 receptors and signaling. HMGB1 signals through RAGE and TLR4 in numerous cell types. In the hepatocyte, HMGB1 signals through RAGE, activating p-p38/p42/44 MAPK, pJNK, and c-Jun. In HSCs, HMGB1 can signal to RAGE to activate first pMEK1/2, pERK1/2, and p-c-Jun and second PI3K and pAKT1/2/3 to increase collagen-I. HMGB1 activates TLR4 on hepatocytes to signal by MyD88-dependent pathways or through pJNK, p-c-Jun, p38 MAPK, pERK to activate NFκB. In Kupffer cells, HMGB1 signals through TLR4, resulting in activation of p38 MAPK, pJNK, and NFκB, leading to induction of IL-1β and TNFα. Abbreviations: MAPK, mitogen-activated protein kinase; pMEK, phospho-mitogen-activated protein kinase kinase.

Because HMGB1 lacks a conventional hydrophobic secretion signal peptide,[78] it is not secreted by conventional pathways but through passive and active mechanisms (Fig. 2). Passive release of HMGB1 typically occurs in injured, necroptotic, or necrotic cells where damage to the plasma and nuclear membranes occurs. In this setting, HMGB1 acts as an intracellular marker detected by the innate immune system that recognizes tissue damage and initiates reparative responses.[12,79] Both *in vitro* induction of apoptosis[80] and injection of mice with the Fas agonistic Jo2 antibody[81] induce HMGB1 release; yet, cells undergoing apoptosis induce negligible inflammation in the surrounding tissue[82] as HMGB1 remains bound to DNA in apoptotic cells.[12]

Active secretion of HMGB1 by secretory lysosomes occurs, for example, in immune cells[9,13,83,84] where it acts as a proinflammatory cytokine during an immunologic challenge. Stimuli for secretion of HMGB1 in these cells include pathogen-associated molecular patterns (PAMPs), cytokines, and oxidant stress, among others. Kupffer cells and monocyte-derived macrophages release HMGB1 in response to LPS translocated from the gut lumen into the portal circulation,[85] a key contributor to ALD and NASH, among others. Likewise, hepatocytes actively secrete HMGB1 in response to hypoxic conditions or oxidative stress induced by ischemia, pro-oxidants, or alcohol consumption, and they appear to be the major cellular source of

HMGB1 in the damaged liver.[41,86–88] Lastly, intestinal epithelial cells respond to local injury by secreting HMGB1[89] (Fig. 3).

Receptors and Signaling

HMGB1 binds multiple receptors, including RAGE, toll-like receptors (TLRs) 2/4/9, Mac-1, syndecan-1, phosphacan protein-tyrosine phosphatase-ζ/β, and clusters of differentiation 24.[11,90–93] Due to the dominant role of RAGE and TLR4 in liver disease, we focus on these two receptors.

RAGE

HMGB1 is a specific and saturable ligand that binds with high affinity to RAGE.[94] The expression of RAGE is mediated, at least in part, through an autoregulatory loop whereby the interaction of HMGB1 with RAGE leads to nuclear factor κB (NFκB) and Sp1-dependent transactivation of the advanced glycosylation end-product receptor (*AGER*) gene.[95–97] RAGE is expressed on hepatocytes, HSCs, sinusoidal endothelial cells, Kupffer cells, oval cells, and monocyte-derived macrophages.[44,49,59,60,98–100] RAGE activation leads to induction of NFκB-dependent proinflammatory genes[101] through a number of different pathways (Figure 3): mitogen-activated protein

kinase, cell division control protein 42/Rac, phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/pAkt, extracellular signal regulated kinase (ERK)/c-Jun, and c-Jun N-terminal kinases (JNK), all inevitably leading to an inflammatory and fibrogenic signaling cascade.[44,102]

