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Attenuation of thioacetamide-induced hepatocellular injury by short-term repeated injections associated with down-regulation of metabolic enzymes and relationship with MHC class II-presenting cells

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ABSTRACT

The liver is the primary organ participating in the metabolism of xenobiotics and is therefore an important target in the safety assessment of drugs, chemicals and environmental toxins. Drug-induced liver injury (DILI) has recently become widely recognized in human medicine as an adverse event. The progression of DILI often involves "damage-associated molecular patterns" (DAMPs) of gene and protein expression such as high-mobility group boxes (HMGBs), S100 proteins and heat shock proteins (Hsp). DAMPs are released from injured or necrotic cells and are bound to Toll-like receptors (TLRs) and modulate inflammatory reactions. Previously, in thioacetamide (TAA; 300 mg/kg body weight, single injection)-induced rat liver, we demonstrated that the expressions of DAMPs, TLR4 and major histocompatibility complex (MHC) class II were simultaneously increased, accompanied with progression of hepatocellular injury and inflammation. Here we investigated the association of DILI and DAMPs, TLRs and MHC class II by using rat livers repeated injections with TAA (100 mg/kg body weight, once, three times). Two days after TAA single injection, centrilobular hepatocellular necrosis with infiltration of mononuclear cells was observed, being paralleled with increase in serum levels of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP). However, two days after duplicate and triplicate injections, only mild degenerative change of hepatocytes and slight infiltration of mononuclear cells were seen in the affected centrilobular area. Serum levels of AST, ALT and ALP were also decreased to the same levels of control. mRNA expressions of DAMPs (HMGBs, S100A4 and Hsp 70-2), TLR4 and MHC class II tended to be increased only on single injection, although the number of MHC class II-positive cells in the centrilobular area was still increased on each examination point. The analysis of enzymes (CYP2E1 and Flavin monooxygenase (FMO) 3), which metabolize TAA in hepatocytes, showed a significant decrease in FMO3 on the duplicate and triplicate injections. Autophagy and regulatory T cells were not significantly changed for the attenuation of hepatocyte injury. Collectively, these results suggest that hepatocytes may adapt accumulation of the toxicant by changing their enzyme functions; furthermore, MHC class II cells, which still showed increased number in the duplicate and triplicate injections, may be related with protection from the toxicant.

1. Introduction

The accumulation of chemical materials, such as heavy metals (lead, copper), pesticides and industrial waste, represent significant environmental problems. These materials induce toxicity after accumulation and metabolism in different organs, particularly in the liver. Accumulation of harmful materials often causes various disorders due to direct tissue injury or oxidative stress (Wasi et al., 2013). In a similar manner, repeated exposure to some drugs can cause liver diseases known as idiosyncratic and allergic drug-induced liver injury (DILI). Specific drugs such as halothane, phenytoin and nitrofurantoin are known to induce DILI; however, the mechanism has not been yet elucidated (You et al., 2006; Navarro and Senior, 2006). Generally, DILI has been reported to be caused by more than 600 medical drugs (Park et al., 2005), including widely available medicines (Chen et al., 2015) such as acetaminophen, which is the most common cause of acute liver failure in the United States (Larson et al., 2005). If a specific drug causes hepatotoxicity, daily/repeated intakes may induce progressive

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liver injury with more severe lesions compared to those seen with a single exposure. Surprisingly, reports of multiple exposure animal models of liver injury are remarkably limited in this context.

