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Development of a cell-based assay system considering drug metabolism and immune- and inflammatory-related factors for the risk assessment of drug-induced liver injury

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HIGHLIGHTS

- Drug-induced liver injury (DILI) is a major safety concern in drug development.
- Four kinds of *in vivo* mouse DILI models we recently established were used.
- Five *in vivo* biomarkers were found and applied to a cell-based assay system.
- Drug metabolism and immune- and inflammatory-related factors were considered.
- We proposed the total sum score of gene expression level for the risk assessment.

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ABSTRACT

Drug-induced liver injury (DILI) is a major safety concern in drug development and clinical pharmacotherapy. However, prediction of DILI is difficult because the underlying mechanisms are not fully understood. To establish a novel cell-based screening system to suggest drugs with hepatotoxic potential in preclinical drug development, comprehensive gene expression analyses during *in vivo* DILI are necessary. Using *in vivo* mouse DILI models and 4 sets of hepatotoxic positive and non-hepatotoxic drugs, we found that the hepatic mRNA levels of S100A8; S100A9; "NATCH, LRR, and pyrin domain-containing protein 3" (NALP3); interleukin (IL)-1β; and the receptor for advanced glycation endproducts (RAGE) were commonly increased in hepatotoxic drug-administered mice compared to non-hepatotoxic drug-administered mice. To clarify whether these 5 *in vivo* biomarkers can be applied to a cell-based screening system, we adapted human liver microsomes (HLM) in the presence of NADPH to assess the metabolic activation reaction, and we also adapted human monocytic leukemia cells HL-60, K562, KG-1 and THP-1 to assess the effects on mRNA expression of immune- and inflammatory-related factors. We investigated 30 clinical drugs with different safety profiles with regard to DILI and found that the total sum score of gene expression levels of S100A8, S100A9, RAGE, NALP3 and IL-1β mRNA in HL-60 or K562 cells incubated with HLM, could identify drugs at high risk for hepatotoxicity. We proposed the use of the total sum score of gene expression level for assessing metabolic activation by drug-metabolizing enzymes and immune- and inflammatory-related factors for the risk assessment of DILI in preclinical drug development.

Abbreviations: ALT, alanine aminotransferase; AMP, ampicillin; APAP, acetaminophen; AST, aspartate aminotransferase; CXCL1, chemokine (C-X-C motif) ligand 1; CYP, Cytochrome P450; DAMP, damage-associated molecular pattern molecule; DIC, diclofenac; DILI, Drug-induced liver injury; DIX, dicloxacillin; ELISA, enzyme-linked immunosorbent assay; FasL, Fas ligand; FLU, flutamide; GATA-3, GATA-binding domain-3; HAL, halothane; HLM, human liver microsomes; HMGB1, high-mobility group box 1; HSP70, heat shock protein 70; IBU, ibuprofen; IFN-γ, interferon-γ; IL, interleukin; ISO, isoflurane; KC, Kupffer cell; LPS, Lipopolysaccharide; MBZ, mebendazole; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; NALP3, NATCH, LRR, and pyrin domain-containing protein 3; NK cell, natural killer cell; NKT cell, NK cell with T cell receptors; RAGE, receptor for advanced glycation endproducts; ROR-γt, retinoid-related orphan receptor-γt; STAT, signal transducer and activators of transcription factor; T-bet, T-box expressed in T cells; TIM, T cell immunoglobulin and the mucin domain; TLR, toll-like receptor; TNFα, tumor necrosis factor α; VCAM, vascular cell adhesion molecule.

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1. Introduction

Drug-induced toxicity is an important human health problem. Although toxic candidate compounds are mostly eliminated during preclinical safety studies in drug development, in some cases, toxicity is detected only in late clinical phases or during post-marketing evaluation. Drug-induced liver injury (DILI) is the most frequent reason for the withdrawal of an approved drug from the market and also a major cause of attrition in drug development (Lee, 2003). The drugs nefazodone, troglitazone, and bromfenac were withdrawn from the market because of DILI. For the pharmaceutical industry, it is important that drugs with the potential risk of DILI are screened out in the early phase of discovery and development process. However, in most cases of DILI, the mechanism is largely unknown despite many efforts to clarify them. The low incidence of DILI in humans together with the very large inter- and intra-individual variability of drug metabolism abilities in humans has hampered detailed mechanistic studies of DILI (Evans et al., 2004; Walgren et al., 2005; Takakusa et al., 2008).

Although the mechanisms of DILI are not fully understood, it is generally believed that one of the triggers of the pathogenesis of DILI is that the drug and/or its metabolites are chemically reactive. Cytochrome P450 (CYP) enzymes play an important role in metabolic activation and may generate reactive metabolites. Because a high proportion of drugs are capable of generating reactive metabolites, which covalently bind to various target macromolecules through nucleophilic substitution, it is thought that metabolic activation of a drug might be a necessary first step in DILI in many cases (Uetrecht, 1999; Walgren et al., 2005).