RAGE mediates acute liver injury. In fact, during liver I/R, HMGB1 activates RAGE, leading to JNK and c-Jun activation with subsequent ergosterol-1 up-regulation.[103] In the context of APAP intoxication, HMGB1 acted through RAGE to trigger neutrophil recruitment during liver necrosis, which mediated subsequent liver damage.[23] RAGE-deficient neutrophils had 80% reduction in migration toward necrotic liver tissue. Furthermore, mice lacking *Rage* or *Hmgb1* had reduced neutrophil recruitment, overall proinflammatory gene expression, and liver injury after APAP exposure. RAGE expressed on bone marrow-derived cells was responsible for this effect as either global or bone marrow-specific *Rage* null mice produced the same effect on neutrophil infiltration and liver injury.[23] Of note, *Tlr4* null mice had no difference in neutrophil migration or liver injury.

In the setting of chronic liver disease, our laboratory has shown an association between HMGB1 and RAGE signaling in ALD and fibrosis. Mice lacking *Rage* in myeloid cells were protected from alcohol-induced steatosis, hepatocyte ballooning degeneration, inflammation, and liver injury (unpublished observations). Arriazu et al.[44] demonstrated that in liver fibrosis, HMGB1 signals via RAGE through the PI3K-pAkt1/2/3 pathway in HSCs. This was demonstrated by treating *Rage*-ablated HSCs with recombinant HMGB1 and observing reduced collagen-I expression along with blockade of PI3K and pAkt1/2/3 activation. Moreover, a neutralizing RAGE antibody prevented CCl_4-induced fibrosis *in vivo*. Further experiments from our laboratory have identified HMGB1 signaling by RAGE through phospho-mitogen-activated protein kinase kinase (pMEK) 1/2-pERK1/2-p-c-Jun in HSCs, which appears upstream of the PI3K-pAkt1/2/3 pathway in regulating collagen-I production.[49] Lastly, in the setting of HCC, HMGB1 signaling by RAGE evoked NFκB activation in HCC cells.[104] *Rage*$^{-/-}$ bone marrow-derived macrophages had 70% lower tumor necrosis factor-α (TNFα), interleukin (IL)-1β, and IL-6 production after stimulation with HMGB1 than cells expressing RAGE, indicating the importance of HMGB1 signaling by RAGE in these immune cells.

TLR4

TLRs recognize danger signals, such as PAMPs from bacteria or DAMPs, and subsequently activate the innate immune system. TLR4 is expressed on hepatocytes, Kupffer cells, HSCs, and sinusoidal endothelial cells.[105-107] HMGB1 activation of TLR4 mainly occurs in myeloid cells,[108-110] although the binding site in these and other cells has yet to be identified.

In Kupffer cells, HMGB1 signals through TLR4 to activate p38/pJNK and NFκB, which results in the production of the proinflammatory cytokines IL-1β and TNFα.[111] In experimental I/R models, HMGB1 signals via TLR4, phosphorylating JNK and activating NFκB.[24] This will be discussed in detail later; however, other receptors have also been found to be involved.[112,113] In mouse models of nonalcoholic fatty liver disease (NAFLD), HMGB1 appears to signal through TLR4 and NFκB because primary hepatocytes treated with free fatty acids show HMGB1 activation of the TLR4/myeloid differentiation primary response gene-88 (MyD88) pathway.[114] Conversely, together with HMGB1 signaling through TLR4, some studies show that TLR4 signaling is likely involved in increasing HMGB1 expression in liver injury[83,115-117]; nevertheless, additional studies are required to clarify this.

While, as described above, the literature points to a major role for HMGB1 signaling via RAGE, careful binding studies using surface plasmon resonance and/or nuclear magnetic resonance are needed to understand the impact of the HMGB1 PTMs on the binding. Should it be the case that one HMGB1 isoform binds RAGE faster than and/or dissociates slower to TLR4 or the reverse, this could represent a mechanism whereby these receptors regulate each other's signaling, a synergism or an alternative pathway that becomes activated (similar to an on/off system) when one receptor is saturated by its own ligand or an antagonist (e.g., LPS complex on TLR4). Future research should clarify this.

