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A novel cell-based assay for the evaluation of immune- and inflammatory-related gene expression as biomarkers for the risk assessment of drug-induced liver injury

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HIGHLIGHTS

- Drug-induced liver injury (DILI) is a major safety problem in drug development.
- A novel cell-based assay was developed based on immune and inflammatory factors and drug metabolism to detect drugs with a propensity to cause DILI.
- The IL-1β, IL-8, and S100A9 mRNA levels were acceptable indicators for the risk assessment of DILI.
- An integrated score of the three factors showed a superior discriminative ability.
- Our method considering immune and inflammatory factors and drug metabolism is useful for detecting DILI-positive drugs.

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ABSTRACT

Drug-induced liver injury (DILI) is a major problem in drug development. Although some *in vitro* methods assessing DILI risk that utilize hepatic cell death or cellular stress as markers have been developed, the predictive ability of these tests is low. In this study, we sought to develop a novel cell-based assay for the risk assessment of DILI that considers drug metabolism as well as immune- and inflammatory-related gene expression. To accomplish this goal, human hepatoma HepaRG or HepG2 cells were treated with 96 drugs with different clinical DILI risks. The conditioned media were subsequently used to treat human promyelocytic leukemia HL-60 cells, and the mRNA expression levels of immune- and inflammatory-related genes in the cells were measured. An area under the receiver operating characteristic curve (ROC–AUC) was calculated to evaluate the predictive performance of the mRNA levels as markers to discriminate DILI risk. The expression of interleukin-8 (IL-8) in HL-60 cells treated with conditioned media from HepaRG cells (HL-60/HepaRG) exhibited the highest ROC–AUC value of 0.758, followed by the expression of IL-1β in HL-60/HepaRG (ROC–AUC: 0.726). Notably, the ROC–AUC values of these genes were higher in HL-60/HepaRG than in HL-60/HepG2, which suggests that HL-60/HepaRG has a higher potential for detecting the metabolic activation of drugs. An integrated score calculated from the levels of S100 calcium-binding protein A9 (S100A9), IL-1β, and IL-8 more precisely determined the DILI risks than individual gene expression did. The developed cell-based assay that utilizes immune-related gene expression would aid in the assessment of potential DILI risks.

Abbreviations: AR, adverse reaction; BBW, black box warning; CCL2, chemokine (C-C motif) ligand 2; CXCL8, chemokine (C-X-C motif) ligand 8; DAMP, damage-associated molecular pattern; DILI, drug-induced liver injury; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FDA, the U.S. Food and Drug Administration; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSH, glutathione; IL, interleukin; LTKB-BD, the liver toxicity knowledge base benchmark dataset; MCP-1, monocyte chemoattractant protein 1; NLRP3, NACHT, LRR, and pyrin domain-containing protein 3; NM, no mention; RAGE, the receptor for advanced glycation endproducts; ROC–AUC, area under the receiver operating characteristic curve; ROS, reactive oxygen species; RT, reverse transcription; S100A9, S100 calcium-binding protein A9; TNF-α, tumor necrosis factor α; WDN, withdrawn; WP, warning and precautions.

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1. Introduction

Drug-induced liver injury (DILI) is a major complication in drug development and drug therapy. In the USA, DILI accounts for more than 50% of acute liver failure, including hepatotoxicity caused by acetaminophen (39%) and toxicity from other drugs (13%) (Lee, 2013). More than 600 drugs have been associated with DILI (Park et al., 2005). Because of the significant patient morbidity and mortality associated with DILI, more than 50 drugs have been withdrawn from the global market in the past half century, and many drugs have been assigned "black box" warnings (Chen et al., 2011). The occurrence of DILI mostly ranges from less than 1 in 10,000 to 1 in 100,000 patients (Larrey, 2002). Due to the low incidence, compounds causing DILI are difficult to identify during drug development and often remain unidentified until the drugs have been released into the market. Therefore, for the pharmaceutical industry, it is imperative that drug candidates with a potential risk for DILI are screened out in the early stage of the drug discovery and development process. However, regulatory animal toxicity studies with two species (rodent and non-rodent) failed to identify the risk of DILI for approximately 45% of drug candidates observed in subsequent clinical trials (Olson et al., 2000).

Although the mechanism of DILI is not fully understood, direct hepatotoxicity and inappropriate immune reactions appear to be involved. It is generally believed that the initiation of DILI involves the bioactivation of drugs to chemically reactive metabolites, which are capable of binding to cellular macromolecules and thus lead to protein dysfunction, lipid peroxidation, DNA damage, and oxidative stress (Holt and Ju, 2006). Additionally, reactive metabolites could induce mitochondrial dysfunction (Kon et al., 2004). This impairment of cellular homeostasis can lead to cell death, apoptosis, or even liver injury. Cellular dysfunction also has the ability to initiate immune responses (Adams et al., 2010; Holt and Ju, 2006). In particular, damage-associated molecular patterns (DAMPs) such as high mobility group box-1, heat shock proteins, S100 calcium-binding protein A8 (S100A8), and S100A9 released from damaged hepatocytes activate hepatic innate immune cells, including Kupffer cells, NK, and NKT cells (Laskin and Laskin, 2001; Martin-Murphy et al., 2010; Masson et al., 2008). This activation leads to hepatic infiltration by immune cells, which may contribute to the progression of liver injury through the production of pro-inflammatory cytokines and chemokines such as interleukin-1β (IL-1β), IL-18, tumor necrosis factor α (TNF-α), IL-8, and monocyte chemoattractant protein 1 (MCP-1) (Chen et al., 2007; Holt and Ju, 2006; Imaeda et al., 2009). These mechanisms associated with DILI could be potential targets for preclinical assessment of the DILI potential of drugs.

