High mobility group box 1 activates toll like receptor 4 signaling in hepatic stellate cells

Zhe Zhang, Chenzhao Lin, Lijun Peng, Yangyang Ouyang, Yirong Cao, Jiyao Wang, Scott L. Friedman, Jinsheng Guo

Aims: The aim of the present study was to investigate the effect of high mobility group box 1 (HMGB1), a damage pattern molecule that signals the presence of necrosis, on TLR4 signaling in hepatic stellate cells (HSC).

Main methods: Immortalized mouse HSC lines JS1, JS2, and JS3 that were either TLR4+/+, TLR4−/−, or MyD88−/− were transfected with NF-κB or AP-1 responsive luciferase reporter plasmids, followed by stimulation with 100 ng/ml lipopolysaccharide (the exogenous TLR4 ligand) or 100 ng/ml HMGB1. The activation of NF-κB or AP-1 activities was determined by a dual-luciferase reporter assay system. The cells were also stimulated with LPS or HMGB1 and collected for the determination of chemotactic cytokine MCP-1 mRNA or protein secretion. In a separate experiment, the cells were co-stimulated with 10 μg/ml TGF-β1 and LPS or HMGB1 and collected for assessment of fibrogenic mRNA and protein expression.

Key findings: HMGB1 stimulation markedly up-regulated MCP-1 mRNA expression and protein secretion, and enhanced TGF-β1-stimulated collagen α2(I) and α-SMA expression in JS1 cells. This was associated with enhanced activation of NF-κB and AP-1 responsive luciferase reporters. On the contrary, JS2 and JS3 cells were hyporesponsive to both LPS and HMGB1 stimulation compared to JS1 cells.

Significance: As an endogenous ligand of TLR4, HMGB1 activates TLR4 signaling in HSCs to enhance their inflammatory phenotype, indicating that TLR4 signaling need not rely solely on gut-derived LPS for activation during liver injury. HMGB1 also has a synergistic effect with TGF-β1 to stimulate fibrogenic protein expression, which is likely to be TLR4 dependent.

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of HMGB1 is increased in the serum of chronic hepatitis patients (Albayrak et al., 2010), and experimental model of liver fibrosis (Han et al., 2008). The increased level of HMGB1 is closely associated with the severity of inflammation and fibrosis, indicating a role in the initiation and development in liver fibrosis.

TLR4 signaling has been identified in HSC, the major fibrogenic cell type in injured liver, which mediates the cell’s inflammatory phenotype, and fibrogenic and anti-apoptotic properties (Seki et al., 2007; Guo et al., 2009). In response to LPS, HSC signal through adaptor protein myeloid differentiation factor 88 (MYD88), activating of transcription factors nuclear factor-κB (NF-κB) and activator protein-1 (AP-1), to produce inflammatory and chemotactic cytokines including monocyte chemotactic protein-1 (MCP-1) (Guo et al., 2009; Paik et al., 2003). However, endotoxemia and increased production of LPS in the intestine are only significant in fulminant liver failure and in the decompensated stage of liver cirrhosis, but not in the earlier stage of chronic viral hepatitis (Yajima et al., 1985). In contrast, endogenous ligands such as HMGB1 are released in significant amounts during liver injury, inflammation and fibrogenesis and therefore could account for TLR4 signaling in HSCs well before altered gut permeability leads to increased LPS presentation to the liver.

HMGB1 reportedly activates TLR4 in macrophages, monocytes and neutrophils (Park et al., 2004; Yu et al., 2006; Park et al., 2006). The aim of the present study was to investigate the impact of HMGB1, a damage pattern molecule that signals the presence of necrosis, on TLR4 signaling in HSC and its effect on the cell biology using immortalized wild type (JS1), TLR4<sup>−/−</sup> (JS2), and MyD88<sup>−/−</sup> (JS3) mouse HSC lines that have been generated in our previous study (Guo et al., 2009).