Posttranslational Modifications

HMGB1 can undergo multiple PTMs, including cysteine oxidation,[41,118-121] lysine N-acetylation,[41,122-130] serine phosphorylation,[41,125,131-134] lysine methylation,[135,136] serine adenosine diphosphate

ribosylation,[137] and asparagine, threonine, and lysine glycation.[138] Prediction analysis suggests that HMGB1 may also undergo ubiquitination in lysines 146 and 147; however, this has not been experimentally confirmed. Some of these modifications influence DNA binding and stability, regulation of transcription, intracellular protein localization, secretion, cell motility, and the proinflammatory or profibrogenic effects.[4] Of these, oxidation, acetylation, and phosphorylation appear to play a significant role in liver disease[41,44] (see modified residues in Fig. 1).

HMGB1 contains three conserved redox-sensitive cysteines (23, 45, and 106), and their oxidation determines the bioactivity of extracellular HMGB1. Cells exclude HMGB1 from the nucleus by acetylating lysines, thereby neutralizing their basic charge and rendering them unable to function as NLSs. Although HMGB1 contains 43 lysines, only 21 can undergo acetylation, but only those in two key clusters appear to play a major role in preventing HMGB1 nuclear re-entry due to acetylation, lysines 28-30 and 43-44 (representing a classic bipartite and functional NLS1[9,139]), and lysines 180 and 182-185 within NLS2.[9,139] Histone N-acetyltransferase can acetylate sites within HMGB1 that are structurally similar to those in histones, even though HMGB1 binds the enzyme with lower affinity.[140] While a significant body of literature exists on HMGB1 oxidation, phosphorylation, and acetylation, the majority derives from *in vitro* experiments using non-liver related cell lines; therefore, their relevance in acute and chronic liver disease needs to be demonstrated *in vivo*.

Acute Liver Injury

HMGB1 has been implicated in I/R injury, [15–27] sepsis,[23,28–32] and APAP-induced liver injury.[23,33–40]

I/R

In I/R injury, organ damage occurs due to the return of oxygen to a previously hypoxic tissue.[141] Warm I/R occurs during liver resection, injury, or trauma as a result of low blood flow. Cold I/R occurs when a liver graft is preserved in the University of Wisconsin solution prior to liver transplant and reperfusion.

Warm I/R

Tsung et al.[24] identified HMGB1 as an early mediator of inflammatory organ damage, where levels increased within 1 hour and up to 24 hours after I/R injury in mice. Inhibition of HMGB1 with a neutralizing antibody decreased liver damage through phosphorylation of JNK and increased NFκB binding. TLR4 was identified as being responsible for the effect of HMGB1 because global $Tlr4^{-/-}$ mice showed lessened I/R injury.[24] Other studies support that an HMGB1 antibody protects the liver from I/R injury.[25] HMGB1 was released from necrotic cells within the first 12 hours of injury and during the inflammatory phase in the subsequent 12 hours.[25,26] Further work revealed that HMGB1 was actively released from hypoxic hepatocytes through regulation by reactive oxygen species (ROS) that required TLR4 signaling; this was confirmed in $Tlr4$ null hepatocytes under hypoxia as they released less ROS and HMGB1.[88] Furthermore, global $Tlr4^{-/-}$ mice subjected to I/R had less liver injury and inflammatory cytokine production that could not be further reduced by the antioxidant N-acetylcysteine. This suggests that hepatocytes release ROS during I/R and HMGB1 is regulated by TLR4 signaling, which leads to liver injury and inflammation.[88] However, TLR4 is not the only receptor involved in HMGB1-mediated I/R injury as both TLR9 and RAGE inhibition protect from I/R injury in mice.[112,113]

