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Involvement of oxidative stress and immune- and inflammation-related factors in azathioprine-induced liver injury

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HIGHLIGHTS

- We developed a mouse model of AZA-induced liver injury.
- The mechanism of AZA-induced liver injury was investigated.
- Oxidative stress is involved in AZA-induced liver injury.
- Inflammatory response contributes in the exacerbation phase of liver injury.
- Activation of the innate immune system contributes to the exacerbation phase.

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ABSTRACT

Drug-induced liver injury (DILI) is a growing concern in the fields of drug development and clinical drug therapy because numerous drugs have been linked to hepatotoxicity. However, it is difficult to predict DILI in humans due to the lack of experimental animal models. Although azathioprine (AZA), which is a widely used immunosuppressive drug, is generally well tolerated, a small number of patients prescribed AZA develop severe hepatitis. However, the mechanism underlying this process has not yet been elucidated. In this study, we developed a mouse model of AZA-induced liver injury and investigated the mechanisms responsible for the hepatotoxicity of AZA. Female BALB/c mice were orally administered AZA. After AZA administration, the plasma levels of alanine aminotransferase and aspartate aminotransferase were increased, and liver damage was confirmed through a histological evaluation. In addition, the hepatic glutathione levels and superoxide dismutase activity were significantly decreased. The plasma levels of reactive oxygen species were significantly increased during the early phase of AZA-induced liver injury, and the hepatic mRNA levels of immune- and inflammation-related factors were also significantly changed. In conclusion, oxidative stress and the subsequently activated immune- and inflammation-related factors are involved in AZA-induced liver injury.

1. Introduction

Drug-induced liver injury (DILI) is the most frequent reason for the withdrawal of a drug from the market and the cessation of new drug development by pharmaceutical companies. Due to their association with significant patient morbidity and mortality, several drugs, including bromfenac, ebrotidine, and troglitazone, have been removed from the pharmaceutical market (Holt and Ju, 2006). In most cases, the mechanisms of hepatotoxicity are unknown, and predictive experimental animal models are lacking.

Azathioprine (AZA) is an immunosuppressive drug that is often used to treat inflammatory bowel disease and autoimmune conditions, such as rheumatoid arthritis, and is often used after transplantation to avoid organ rejection (Dejaco et al., 2003; Maltzman and Koretzky, 2003; Dubinsky, 2004). However, its therapeutic potential is limited by its high incidence (15–28%) of adverse reactions, such as hepatotoxicity, bone marrow suppression, and gastrointestinal symptoms (Marinaki et al., 2004; Takatsu et al., 2009). In a prospective cohort study, the hepatotoxicity of AZA was recognized in approximately 2% of rheumatoid arthritis and psoriatic arthritis patients (Aithal, 2011). The hepatotoxicity of AZA was demonstrated *in vivo* in rats, which exhibited a less than

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two-fold increase in the ALT level compared with the normal level (El-Beshbishy et al., 2011; Amin and Hamza, 2005).

AZA is rapidly and almost completely converted into 6-mercaptopurine (6-MP) in the liver, and this compound is further metabolized by three enzymatic pathways (Wong et al., 2007; Hisamuddin et al., 2007). The methylation of 6-MP to the inactive 6-methyl mercaptopurine *via* thiopurine methyltransferase (TPMT) is the first pathway. The second pathway involves the metabolization of 6-MP into 6-thiouric acid, which is an inactive metabolite, by xanthine oxidase (XO). The third pathway converts 6-MP into 6-thioinosine 5-monophosphate *via* hypoxanthine guanine phosphoribosyl transferase, and this intermediate is then metabolized into active 6-thioguanine nucleotides. It has been reported that XO has the potential to generate reactive oxygen species (ROS) in human hepatocytes (Petit et al., 2008) and that the oxidation of 6-MP by XO is involved in the AZA-induced liver injury in patients with inflammatory bowel disease (Ansari et al., 2008).

In addition, AZA causes fever and rash, which suggests that inflammation-related mechanisms underlie the AZA-induced liver injury (Jeurissen et al., 1990). However, at present, the involvement of immune- and/or inflammation-related reactions in the AZA-induced liver injury has not been reported. Toll-like receptors (TLR) and the receptors for advanced glycation end products (RAGE), which are expressed on multiple innate immune cells, such as macrophages and dendritic cells, contribute to the activation of the innate immune system (Hennessy et al., 2010; Thornalley, 1998). It was recently reported that damage-associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1) and S100 proteins, which are the ligands of TLR and RAGE, are induced by ROS (Yao and Brownlee, 2010). The relationship between the activation of TLR4 or RAGE and DILI has been reported in acetaminophen- and carbamazepine-induced liver injury (Antoine et al., 2009; Higuchi et al., 2012b), which suggests that the pathogenesis of DILI involves the activation of the inflammatory system. However, only a few studies have investigated the mechanisms of immune- and inflammation-mediated DILI.

Cytokines and chemokines, which result in inflammation or infiltration of lymphocytes into the hepatocytes, are induced through the activation of TLR or RAGE (Lotze et al., 2007). Alternatively, cytokines are secreted by several immune cells, such as macrophages and T cells (Kita et al., 2001; Oo and Adams, 2010). Helper T (Th) cell-mediated immune responses play pivotal roles in the pathogenesis of a variety of human liver disorders (Kita et al., 2001). Th cells are subdivided into Th1, Th2, and Th17 subsets by their unique production of cytokines and characteristic transcription factors. Th1 cells require T-box expressed in T cells (T-bet) and secrete interferon (IFN)-γ. Th2 cells require the presence of GATA-binding domain (GATA)-3 and produce interleukin (IL)-4 and IL-5. Retinoid-related orphan receptor (ROR)-γt is indispensable for the differentiation of Th17 cells, which mainly secrete IL-17 (Kidd, 2003; Steinman, 2007). We previously reported the relationship between Th cell-related factors and the DILI induced by halothane (Kobayashi et al., 2009), α-naphthylisothiocyanate (Kobayashi et al., 2010), dicloxacillin (Higuchi et al., 2011), diclofenac (Yano et al., 2012), carbamazepine (Higuchi et al., 2012b), flutamide (Higuchi et al., 2012a), and methimazole (Kobayashi et al., 2012).