Material and methods

Assessment of HMGB1 responsiveness of TLR4 signaling in HSC

Immortalized wild type (JS1), TLR4<sup>−/−</sup> (JS2), and MyD88<sup>−/−</sup> (JS3) mouse HSC lines have been generated in our previous study (Guo et al., 2009). They were sub-cultured in 24, 12 or 6 well plates and divided into the negative control, LPS (positive control) and HMGB1 treated groups. The cells were treated with normal saline solution, 100 ng/ml LPS (Sigma, St. Louis, MO, USA), or 100 ng/ml HMGB1 (Sigma), respectively, and collected at 12 h after treatment for RNA analysis. Alternatively, the culture supernatant of each group of cells was collected at 24 h after treatment for the determination of MCP-1 by enzyme-linked immunosorbent assay (ELISA). For the assessment of transcription factor NF-κB or AP-1 responses, the cells were transfected with NF-κB or AP-1 responsive firefly luciferase reporter plasmids along with a Renilla luciferase expression control construct (Promega, Madison, WI, USA) in a ratio of 1:0.005 for 12 h, mediated by lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA). The transfected cells were recovered in fresh medium for 12 h, followed by treatment with HMGB1, LPS or vehicle saline. The cells were collected at 12 h thereafter and the activation of NF-κB or AP-1 activities was detected by a dual-luciferase reporter assay system as described below.

Effect of HMGB1 on TGF-β1 stimulated fibrogenic activity in HSC

In order to test the effect of HMGB1 stimulation on the fibrogenic activity of HSC, JS1, JS2 and JS3 cells were sub-cultured in 12 or 6 well plates and divided into the negative control, LPS, HMGB1, TGF-β1, TGF-β1 combined with HMGB1 or TGF-β1 combined with LPS treated groups. The cells were serum starved for 12 h and then treated with normal saline vehicle, 100 ng/ml LPS, 100 ng/ml HMGB1, TGF-β1 (10 ng/ml) or co-treated with TGF-β1 and HMGB1, or TGF-β1 and LPS, respectively. The cells were collected at 12 h of treatment for RNA analysis, or 24 h for protein assay by Western blot.

**Table 1**

<table>
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<th>Primers for real time quantitative PCR.</th>
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<tr>
<td>MCP-1</td>
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<td>α-SMA</td>
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<td>α2(I) collagen</td>
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<td>GAPDH</td>
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NF-κB luciferase reporter assay

Cell lysates were prepared using passive lysis buffer provided by the dual-luciferase reporter assay kits (Promega). Changes in firefly luciferase activity were normalized with Renilla luciferase activity and fold-changes of NF-κB or AP-1 activities with or without HMGB1 or LPS stimuli of each HSC line were compared.

Reverse-transcription and real-time quantitative PCR (qPCR)

The induction of monocyte chemotactic protein-1 (MCP-1) mRNA in these three cell lines after HMGB1 or LPS stimuli, and α-SMA, α2(I) collagen mRNA after co-stimulation of TGF-β1 and HMGB1 or LPS was determined by real time quantitative PCR (RT-qPCR). mRNA was extracted from the cells and reversely transcribed into cDNA using RNeasy® kit (Qiagen, Valencia, CA) and Omniscript RT Kit (Qiagen), respectively, and analyzed by qPCR using SYBR green qPCR Master Mix (Roche, Indianapolis, IN, USA) on the ABI 7500 system (Applied Biosystems, Foster City, CA, USA). Data are represented as the fold induction of MCP-1 by LPS or HMGB1 relative to cells not exposed to LPS or HMGB1, or fold induction of α-SMA, α2(I) collagen by TGF-β1, with or without cotreatment with HMGB1 and LPS. The primers used are listed in Table 1.

Cytokine assay

The protein level of MCP-1 in the culture supernatants was determined by ELISA. The culture supernatants were collected from cells in 12-well plates used for mRNA analysis at 12 h post-LPS stimulation. MCP-1 secretion was measured using ELISA kits (Biosource, Invitrogen) according to the manufacturer’s instructions.