Numerous studies show that blocking TLR4, either ablating it in hepatocytes or using an inhibitor, attenuated liver injury, inflammatory pathways, and HMGB1 serum levels in I/R injury mouse models.[20,142] The TLR4, JNK, and p38 signaling pathway was activated during hepatocyte hypoxia in which JNK inhibition decreased HMGB1 release. Taken together, this indicates that TLR4 mediated proinflammatory signaling and that HMGB1 release from hepatocytes plays a harmful role in I/R injury.[142] Ablation of $Hmgb1$ in hepatocytes reduced the number of infiltrating neutrophils and proinflammatory gene expression following I/R and ameliorated liver injury at later time points.[23]

However, HMGB1 plays a more complex role than first anticipated and could potentially be protective to hepatocytes rather than deleterious.[143] In certain studies, mice with $Hmgb1$ deleted from hepatocytes had significantly greater hepatocellular injury after I/R and greater DNA damage, contrasting its well-known role in promoting inflammation and tissue damage. Huang et al.[143] observed increased liver damage plus

serum TNFα and IL-6, and 1 hour after I/R, pJNK, p38, pERK, and NFκB were increased. This correlated with more recruitment of immune cells to the liver and enhanced chemokine expression, indicating a greater inflammatory response when *Hmgb1* is ablated from hepatocytes. Lack of HMGB1 increased poly adenosine diphosphate-ribose polymerase-1 activation, depleting nicotinamide adenine dinucleotide+ and adenosine triphosphate stores, and resulting in increased mitochondrial injury, oxidative stress in the hepatocytes, and subsequent cell death. Further evidence shows that pretreatment of mice with HMGB1 before I/R protects against injury. Cytokine levels were decreased and NFκB DNA binding reduced in the pretreated group.[144] Higher hepatic IL-1 receptor-associated kinase-M levels, a negative regulator of TLR signaling, reflected the reduced IL-1 receptor-associated kinase-1 levels observed. This protection by HMGB1 was blocked in *Tlr4* null mice; hence, HMGB1 plays a protective role during I/R through down-regulation of TLR4 signaling.[144]

Additional support for the role of HMGB1 in warm I/R comes from the plethora of compounds targeting HMGB1 signaling that have been found to successfully ameliorate injury.[32,145-150] Thus, the role of hepatic HMGB1 in warm I/R is still contradictory. To clarify whether it amplifies or ameliorates injury or has no involvement whatsoever, as one study claims,[74] more research is essential.

Cold I/R

Research into the role of cold I/R has been less extensive, but there is evidence to suggest a role for HMGB1. Serum HMGB1 increased in serum of liver transplant recipients after graft reperfusion for only 10 minutes, with continued release of HMGB1 as well as up-regulation in the hepatocytes.[27] This increase in HMGB1 secretion and expression correlated with alanine aminotransferase activity; nonetheless, HMGB1 levels decreased 1-2 hours after surgery. Remarkably, HMGB1 levels did not correlate with TNFα or IL-6 and neutrophils had low HMGB1 expression. It is still unknown if hepatocytes, macrophages, HSCs, neutrophils, or sinusoidal endothelial cells were the key source of HMGB1; the exact mechanism for the role of HMGB1 has yet to be determined. These results do, however, highlight HMGB1 as a potential biomarker of liver injury after transplant.

SEPSIS

HMGB1 has been identified as a mediator of systemic inflammation leading to multiorgan dysfunction in sepsis. In 2003, a role for HMGB1 in the pathogenesis of sepsis-induced multiple organ failure was first described.[31] In 2009, Nagano et al.[30] showed that recombinant soluble thrombomodulin decreased HMGB1, improving acute liver injury and survival rates in experimental endotoxemia. Later studies by Ye et al.[29] showed that human hematopoietic cells are required to induce sepsis-induced mortality following cecal ligation and puncture in severely immune-deficient, nonobese, diabetic, NOD scid gamma mice and that small interfering RNA treatment to inhibit HMGB1 release by human macrophages and dendritic cells dramatically reduced sepsis-induced mortality. Work from Wang et al.[28] demonstrated that glycyrrhizin is a potential agent for the treatment of sepsis as it reduces the serum level and gene expression of HMGB1 and other proinflammatory cytokines. However, more recent studies indicate that in LPS-induced shock, HMGB1 ablation did not ameliorate inflammation or lethality, despite efficient reduction of HMGB1 serum levels.[23] Hence, additional work is needed to further clarify the role of HMGB1 in sepsis.