Damage-associated molecular patterns (DAMPs) are endogenous molecules that may function as cellular “danger signals”. Examples include high mobility group box (HMGB) proteins, S100 proteins and heat-shock proteins (HSPs) (Bianchi, 2007; Tsung et al., 2007; Jaeschke et al., 2012; Chen et al., 2015). DAMPs are released from injured or necrotic cells and activate the innate immune system through various receptors, such as toll-like receptors (TLRs) (Seki et al., 2007; Chen et al., 2014). In the liver, DAMPs and TLRs are up-regulated in murine models of ischemia/reperfusion (Yu et al., 2010), while there are few studies involving DILI in rats. Recently, major histocompatibility complex (MHC) class II antigen has been identified as a receptor for endogenous antigens (Leung, 2015). In our previous studies using a thioacetamide (TAA)-induced acute liver injury model (a single injection), it was shown that the expression of DAMPs was increased. This was accompanied by increased TLR4 and MHC class II expressions (Kuramochi et al., 2016). These results suggested that overdose of specific drugs may induce severe hepatocellular injury and inflammation due to increased DAMP expression and subsequent progressive liver failure. In the present study, we therefore hypothesized that repeated-injections with TAA should result in more severe injury due to the increase in DAMPs released, followed by enhanced expression of TLRs and MHC class II. To begin to address this hypothesis, we investigated relationships between DAMPs, TLRs and MHC class II expression using a rat model of liver injury induced by repeated injections of TAA. Surprisingly, liver injury was actually attenuated by repeated injections of TAA.

2. Materials & methods

2.1. Animals

Six-week-old male F344 rats (109–121 g body weight; Charles River Japan, Yokohama, Japan) were injected intraperitoneally with TAA (100 mg/kg body weight; Wako Pure Chemicals, Osaka, Japan) dissolved in saline, once to three times, at three days interval for each group ($n = 4$ in each group); the dose (100 mg/kg body weight) of TAA was determined by a preliminary experiment showing mild lesion of hepatocellular injury as compared with lesions induced by a dose of LD50 (around 300 mg/kg body weight for rats) (Kuramochi et al., 2016). Control rats were treated with an equal volume of saline. Animals were housed in an animal room at a controlled temperature 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 after which blood (from the abdominal artery) and liver samples were collected, two days after each TAA injection. 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 for Animal Care at Osaka Prefecture University.

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 3 μm in thickness, were stained with hematoxylin and eosin (HE) for histopathologic examination.

PLP-fixed sections were used for immunohistochemistry with mouse monoclonal antibody against MHC class II (clone OX-6; 1:500; Serotec, Kidlington, UK). After pretreatment in a 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 (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 was counted in three different areas from 4 different rats using a WinRoof software (Mitani Corp., Fukui, Japan). Data are expressed as the number of positive cells per unit area (cells/mm^2).

2.4. Real-time PCR

Four rat liver samples in each group were used for real-time PCR. Liver samples from the right medial lobe were immersed in RNAlater reagent (Qiagen GmbH, Germany) at 4 °C and then stored at −80 °C. Total RNA was extracted using the SV total RNA isolation system (Promega Corporation, Madison, 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 a PikoReal Real-Time 96 PCR System (Thermo Scientific, Massachusetts, USA). Details of primers and probes are listed in Table 1. Eukaryotic 18sRNA was used as internal control. The data were analyzed with the $2^{-\Delta\Delta C}{}_{T}$ method.

2.5. Western blot

Three rat liver samples in each group were used for Western blot. 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 heated to 95 °C for 5 min. Samples were separated on 10% polyacrylamide gels and transferred to polyvinylidene difuorude (PVDF) membranes (BioRad, CA, USA). Membranes were incubated overnight at 4 °C with rabbit anti-CYP2E1 (Novus Biologicals, USA), mouse anti-flavin monooxygenase 3 (anti-FMO3; Abcam, Cambridge, UK) and mouse anti-β-actin antibodies (Sigma-Aldrich Co., St. Louis, MO, USA), followed by an incubation with peroxide-conjugated secondary antibody (Simplestain, MAX-PO; Nichrei) for 30 min at room temperature. Signals were visualized with ECL prime (GE Healthcare, Little Chalfont, UK), and quantified with a luminescent image analyzer (LAS-3000; GE Healthcare).

Table 1
The list of Taqman probes used for real-time PCR.

Symbol	Gene	Assay ID
Hmgb1	High mobility group box 1	Rn02377062_g1
Hmgb2	High mobility group box 2	Rn00820828_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
MCP-1	Chemokine (C−C motif) ligand 2	Rn00580555_m1
IL-1β	Interleukin 1 beta	Rn00580432_m1
IL-10	Interleukin 10	Rn00563409_m1
TGF-β1	Transforming growth factor, beta 1	Rn00572010_m1
Ribosomal 18s	Eukaryotic 18S rRNA	Hs99999901_s1