Western blot

Western blots of cell extracts were prepared by pelleting the cells with lysis buffer (Roche Applied Sciences) complemented with protease inhibitor cocktail (Roche Applied Sciences) and protein phosphatase inhibitor cocktails (Upstate, Temecula, CA). Protein concentration was determined with the Bradford method using Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA). Equal amounts of protein were separated by SDS-PAGE, transferred to Hybond® membranes, immunoblotted with specific primary antibodies for α2(I) collagen (Rockland Immunochemicals, Inc., Gilbertsville, PA), α-SMA (Promega), and GAPDH (Santa Cruz, Santa Cruz, USA), and visualized using secondary horseradish peroxidase (HRP)-conjugated antibodies (GE Healthcare, Waukesha, WI, USA) and the Western chemiluminescent detection system (Millipore, Billerica MA, USA).

Results

**HMGB1 activates NF-κB and AP-1 activities in JS1 but not JS2 and JS3 HSC**

The effect of HMGB1 on the activation of TLR4 downstream transcription factors NF-κB and AP-1 was assessed by a dual-luciferase activity.
reporter assay system. As shown in Fig. 1, HMGB1 significantly activated NF-κB (Fig. 1A) and AP-1 (Fig. 1B) responsive reporter activities in JS1 cells. These effects were less potent than the endogenous TLR4 ligand, LPS. Unlike TLR WT cells, TLR4−/− and MyD88−/− HSCs were hyporesponsive to both LPS and HGMB1 stimulation compared to WT TLR4 HSC.

HMGB1 stimulates MCP-1 expression and secretion in JS1 but not JS2 and JS3 HSC

The inflammatory, chemotactic cytokine MCP-1 was selected as a representative target of TLR4 downstream targets in HSC to assay the effect of HMGB1 on signaling activation. HMGB1 stimulation markedly up-regulated MCP-1 mRNA expression in the cells (Fig. 2A) and secretion of MCP-1 protein in the cell culture supernatants (Fig. 2B). JS2 or JS3 cells which lack TLR4 or MyD88 respectively, were hyporesponsive in the expression and secretion of MCP-1 after HMGB1 or LPS treatment, which was paralleled by reduced activities of NF-κB and AP-1 promoters.

HMGB1 enhances TGF-β1 stimulated fibrogenic gene expression in JS1 HSC

We next examined the effect of HMGB1 stimulation on TGF-β1-induced fibrogenic gene expression in HSC. As shown in Fig. 3 for the mRNA expression of α-SMA (Fig. 3A) and α2(I) collagen (Fig. 3B), and Fig. 4 for these proteins detection by Western blot, TGF-β1 induced a significant up-regulation of α2(I) collagen and α-SMA expression in all three cell lines. Co-stimulation with HMGB1 and TGF-β1 of JS1 cells further enhanced TGF-β1 induced α2(I) collagen and α-SMA expression. This effect was not observed in JS2 or JS3 cells.

Discussion

Toll-like receptor 4 is the first and most important Toll homologue identified, and responds primarily to its ligand, LPS. However, recent studies in monocytes or macrophages indicate that TLR4 signaling can also be activated by endogenous ligands from cellular compartments released and/or increased during tissue injury and matrix degradation. These ligands are collectively referred as ‘damage-associated molecular patterns (DAMPs)’ molecules (review in (Guo and Friedman, 2010)). Previous studies have uncovered an important role of TLR4 signaling in liver fibrogenesis (Seki et al., 2007), and the association of TLR4 polymorphisms with fibrosis risk (Guo et al., 2009). TLR4 signaling is present in activated hepatic stellate cells (HSC), the major fibrogenic cell type in injured liver, and mediates the cell’s inflammatory phenotype and survival. The present study provides direct evidence that HMGB1, a key DAMP molecule, activates TLR4 signaling in HSC, as shown by enhanced activities of downstream transcriptional factors NF-κB and AP-1, and the expression and secretion of the target gene MCP-1 in the wild type mouse HSC line, JS1. These effects were absent in JS2, a TLR4 knockout HSC line, and JS3, a MyD88 knockout line, indicating that the HMGB1 effects are TLR4-MyD88 dependent.