In this study, we established the development of AZA-induced liver injury in wild-type mice and demonstrated that oxidative stress and a set of subsequent inflammation- and immune-related factors are involved in AZA-induced liver injury.

2. Materials and methods

2.1. Chemicals

AZA was purchased from Tokyo Chemical Industry (Tokyo, Japan). Tempol was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Allopurinol was purchased from Woko Pure Chemical Industries (Osaka, Japan). Eritoran was kindly provided by Eisai Co. (Tokyo, Japan). RNAiso was purchased from Nippon Gene (Tokyo, Japan). ReverTra Ace was obtained from Toyobo (Tokyo, Japan). Random hexamer and SYBR Premix Ex Taq were obtained from Takara (Osaka, Japan). All of the primers were commercially synthesized by Hokkaido System Sciences (Sapporo, Japan). Rabbit polyclonal antibody against myeloperoxidase (MPO) was obtained from DAKO (Carpinteria, CA). The HMGB1 ELISA kit II was purchased from Sino-Test Corporation (Tokyo, Japan). The Fuji DRI-CHEM slides of GPT/ALT-PIII and GOT/AST-PIII that were used to measure the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively, were obtained from Fujifilm (Tokyo, Japan). All of other chemicals were of either analytical grade or the highest commercially available grade.

2.2. AZA administration to mice

Female BALB/cCrSlc mice (8 weeks of age, 18–21 g) were obtained from SLC Japan (Hamamatsu, Japan). The mice were housed in a controlled environment (temperature 25 ± 1 °C, humidity 50 ± 10%, and 12-h light/12-h dark cycle) in the institutional animal facility with access to food and water *ad libitum*. The animals were acclimatized before their use in the experiments. Non-fasting mice were orally (*p.o.*) administered AZA (in corn oil) at a dose of 100, 200, and 300 mg/kg on the appropriate days. The blood and liver were collected 24 h after the last administration. A portion of each excised liver was fixed in 10% formalin neutral buffer solution. The degree of liver injury was assessed by hematoxylin–eosin (H&E) staining. The animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, and the protocol was approved by the Institutional Animal Care and Use Committee of Kanazawa University of Japan.

2.3. GSH and GSSG levels

The mouse livers were homogenized in ice-cold 5% sulfosalicylic acid using a glass homogenizer and centrifuged at 8000 × *g* and 4 °C for 10 min. The glutathione (GSH) and glutathione disulfide (GSSG) concentrations in the supernatant were measured as described previously (Tietze, 1969).

2.4. Protein carbonyl contents and SOD activities

Protein carbonyl contents and superoxide dismutase (SOD) activities of liver homogenate were measured using a Protein Carbonyl ELISA kit (Enzo Life Science, Farmingdale, NY) and a Superoxide Dismutase Assay kit (Cayman Chemical, Ann Arbor, MI), respectively.

2.5. Administration of an antioxidant agent

The mice were intraperitoneally (*i.p.*) administered tempol, which is an antioxidant agent (200 mg/kg in PBS) at the same time as the AZA administration (200 mg/kg in corn oil, *p.o.*) during a period of five days. The plasma and liver were collected 24 h after the last AZA administration.

2.6. Administration of a xanthine oxidase inhibitor

The mice were administered allopurinol, which is a xanthine oxidase (XO) inhibitor (30 mg/kg in sterilize PBS, *i.p.*) at the same time as the AZA administration (200 mg/kg in corn oil, *p.o.*) during a period of three days. The plasma was collected 24 h after the last administration.

2.7. Hydrogen peroxide levels

The plasma hydrogen peroxide (H_2O_2) levels were measured using a Hydrogen Peroxide Assay kit (Bio Vision, Milpitas, CA).

2.8. Real-time reverse transcription (RT)-PCR

The RNA from the mouse liver was isolated using RNAiso according to the manufacturer's instructions. The expression levels of TLR2, TLR4, RAGE, S100A8, S100A9, T-bet, GATA-3, ROR-γt, IFN-γ, tumor necrosis factor (TNF)-α, IL-1β, NACHT-LRR-PYD-containing protein 3 (NALP3), and macrophage inflammatory protein (MIP)-2 were quantified by real-time RT-PCR. The primer sequences used in this study are shown in Table 1. For the RT, the total RNA (10 μg) and random hexamer (150 ng) were mixed and incubated at 70 °C for 10 min. The RNA solution was added to a reaction mixture containing 100 units of ReverTra Ace reaction buffer and 0.5 mM dNTPs in a final volume of 40 μl. The reaction mixture was incubated at 30 °C for 10 min, 42 °C for 1 h, and then 98 °C for 10 min to inactivate the enzyme. The real-time RT-PCR was performed using the Mx3000P instrument (Stratagene, La Jolla, CA). The PCR mixture contained 1 μl or 2 μl of template cDNA, SYBR Premix Ex Taq solution, and 8 pmol of the forward and reverse primers. The amplified products were monitored directly by measuring the increase in the intensity of the SYBR Green I dye (Molecular Probes, Eugene, OR).