DRUG-INDUCED LIVER INJURY

Drug-induced liver injury (DILI) is the most common cause of acute liver failure in the United States.[40] Although a plethora of drugs cause severe liver damage as a side effect, APAP is by far the prominent culprit and a significant cause of mortality. As HMGB1 is known to be released from necrotic cells[12] and activated immune cells,[9] it is a likely candidate involved in DILI.

APAP

Antoine et al.[151] found that HMGB1 is released into the serum of mice following an APAP overdose prior to alanine aminotransferase and correlated with the pathology scores of liver injury,[151] suggesting that HMGB1 could be a serum biomarker of DILI. Moreover, neutralization of HMGB1 was therapeutic by reducing the inflammatory reaction initiated by APAP,[118] providing further evidence of the protein's role in DILI. Later, this theory was solidified clinically as concentrations of total and acetylated

HMGB1 were found to increase 10-fold to 180-fold, respectively, in the serum from patients with liver injury after an APAP overdose[152]; this correlated with worse prognosis due to more severe liver injury, failure, or death.[152]

Huebener et al.[23] later showed that mice lacking *Hmgb1* in hepatocytes were protected from a lethal dose of APAP, with 100% of mice surviving compared to only 22% of wild-type (WT) mice. HMGB1 promoted neutrophil migration toward the necrotic tissue, which was blocked in mice lacking *Hmgb1* in hepatocytes. When neutrophil function was inactivated, these mice were protected from APAP-induced injury.[23]

Studies from Lundback et al.[153] revealed that a humanized HMGB1 antibody significantly attenuated APAP-induced liver injury and inflammation in mice, with higher efficacy than treatment with N-acetylcysteine, supporting the idea that HMGB1 could act as a therapeutic target to treat patients with DILI. Moreover, pretreatment or posttreatment with the HMGB1 inhibitor glycyrrhizin, also with antioxidant properties, markedly reduced hepatic inflammation, neutrophil recruitment, and injury caused by APAP in mouse models.[23,154] Despite the overwhelming evidence that HMGB1 is implicated in APAP-induced liver injury, the mechanism by which HMGB1 mediates injury, the receptors involved, and signaling pathways activated remain largely unknown.

Other Hepatotoxins

There is less in-depth research on the role of HMGB1 in DILI caused by other drugs. Mice dosed with flucloxacillin had elevated HMGB1 and other TLR4 ligands in serum, correlating with liver injury and inflammatory markers.[155] D-galactosamine increases HMGB1 release into plasma, correlating with a decrease in nuclear expression in rat liver[156]; and, an HMGB1 neutralizing antibody suppressed plasma HMGB1 and inflammatory cytokines and improved liver injury and survival.[156] Methimazole increased plasma HMGB1 3-6 hours after exposure[157]; this was ameliorated when Kupffer cell activation was blocked, suggesting that HMGB1 may be involved in mediating DILI by Kupffer cells. Rats treated with diclofenac had increased HMGB1 in plasma,[158] and sulfamethoxazole and flucloxacillin stimulated HMGB1 release from human hepatocytes along with markers of cell death and inflammation.[159] Moreover, disulfide HMGB1 stimulated cytokine release from dendritic cells, indicating that it may be involved in

stimulating an immune response to the drugs. There is, however, a lack of data from human patients on the role of HMGB1 in DILI caused by drugs other than APAP; hence, more clinical evidence is needed.

