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The kinetics of damage-associated molecular patterns (DAMPs) and toll-like receptors during thioacetamide-induced acute liver injury in rats

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ABSTRACT

Drug-induced liver injury (DILI) is a common problem in human medicine and it is a major reason to withdraw marketed drugs. However, the mechanism of DILI is still less known. Damage-associated molecular patterns (DAMPs), such as high-mobility group boxes (HMGBs), S100 proteins and heat shock proteins (HSPs), are released from injured or necrotic cells, bind to toll-like receptors (TLRs) and modulate inflammatory reactions. Here we investigated the kinetics of DAMPs, TLRs and MHC class II in a rat model of DILI with thioacetamide (TAA). After TAA administration, extensive necrosis was observed on days 1 and 2, followed by infiltration of inflammatory cells on day 3. The levels of serum liver enzymes also peaked on day 1. Expression of HMGB-1, -2 and S100A4 peaked on day 2. TLR-4 was up-regulated on day 3. The number of MHC class II-positive macrophages increased until day 2. These results suggest that HMGB-1, -2 and S100A4 are associated with hepatocellular necrosis and that DAMPs may activate TLR-4 and MHC class II during TAA-induced liver injury. Our data would contribute to the elucidation of the mechanism of DILI.

1. Introduction

Drug-induced liver injury (DILI) is caused by various drugs and is the major reason of drug withdrawal from the market (Chen et al., 2015). The incidence of DILI is estimated on one in 10,000 to one in 100,000 (Au et al., 2011). Clinically, patients have symptoms of hepatitis, cholangitis or jaundice, and some patients need liver transplantation due to liver failure (Navarro and Senior, 2006). The etiology of DILI is multifactorial (e.g. reactive metabolites, reactive oxygen species and mitochondrial dysfunction) (Shi and Yang 2015; Xuan et al., 2015) and is still largely unknown.

Damage-associated molecular patterns (DAMPs) are endogenous danger signal molecules that are released from injured or necrotic cells and activate the innate immune system through Toll-like receptors (TLRs), nucleotide-binding domain, leucine-rich repeat-containing proteins or the receptor for advanced glycation endproducts (Maher 2009; Tang et al., 2012). In the liver, the relationships between DAMPs and TLRs have been studied in murine models of ischemia/reperfusion (Yu et al., 2010) and acetaminophen (APAP)-induced liver injury (Jaeschke et al., 2012). In these models, DAMPs, such as high mobility group box (HMGB) -1 (Jaeschke et al., 2012; Tsung et al., 2007), S100 proteins (Bianchi, 2007; Chen et al., 2015) and heat shock proteins (HSPs) (Jaeschke et al., 2012), play a role in enhancing liver inflammation and are involved in liver fibrosis. For example, HMGB-1 is related with liver fibrosis through activatied TLR-4, and S100A4 also contributes to liver fibrosis through activating hepatic stellate cells (Chen et al., 2014; Seki et al., 2007). Recently, major histocompatibility complex (MHC) class II molecules have been reported to recognize endogenous antigens (Leung, 2015) and mobilize lymphocytes (Shichita et al., 2012).

Hepatocyte injury by drugs can lead to extracellular release of DAMPs, which activate TLRs, induce the production of inflammatory cytokines and progression of inflammation; however, the relationship between DAMPs, TLRs and MHC class II during DILI is still unclear. In this study, we investigated the pathological roles of DAMPs, TLRs and MHC class II in thioacetamide (TAA)-induced acute liver injury in rats.

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2. Materials and methods

2.1. Animals

Six-week-old, male F344 rats (117–134 g body weight) were purchased from Charles River Japan (Yokohama, Japan). The test group was injected intraperitoneally with thioacetamide (TAA) dissolved in saline (300 mg/kg body weight; Wako Pure Chemicals, Osaka, Japan). The control group was administered an equal volume of saline. These animals were housed in an animal room at a controlled temperature and with a 12-h light-dark cycle; they were provided a standard diet (DC-8; CLEA, Tokyo, Japan) and tap water *ad libitum*. Rats were euthanized by deep isoflurane anesthesia, and blood (from the abdominal artery) and liver were collected at hour 10 and on days 1, 2, 3, 5 and 7 after injection ($n = 4$ in each group). Aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were measured by SRL Inc. (Tokyo, Japan). The animal experiments were conducted under the institutional guidelines approved by the ethical committee of Osaka Prefecture University for animal care.

2.2. Histopathology and immunohistochemistry

Tissues from the left lateral lobe of the liver were fixed in 10% neutral buffered formalin or periodate-lysine-paraformaldehyde (PLP) solutions. These tissues were dehydrated and embedded in paraffin. Deparaffinized sections, cut at 4 μm in thickness, were stained with hematoxylin and eosin (HE) for histopathologic examination. PLP-fixed sections were used for immunohistochemistry with mouse monoclonal antibodies against MHC class II (clone OX-6; 1:500; Serotec, Kidlington, UK). After pretreatment by microwave for 20 min in 0.01 M citrate buffer (pH 6.0), sections were incubated with each primary antibody for 1 h at room temperature, followed by 1 h incubation with peroxidase-conjugated secondary antibody (Histofine Simple Stain MAX- PO; Nichirei, Tokyo, Japan). Positive reactions were detected with 3, 3′-diaminobenzidine (DAB Substrate Kit; Nichirei). Sections were counterstained lightly with hematoxylin.