Indeed, several preclinical tests, including cell viability (Cosgrove et al., 2009; O'Brien et al., 2006), reactive oxygen species (ROS) production (Cosgrove et al., 2009), the assessment of reactive metabolites through covalent binding to glutathione (GSH) or protein (Usui et al., 2009) have been developed to detect the DILI potential of compounds. Although the HepG2 cell line is frequently used to study the general cytotoxic potential of drugs, these cells do not express significant amounts of phase I drug-metabolizing enzymes such as cytochrome P450s (Wilkening et al., 2003), which has limited the detection of the metabolism-dependent toxicity of drugs (Hosomi et al., 2011; Iwamura et al., 2011). HepaRG cells, which are from an established hepatoma line, are capable of differentiating into hepatocyte-like and biliary epithelial-like cells with a high expression of drug-metabolizing enzymes that is comparable to primary human hepatocytes (Aninat et al., 2006). Several studies have demonstrated the utility of HepaRG cells in detecting the toxicity of compounds, such as acetaminophen, aflatoxin B_1, and chlorpromazine, that are known to produce chemically reactive metabolites (Aninat et al., 2006; McGill et al., 2011; Mueller et al., 2014). However, these studies mostly evaluate events occurring within hepatic parenchymal cells without considering immune-mediated mechanisms. Our previous study found that immune- and inflammatory-related genes, including S100A8, S100A9, IL-1β, "NACHT, LRR, and pyrin domain-containing protein 3" (NLRP3), and the receptor for advanced glycation endproducts (RAGE), were more highly expressed in the livers of mice that received hepatotoxic drugs compared to mice that received non-hepatotoxic drugs (Yano et al., 2014). Among several immune cell lines, we observed that HL-60 cells responded well to hepatotoxic drug treatment in terms of the induction of the aforementioned immune and inflammatory genes. These results raised the possibility that an *in vitro* assay considering inflammation and immune reactions could improve or aids the prediction of clinical DILI.

In this study, we developed a novel cell-based assay system considering drug metabolism as well as immune- and inflammatory-related genes and evaluated to what extent the expression levels could predict clinical DILI. Furthermore, we sought to evaluate the utility of a score generated by combining the expression levels of several genes. We found that the score could separate drugs with a propensity to cause DILI from those with less DILI potential.

2. Materials and methods

2.1. Chemicals and reagents

The supplier and the catalog number for each of the test drugs are shown in Supporting information Table S1. Fetal bovine serum (FBS), nonessential amino acid, Williams' E medium, and penicillin-streptomycin (100×) were purchased from Life Technologies (Gaithersburg, MD). The hydrocortisone 21-hemisuccinate sodium salt and insulin solution from bovine pancreas was purchased from Sigma–Aldrich (St. Louis, MO). RNAiso PLUS and SYBR *Premix Ex Taq* were obtained from Takara (Ohtsu, Japan). The ReverTra Ace qPCR RT kit was obtained from Toyobo (Osaka, Japan). The cell counting kit-8 (CCK-8) was purchased from Dojin Chemistry (Kumamoto, Japan). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). The other chemicals or solvents were the highest grade that was commercially available.

2.2. Test drugs

A total of 96 drugs were examined in this study. Information on the clinical DILI risk for the test drugs was collected from the U.S. Food and Drug Administration (FDA)-approved prescription drug labels cited in DailyMed (http://dailymed.nlm.nih.gov/dailymed/index.cfm) and the Liver Toxicity Knowledge Base Benchmark Dataset (LTKB-BD; http://www.fda.gov/ScienceResearch/BioinformaticsTools; developed by FDA (Chen et al., 2011)). FDA-approved prescription drug labels classify DILI risks into five categories as follows: withdrawn from the market (WDN), black box warning (BBW), warning and precautions (WP), adverse reactions (AR), or no mention (NM). Drug labeling is regulated by law under the Code of Federal Regulation (CFR) Title 21 Part 201 (21CFR201.56) (http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfCFR/CFRSearch.cfm?fr=201.57). Drug labeling implicitly balances the information of causality, incidence and severity based on data from controlled trials, published literature reports and spontaneous reports to AERS (adverse event reporting systems) (more information in Chen et al., 2011). LTKB-BD classifies DILI risks by considering the clinical severity of DILI and labeling approved by FDA to place drugs in one of three categories as follows: most-, less-, or no-DILI-concern. The classifications are summarized in Table 1.