Chronic Liver Disease

ALD

Ge et al.[41] demonstrated that liver biopsies from patients with ALD showed a robust increase in HMGB1 expression and translocation that correlated with disease stage compared to healthy explants. This was also accompanied by enhanced HMGB1 levels in the serum compared to healthy individuals. Similar findings were observed in chronic ethanol-fed WT mice. Using primary cell culture, our laboratory validated the ability of hepatocytes from ethanol-fed mice to translocate HMGB1 from the nucleus to the cytoplasm and ultimately secrete a large concentration of HMGB1 that was far superior to that from Kupffer cells. Of note, HMGB1 secretion was time and dose dependent and responsive to pro-oxidants and blocked by antioxidants. We further demonstrated that selective ablation of *Hmgb1* in hepatocytes protected from alcohol-induced liver injury in mice due to the increase in key enzymes involved in fatty acid β-oxidation. Likewise, there was a more efficient low-density lipoprotein plus very low-density lipoprotein export from the liver into the circulation that largely prevented steatosis and ultimately liver injury.[41]

In subsequent studies, we detected native and posttranslationally modified HMGB1 in humans and in mice with ALD. Specifically, in liver and in serum from control mice and in serum from healthy volunteers, the lysine residues within NLS1 and NLS2 were nonacetylated and all cysteine residues were reduced. However, in livers from ethanol-fed mice, in addition to all thiol/nonacetylated isoforms of HMGB1, we observed acetylated NLS1 and NLS2, a unique phosphorylation site in serine 35, and an increase in oxidation of HMGB1 to the disulfide isoform. In serum from ethanol-fed mice and from patients with ALD, there was disulfide-bonded hyperacetylated-HMGB1, disulfide-bonded nonacetylated-HMGB1, and phosphorylated HMGB1 in serine 35. Hepatocytes appeared to be a major source of all these HMGB1 isoforms. Thus, hepatocyte HMGB1 participates in the pathogenesis of ALD and undergoes PTMs that could condition its toxic effects. Kupffer cells, however, produced mostly the all thiol/nonacetylated and acetylated

versions of the protein.[41] Current studies from our laboratory using genetic approaches are focusing on trying to understand the specific role of each of these PTMs and the contribution of HMGB1 acetylation in Kupffer cells to the pathogenesis of ALD.

FIBROSIS

Liver fibrosis is a frequent life-threatening complication of most chronic liver diseases. Our laboratory observed that liver HMGB1 protein expression correlated with fibrosis stage in patients with chronic hepatitis C virus infection and primary biliary cirrhosis.[49] Serum levels of HMGB1 were also increased in these patients compared to healthy controls, suggesting protein secretion. These findings were equally replicated in four mouse models of liver fibrosis due to DILI caused by chronic CCl_4 injections or thioacetamide administration, cholestasis triggered by common bile duct ligation, and NASH induced by feeding a methionine- and choline-deficient diet. Overall, these data suggested that HMGB1 has a key role in liver fibrosis.[49]

Studies initiated by Arriazu et al.[44] demonstrated that, while HSC secretion of HMGB1 is minimal, in response to a noxious stimuli, HSCs acetylated HMGB1 *in vitro,* which contributed to collagen-I deposition. In a follow-up study, Ge et al.[49] showed that HMGB1 also stimulated HSC migration *in vitro* and *in vivo*, a critical event driving the progression of liver fibrosis. Importantly, the authors demonstrated that ablation of *Hmgb1* in HSCs *in vivo* reduced fibrosis about 25%. This finding unlocked the possibility that additional cellular sources of HMGB1 may have a more meaningful impact in liver fibrosis.