In addition, there is some evidence that immune- and inflammatory-related factors are involved in the pathogenesis of DILI (Ramaiah and Jaeschke, 2007). The liver is selectively enriched in Kupffer cells (KCs), natural killer (NK) cells, and NK cells with T cell receptors (NKT cells), which are key components of the innate immune system that can develop intracellular networks mediated by cytokine and chemokine signaling (Racanelli and Rehermann, 2006). Several mediators have been suggested to induce hepatotoxicity, including tumor necrosis factor (TNF) α, interleukin (IL)-1β, high-mobility group box (HMGB) 1, and IL-17 (Ishida et al., 2004; Tukov et al., 2007; Antoine et al., 2010; Kobayashi et al., 2009). These mediators can induce hepatocyte cell death by causing an excessive inflammatory response. Thus, comprehensive analyses of gene expression are necessary to establish a new *in vitro* screening system to identify drugs with hepatotoxic potential.

We recently reported the development of mouse *in vivo* DILI models of dicloxacillin (DIX), flutamide (FLU), halothane (HAL), and diclofenac (DIC) using wild type Balb/c mice (Higuchi et al., 2011, 2012a; Kobayashi et al., 2009; Yano et al., 2012). FLU- and HAL-induced liver injury were extensively studied in mice and rats (Deng et al., 2006, 2008; Dugan et al., 2010, 2011; Cheng et al., 2010; You et al., 2006). In the present study, we conducted analyses of hepatic mRNA expression profiles in the hepatotoxic drugs-induced *in vivo* mouse model, using non-hepatotoxic drugs as negative controls, to determine the common risk biomarkers of DILI. The pharmacological properties and chemical structures of the controls are similar to those of the hepatotoxic drugs. Next, we used 17 representative hepatotoxic and 13 non-hepatotoxic drugs to investigate the application of the risk biomarkers to an *in vitro* cell-based assay system to determine the risk of DILI for each of these drugs. Taking drug metabolism reactions and immune- and inflammatory-related reactions in human into consideration, we proposed the calculation of a "total sum score of gene expression level" for risk assessment of DILI.

2. Materials and methods

2.1. Materials

Caffeine, DIC, DIX, lipopolysaccharide (LPS), mebendazole (MBZ), procainamide, valproic acid, warfarin, and zomepirac were purchased from Sigma-Aldrich (St. Louis, MO). HAL and isoflurane (ISO) were kindly provided from Takeda Yakuhin (Osaka, Japan) and Abbott Japan (Tokyo, Japan), respectively. Ampicillin (AMP), APAP, ibuprofen (IBU), FLU, aminopyrine, carbamazepine, ticlopidine, clopidogrel, erythromycin, furosemide, indomethacin, phenytoin, sulfamethoxazole, tacrine, acetylsalicylic acid, dexamethasone, losartan, pravastatine, and theophylline were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Bicalutamide (BIC) was obtained from Enzo Life Sciences (Farmingdale, NY). Rosiglitazone was obtained from LKT Labs (St. Paul, MN). Olanzapine, pioglitazone and valsartan were purchased from Toronto Research Chemicals (Toronto, Canada). Levofloxacin was purchased from BioChemika (Buchs, Switzerland). β-NADPH and glutathione reductase were from Oriental Yeast (Tokyo, Japan). Troglitazone and eritoran were kindly provided by Daiichi–Sankyo (Tokyo, Japan) and Eisai (Tokyo, Japan), respectively. The Fuji DRI-CHEM slides of GPT/ALT-PIII and GOT/AST-PIII used to measure alanine aminotransferase (ALT) and aspartate aminotransferase (AST), respectively, were purchased from Fujifilm (Tokyo, Japan). RNAiso, random hexamer, and SYBR Premix Ex Taq were purchased from Takara (Ohtsu, Japan). ReverTra Ace was purchased from Toyobo (Osaka, Japan). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Monoclonal anti-mouse/rat RAGE antibody and rat IgG2a isotype (used as a control) were obtained from R&D systems (Abingdon, UK). The HMGB1 enzyme-linked immunosorbent assay (ELISA) kit II was purchased from Sino-Test Corporation (Tokyo, Japan). Pooled HLMs ($n = 50$, testosterone 6β-hydroxylase activity of 5700 pmol/mg/min) were purchased from BD Gentest (Woburn, MA). All other chemicals were either analytical grade or the highest commercially available grade.

2.2. Drug-induced liver injury mouse models

Female BALB/cCrSlc mice (8 weeks old) were obtained from SLC Japan (Hamamatsu, Japan). 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*. Animals were acclimatized before the experiments. HAL or ISO (30 mmol/kg in 2 μL olive oil, *i.p.*), DIC or IBU (150 mg/kg in saline, *i.p.*), FLU or BIC (1500 mg/kg in 0.5% carboxymethylcellulose (CMC), *p.o.*), and DIX (600 mg/kg in saline, *i.p.*) or AMP (1000 mg/kg in saline, *i.p.*) were administered to mice in a non-fasting condition. APAP (300 mg/kg in saline, *i.p.*) was administered to mice in an overnight fasting condition. Blood samples from the inferior vena cava and the largest lobe of the liver were collected 1, 3, 6 and 24 h after drug administration under diethyl-ether anesthesia ($n = 5$ for each drug-treated group). As previously reported by our group (Higuchi et al., 2011, 2012a; Kobayashi et al., 2009; Yano et al., 2012), the liver damage was confirmed by histopathology in APAP, HAL, DIC, FLU, and DIX-administered mice. Animals were treated and maintained in accordance with the Japan National Institutes of Health Guide for Animal Welfare, and the animal protocols were approved by the Institutional Animal Care and Use Committee of Kanazawa University, Japan (AP-#111985).

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

RNA from the mouse liver or human monocytic leukemia cells were isolated using RNAiso according to the manufacturer's