HMGB1 is a highly conserved nuclear non-histone DNA-binding protein that functions as a structural co-factor critical for proper transcriptional regulation in somatic cells. It induces bends in the helical DNA structure to facilitate multiple physical interactions of DNA with transcription factors, recombinases and steroid hormone
HMGB1 contributes to ischemia reperfusion (I/R) injury and other pathological processes, such as liver fibrosis (Albayrak et al., 2010), implicating a role in the initiation and development of liver fibrosis. Key studies have demonstrated that TLRs, especially TLR4 signaling, participate in fibrosis pathogenesis in liver diseases of various etiologies (Seki et al., 2007; Guo and Friedman, 2007, 2010), including drug-induced liver injury (Yohe et al., 2006), nonalcoholic steatohepatitis (Shi et al., 2006; Spruss et al., 2009; Thuy et al., 2008) and alcoholic liver disease (Uesugi et al., 2001; Mandrekar and Szabo, 2009). As an endogenous ligand of TLR4, HMGB1 may be released during liver injury and signal through TLR4 to participate in the development of liver fibrosis in these diseases.

It has been reported that HMGB1 may act as a chemotactic factor in fibrocytes, endothelial cells, and smooth muscle cells (Raucci et al., 2007; Mitola et al., 2006), which take part in injury and fibrogenic responses. HMGB1 may stimulate fibrocyte proliferation and contribute to the development of lung fibrosis (Hamada et al., 2008). Direct effects of HMGB1 on fibrogenic activity of HSC has been observed (Kao et al., 2008), indicating that HMGB1 stimulates HSC proliferation and the expression of α-SMA in vitro. In the present study we document the stimulation by HMGB1 on TGF-β1-induced α(SMA) collagen and α-SMA mRNA expression, which were attenuated in TLR4−/− and MyD88−/− HSCs, implicating TLR4 signaling in this response. This effect is presumably due to a synergistic interaction of TLR4 downstream transcription factors NF-κB and AP-1 with Smads, especially Smad2 and 3, the downstream transcriptional factors of TGF-β1 signaling. This finding is important because TLR4 activation via LPS may only occur in advanced stages of liver injury even at early stages of the disease. Inhibition of HMGB1 and TLR4 signaling in stellate cells means that TLR4-mediated stimulation of fibrogenic activity of HSC has been observed (Kao et al., 2008)

receptors, and thus allows transcription and other nuclear events to take place (Ellerman et al., 2007). In addition to its transcription factor-like function, HMGB1 also has cytokine-like effects in promoting tumor metastasis and inflammation, which require its presence in the extracellular space. The release of HMGB1 into the extracellular space is mediated by acetylation of many of lysine residues of HMGB1 that lie in proximity to its two nuclear-localization signals, thus reducing interactions with the nuclear import protein complex, and preventing nuclear re-entry while promoting secretion of HMGB1.

In addition to this active HMGB1 secretion pathway in inflammatory cells (Semino et al., 2005; Bonaldi et al., 2003; Wang et al., 1999; Dumitriu et al., 2005; Abraham et al., 2000; Lotze and Tracey, 2005), HMGB1 passively diffuses from cells undergoing necrosis (Scalfi et al., 2002). The released HMGB1 may signal through its receptors to produce biological effects, mediating early (Tsung et al., 2005) and late (Wang et al., 1999) non-specific inflammatory reactions, and serving as an endogenous ‘danger signal’ to activate antigen processing cells in initiating and enhancing specific immune responses. HMGB1 contributes to ischemia reperfusion (I/R) injury and other pathological process of major organs. In particular, serum levels of HMGB1 are significantly increased in patients with chronic hepatitis and correlate well with the extent of inflammation and fibrogenesis (Albayrak et al., 2010), implicating a role in the initiation and development of liver fibrosis. Key studies have demonstrated that TLRs, especially TLR4 signaling, participate in fibrosis pathogenesis in liver diseases of various etiologies (Seki et al., 2007; Guo and Friedman, 2007, 2010), including drug-induced liver injury (Yohe et al., 2006), nonalcoholic steatohepatitis (Shi et al., 2006; Spruss et al., 2009; Thuy et al., 2008) and alcoholic liver disease (Uesugi et al., 2001; Mandrekar and Szabo, 2009). As an endogenous ligand of TLR4, HMGB1 may be released during liver injury and signal through TLR4 to participate in the development of liver fibrosis in these diseases.