The role of HMGB1 was later proven as neutralization of HMGB1 protected liver fibrosis whereas injection of HMGB1 promoted liver fibrosis. The authors observed that HMGB1 was up-regulated and secreted mostly by hepatocytes and also Kupffer cells along with infiltrating macrophages following chronic CCl_4 treatment. Moreover, *Hmgb1* ablation in hepatocytes or myeloid cells was also partially protective; more notably, ablation in both reduced fibrosis about 75% compared to WT mice, thus indicating a stronger contribution of HMGB1 from these two cell types to liver fibrosis.[49]

In line with the *in vivo* data, coculture with hepatocytes or Kupffer cells from CCl_4-injected WT mice up-regulated collagen-I production by HSCs; however, coculture with hepatocytes from CCl_4-injected conditional *Hmgb1* knockout mice or with Kupffer cells from CCl_4-injected conditional *Hmgb1* knockout mice partially blunted this effect. Overall, the data suggested that signaling from hepatocytes and Kupffer cells by HMGB1 was driving collagen-I production by HSCs.[49]

Our laboratory also investigated whether induction of HMGB1 could participate in the pathogenesis of liver fibrosis by RAGE cell-specific signaling mechanisms.[44,49] Notably, while *Rage* ablation in hepatocytes and myeloid cells did not cause significant protection, *Rage* ablation in HSCs and RAGE neutralization almost fully prevented liver fibrosis. Mechanistically, we then demonstrated that HMGB1 signaled through RAGE to up-regulate collagen-I expression by activating the pMEK 1/2/pERK1/2/p-c-Jun signaling pathway upstream of PI3K/pAkt, thereby contributing to scarring.[49]

While two studies[59,60] did not find a role for HMGB1 in liver fibrosis, work from Kao et al.[160] found that HMGB1 up-regulated α-smooth muscle actin expression and suppressed the activity of the collagen-degrading matrix metalloproteinases 2, leading to extracellular matrix accumulation. Likewise, it was shown that an Hmgb1 small interfering RNA inhibited the synthesis of α-smooth muscle actin and collagen in rat fibrosis.[161] Although the role of HMGB1 in fibrosis progression appears clear, the contribution of the various HMGB1 isoforms as well as their potential role in fibrosis regression and the receptor involved during the resolution phase remain open questions and may clarify some aspects of the role of HMGB1 in liver fibrosis.

NASH

NAFLD is the most common cause of chronic liver disease in developed countries and particularly in the United States due to the major consumption of junk food.[162] In 2018, work from Chen et al.[51] proved that HMGB1 expression was detected in high-fat diet-induced NAFLD in mice and that liver injury was mitigated by an HMGB1 neutralizing antibody. In this study, HMGB1 expression was regulated by both the JNK1/2-activating transcription factor 2 axis and the microRNA-200 family. Li et al.[114] later established that HMGB1 release from hepatocytes in response to free fatty acid infusion was vital for TLR4/MyD88 activation and cytokine expression both *in vitro* and *in vivo*. They then treated mice with a neutralizing

antibody to HMGB1 that protected against free fatty acid-induced TNFα and IL-6 production. They concluded that TLR4/MyD88 signaling in liver parenchymal cells played a pivotal role during the early progression of NAFLD in which free HMGB1 served as a ligand for TLR4 activation.[114]

Chandrashekaran et al.[163] identified HMGB1 as a key mediator of intestinal inflammation in NAFLD that is RAGE and redox signaling dependent and promotes ectopic intestinal inflammation. These studies point to HMGB1 as an important mediator of liver injury in the early stages of NAFLD. Nonetheless, the key receptor participating in the later stages of the disease as well as during the resolution phase and the HMGB1 isoforms involved remain elusive. Likewise, the contribution of HMGB1 from intestinal origin to the overall pathogenesis of NASH is unknown.

HCC

HMGB1 induces chronic inflammation and extracellular matrix accumulation, creating a prime microenvironment for the development of HHC. In 2008, serum HMGB1 levels were found to be strongly increased in patients with HCC and correlated with clinicopathologic features, such as with α-fetoprotein levels and tumor size,[164] and Masuda et al.[56] established that serum HMGB1 predicted worse clinical prognosis in patients with HCC.