2.3. Cell count

The number of MHC class II-positive cells around the central vein of the hepatic lobule is counted in three different areas of 4 different rats using WinRoof software (Mitani Corp., Fukui, Japan) and are expressed as the number of positive cells per unit area (cells/mm^2).

2.4. Real-time PCR

Liver samples from the right medial lobe were immersed in RNAlater reagent (Qiagen GmbH, Germany) overnight at 4 °C and stored at −80 °C. Total RNA was extracted by SV total RNA isolation system (Promega Corporation, Wisconsin, USA). Two μg of total RNA was reverse-transcribed with Superscript VILO reverse transcriptase (Life Technologies, CA, USA). Real-time PCR was performed with TaqMan gene expression assays (Life Technologies) in PikoReal Real-Time 96 PCR System (Thermo Scientific, Massachusetts, USA). Details of primers and probes are listed in Table 1. The 18s ribosomal RNA was used as the internal control. The data were analyzed with the $2^{-D\Delta C}{}_{T}$ method.

Table 1
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Symbol	Gene	Gene ID
Hmgb1	High mobility group Box 1	Rn02377062_g1
Hmgb2.	High mobility group Box 2	Rn0082028_g1
S-100 A4	S-100 calcium binding protein A4	Rn01451938_m1
Hsp70-2	Heat shock 70 kDa protein 1b	Rn02532795_s1
Tlr2	Toll-like receptor 2	Rn02133647_s1
Tlr4	Toll-like receptor 4	Rn00569848_m1
RT1-Ba	RT1 class II,locus Ba	Rn01428452_m1
Ribosomal 18s	Eukaryotic 18S rRNA	Hs99999901_s1

2.5. Western blots

Liver samples from the right medial lobe were homogenized in RIPA buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.1% deoxycholate, 0.1% SDS, 1 mM NaF, 0.1 mM Na_3VO_4, 1 mM PMSF and proteinase inhibitor cocktail; Nacalai tesque, Kyoto, Japan). After centrifugation at 13,000 × *g* for 10 min, the supernatant was mixed with an equal volume of 2 × SDS sample buffer (125 mM Tris-HCl pH 6.8, 4% SDS, 30% glycerol and 10% 2-mercaptoethanol) and then boiled at 95 °C for 5 min. Samples were separated on 10% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (BioRad, CA, USA). Membranes were incubated overnight at 4 °C with mouse anti-TLR-4 (Novus Biologicals, USA) and mouse anti-β-actin antibodies (Sigma-Aldrich Co., MO, USA), followed by an incubation with peroxide-conjugated secondary antibody (Simplestain, MAX-PO; Nichrei) for 30 min. Signals were visualized with ECL prime (GE Healthcare, Little Chalfont, UK), and quantified with a luminescent image analyzer (LAS-3000; GE Healthcare).

2.6. Statistics

Data are represented as mean ± standard deviation (SD). Statistical analyses were performed using Dunnett's test. Significance was accepted at $P < 0.05$.

3. Results

3.1. Hepatocyte injury peaks on days 1 and 2 post injection

In control liver, no pathological change was seen (Fig. 1a). At hour 10 after injection, some hepatocytes around the central veins showed slight degenerative changes (Fig. 1b, arrow). On day 1, coagulation necrosis was seen in the centrilobular area (Fig. 1c), which was more severe on day 2 (Fig. 1d). On day 3, infiltration of mononuclear cells was seen in the injured areas (Fig. 1e). After day 5, these lesions were decreased in severity and tended to be recovered (Fig. 1f). Consistent with the histopathologic lesions, AST and ALT values significantly increased on days 1 and 2, and decreased from day 3 on (Fig. 2a–c). ALP value gradually increased to day 2, and decreased from day 3.

3.2. DAMPs and TLRs increase after hepatocyte injury

To investigate the kinetics of DAMPs during TAA-induced acute liver injury, real-time PCR was performed using liver samples. mRNA expression of HMGB-1, -2 and S100A4 significantly increased on day 2 (Fig. 3a–c); HMGB-1 also increased on day 3. On the other hand, HSP70-2, which reacts to cell stress (Yashin et al., 2011), increased at hour 10 and then decreased (Fig. 3d). As HMGB-1, -2 and S100 proteins are ligands of TLR-2 or TLR-4, we investigated the expression of these receptors. TLR-2 tended to increase on day 2 (Fig. 3e); TLR-4, a receptor for HMGB-1, increased