Conclusion

By using immortalized HSCs lines in vitro the present study provides further evidence that TLR4 signaling is a key inflammatory and fibrogenic signal in HSC and hepatic fibrogenesis. Importantly, TLR4 responds in this cell type to HMGB1, a signature damage-associated molecular pattern that signals the presence of necrosis, and that subsequently triggers inflammation via downstream signaling. This finding is important because TLR4 activation via LPS may only occur in advanced stages of liver disease when gut permeability is compromised. The identification of a physiologically relevant endogenous ligand of TLR4 signaling in stellate cells means that TLR4-mediated stimulation of fibrogenesis and inflammation is likely to be contributing to the cascade of injury even at early stages of the disease. Inhibition of HMGB1 and TLR4 signaling activity may therefore be important targets for anti-fibrotic therapy, which warrants further investigation in primary isolated HSCs in vitro and in vivo in future studies.

Abbreviations

HSC hepatic stellate cells  
ECM extracellular matrix  
MFB myofibroblasts  
TLR4 Toll-like receptor 4  
LPS lipopolysaccharide  
HMGB1 high mobility group box 1  
DAMP damage associated molecular pattern molecule  
MyD88 myeloid differentiation factor 88  
NF-κB nuclear factor-κB  
AP-1 activator protein-1  
MCP-1 monocyte chemotactic protein-1  
TGF-β1 transforming growth factor β1  
α-SMA α smooth muscle actin  
RT-qPCR real time quantitative PCR
Fig. 4. Effect of HMGB1 on TGF-β1 stimulated α2(I) collagen and α-SMA protein expression in HSC. Mouse HSC lines were serum starved for 12 h. They were then treated with HMGB1 (100 ng/ml), LPS (100 ng/ml) or vehicle saline, with or without co-treatment with TGF-β1 (10 ng/ml). The cells were collected at 24 h later for the determination of α2(I) collagen and α-SMA protein levels in the cells by Western blot. (A) Representative Western blot profile of α2(I) collagen and α-SMA protein expression in the HSCs. (B) Semi-quantitative analysis of α2(I) collagen and α-SMA protein signals using densitometric scanning. Each α2(I) collagen and α-SMA protein signal was standardized against the corresponding GAPDH signal and the results are expressed as ratio of α2(I) collagen or α-SMA to GAPDH. Data are expressed as mean ± S.E.M of three independent experiments. *P<0.05 when compared with the NC group of the same HSC line. #P<0.05 when compared with the TGF-β1 treated group of the same HSC line. HMGB1 enhanced TGF-β1 stimulated protein synthesis of α2(I) collagen and α-SMA in JS1 cells (lane HMGB1 + TGF-β1 vs TGF-β1). This effect was not observed in JS2 or JS3 cells. JS1: wild type mouse HSC; JS2: TLR4−/− mouse HSC; JS3: MyD88−/− mouse HSC; NC: negative control group; HMGB1: HMGB1 treated group; TGF-β1: TGF-β1 treated group; HMGB1 + TGF-β1: HMGB1 and TGF-β1 co-treated group; LPS + TGF-β1: LPS and TGF-β1 co-treated group; LPS: LPS treated group.

Conflict of interest statement

The authors have declared no conflicting interests.

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References