Likewise, Dong et al.[165] found that HMGB1 was highly expressed in human liver cancer compared with normal tissue and was positively associated with pathologic grade and distant metastases of liver cancer. They further demonstrated that knockdown of *Hmgb1* down-regulated the expression of pAKT, Ki67, and matrix metalloproteinases 2; inhibited the proliferation and metastatic potential of SMMC-7721 cells; induced cell cycle arrest and apoptosis; and slowed the growth of xenograft tumors in mice. Thus, this study suggested that HMGB1 may be involved in liver cancer development and progression, representing a potential therapeutic target for this aggressive malignancy.

Later, Chen et al.[53] showed that HMGB1 plays a role in the regulation of the Hippo pathway during liver tumorigenesis as binding of HMGB1 to GA-binding protein α promoted the expression of Yes-associated protein (the downstream effector of the Hippo pathway) to induce liver tumors in mice. Additionally, HMGB1 depletion in hepatocytes blocked diethylnitrosamine-induced liver cancer initiation and short

hairpin RNA-mediated gene silencing of *Hmgb1* inhibited HCC cell proliferation.

Two of our studies showed that *Hmgb1* ablation prevents ductular reaction, a major mechanism contributing to portal fibrosis.[44,49] Recently, Kambu et al.[60] reported that HMGB1 release is a critical mechanism in hepatic pathogenesis in autophagy-deficient conditions and leads to ductular reaction, hepatic progenitor cell expansion, and tumor development. A similar conclusion was put forward by Hernandez et al.,[59] pointing to HMGB1 as linking hepatocyte death to ductular reaction, progenitor signature, and hepatocarcinogenesis in chronic liver disease.

Regarding the receptors involved, Yaser et al.[166] showed that RAGE is overexpressed in primary HCC compared to adjacent nontumor liver samples and demonstrated that it regulates cellular proliferation in HCC due to HMGB1 binding. The Tsung laboratory showed that in hypoxic HCC cells, HMGB1 activates TLR4- and RAGE-signaling pathways to induce caspase-1 activation with the ensuing production of multiple inflammatory mediators, which in turn promote cancer invasion and metastasis[167]; yet, the authors did not discuss the potential effect of caspase-1 cleavage of HMGB1. In a follow-up publication, they established that tumors lacking HMGB1 had a significant reduction in mitochondrial biogenesis and increased mitochondrial dysfunction. In these studies, they showed that in hypoxic conditions HMGB1 that translocated from the nucleus to the cytoplasm interacted with TLR9, activated p38, and phosphorylated peroxisome proliferator-activated receptor gamma coactivator 1α with subsequent up-regulation of mitochondrial biogenesis.[57]

An area that remains to be investigated is the likely possibility that specific HMGB1 isoforms may have a dominant role in the onset and progression of HCC. Likewise, the signaling cascades that they could activate and whether they drive the phenotype of cancer stem cells remain elusive.

Therapeutic Implications

HMGB1 orchestrates the regulation of both inflammation and wound healing.[44,64,116,168,169] There is significant evidence that HMGB1 plays a role in the initiation, exacerbation, or prolongation of acute liver injury and chronic liver disease, although the receptors involved may vary among the clinical conditions and their activation is likely regulated by the presence of various HMGB1 isoforms or even HMGB1

protein complexes, some of which may have significant immune effects. HMGB1 has promise as a target for treatment as well as potential to be used as a biomarker. Nevertheless, additional research into the molecular mechanisms underlying its effects, the overall contribution of the PTMs, and if one isoform dominates or regulates the others is essential in order to elucidate the importance of HMGB1 in liver disease and to design drugs or develop antibodies that specifically target each HMGB1 isoform. It is likely that the isoforms play different roles in liver disease, perhaps by binding with different affinity to key receptors.

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