130 represses PPARγ, thereby controlling adipocyte gene expression programs (Lee et al., 2010). Interestingly, an inverse correlation was observed in which PPARγ mRNA was high in obese women but low in lean women, whereas miR-130 was low in obese women but high in lean women. Since it was reported that PPARγ agonists such as rosiglitazone and pioglitazone increased the expression of CYP26 (retinoic acid-metabolic enzyme) in HepG2 cells (Tay et al., 2010), it would be interesting and worth investigating whether the miRNA-dependent regulation of PPARγ affects retinoid metabolism.

Lin et al. (2009) have reported, using mouse embryonic fibroblast-derived 3T3-L1 preadipocytes, that overexpression of miR-27a or miR-27b decreased the expression of C/EBPα, which is involved in the regulation of some P450s (Akiyama & Gonzalez, 2003). Pan et al. (2009a) have reported, using LS180 and PANC1 cells, that overexpression of miR-27b decreased the expression of VDR. Ji et al. (2009) have reported, using rat hepatic stellate cells, that the inhibition of miR-27a and miR-27b increased the expression of retinoid X receptor α (RXRα), a heterodimer partner of various nuclear receptors such as PXR, VDR, CAR, PPAR, farnesoid X receptor, and liver X receptor. They described that the sequences of MRE on the RXRα mRNA are highly conserved, implying that human RXRα may also be regulated by miR-27. Taken together, miR-27 seems to regulate a wide variety of key transcriptional factors that are involved in the regulation of various drug metabolizing enzymes. It would be interesting to investigate the impact of miR-27 on the metabolism of xenobiotics/endobiotics in human livers, in addition to our finding that CYP1B1 is a direct target of miR-27b.

Vreugdenhil et al. (2009) have reported that rat and human glucocorticoid receptors (GR) are regulated by miR-18 and miR-124a. Uchida et al. (2008) also reported that rat GR is regulated by miR-18 and that the sequences of MRE are well conserved among rats, mice, and humans. Although the expression of miR-124a is restricted to brain, miR-18 is widely expressed throughout the body. Since GR is involved in the regulation of CYP2B6, CYP2C9, CYP3A4, PXR, and CAR (Rezen et al., 2011), additional studies are needed to determine whether the miRNA-dependent regulation of GR might affect drug metabolism.

Collectively, it has been revealed that various nuclear receptors are regulated by miRNAs. The regulation of nuclear receptors by miRNA results in changes in the expression of a variety of target genes, constructing complex regulatory networks. Since this knowledge has only recently been taken into consideration in drug metabolism, further studies are warranted to clarify the clinical significance of miRNAs in the control of pharmacokinetics for better understanding of inter- and intraindividual differences in drug responses and adverse reactions.

5. Modulation of miRNA expression

5.1. Modulation of miRNA expression by chemicals, drugs, and hormones

Similar to coding RNA, miRNAs are controlled by transcription factors (Krol et al., 2010). There are several reports regarding the changes of miRNA expression through ligand-activated nuclear receptors. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a potent environmental toxicant, is a ligand of aryl hydrocarbon receptor (AhR), which forms a heterodimer with AhR nuclear translocator (ARNT) and binds to xenobiotic response elements on the promoter of many kinds of drug-metabolizing enzymes such as CYP1A, alcohol dehydrogenase (ALDH), and glutathione S-transferase (GST). It is well known that TCDD causes vast changes in mRNA expression, but the administration of TCDD to mice and rats caused few changes in miRNA (45 and 17 miRNAs, respectively) levels in liver (Moffat et al., 2007). Mouse and rat hepatoma cells in culture also exhibited few changes in miRNAs in response to TCDD (Moffat et al., 2007). A recent study has reported that the oral administration of benzo(a)pyrene, another ligand of AhR, to mice caused no changes in miRNA, in spite of widespread changes (>400 genes) in mRNA expression (Yauk et al., 2011).

It has been demonstrated that the administration of Wy-14,643, a specific PPARα agonist, to mice for 2 weeks caused changes (>1.5-fold) in the expression of 27 miRNAs (12 miRNAs were decreased and 15 miRNAs were increased) (Shah et al., 2007), but greater changes in 707 mRNAs (671 mRNAs were decreased and 36 mRNAs were increased, >4-fold). When rat primary thymocytes were treated with dexamethasone, a synthetic glucocorticoid that binds to GR, the expression of 56 out of 350 miRNAs was altered (44 miRNAs were decreased and 12 miRNAs were increased) (Smith et al., 2010). Testosterone, which regulates gene expression through androgen receptor (AR), affects the expression of 6 miRNAs in female mouse liver (Delić et al., 2010). Treatment of estradiol, a ligand of ERα, caused changes in the expression of a subset of miRNAs in mice, rats, and human breast cancer-derived MCF-7 cells (Klinge, 2009). Taken together, the expression of miRNAs actually changes in response to chemicals and steroid hormones, but the changes are likely smaller than those of mRNA expression.

Various stressors also affect miRNA expression. Arsenite, which is known to activate nuclear factor-erythroid 2-related factor 2 (Nrf2) (Aono et al., 2003), affects miRNA expression in human lymphoblastoid TH-6 cells (Marsit et al., 2006). Cigarette smoking causes the down-regulation of many miRNAs in the lungs of both mice and rats (Izzotti et al., in press) as well as human airway epithelial cells (Schembri et al., 2009). The administration of the hepatotoxicants acetaminophen or carbon tetrachloride to rats caused changes in the expression of some miRNAs (there were only 13 commonly changed miRNAs in the two groups) in liver (Fukushima et al., 2007). As described above, the chronic administration of NNK, a tobacco-specific carcinogen, to rats reduced the expression of several miRNAs in lung (Kalscheuer et al., 2008). There is accumulating evidence that these up-regulated or down-regulated specific miRNAs could alter the expression of target mRNAs and lead to some phenotypic changes (Shah et al., 2007; Kalscheuer et al., 2008; Maillot et al., 2009; Ribas et al., 2009).

Interestingly, it has been demonstrated that enoxacin, an antibiotic, affects the processing of miRNAs by facilitating the interaction between TRBP and RNA (Shan et al., 2008). Yamagata et al. (2009) reported that the ligand-activated ERα inhibits the Drosha processing of pre-miRNA. Smith et al. (2010) have reported that the expression of miRNA-processing enzymes such as Dicer, Drosha, and DGCR8 were significantly reduced at the mRNA and protein levels during glucocorticoid-induced apoptosis. Thus, it is likely that chemicals and steroids modulate the expression of miRNAs not only transcriptionally but also post-transcriptionally.

5.2. Modulation of miRNA expression in disease

A growing number of reports have shown that aberrant miRNA expression is a common feature of human diseases such as cancer, Alzheimer's disease, cardiovascular disease, and schizophrenia. Since miRNAs regulate cellular functions, it is not surprising that miRNAs are implicated in a wide variety of diseases. We can refer to a database for miRNA–diseases associations, the Human MiRNA & Disease Database (HMDD) (http://202.38.126.151/hmdd/mirna/md/) (Lu et al., 2008). Because of the growing evidence that a variety of diseases are associated with the dysregulation of miRNAs, miRNAs are now considered as a new tool for diagnosis and therapy, as described below in detail (Calin & Croce, 2006). Our great concern is that such dysregulation of specific miRNAs in diseases or miRNA manipulation in therapy may lead to changes in drug responses in patients, although it has never been noticed so far.

6. Potential therapeutic applications of miRNAs

Since miRNAs are differently expressed in diseases and they play critical roles in various biological pathways, miRNAs are expected to be potential targets of therapeutics. The modulation of the levels of

miRNAs can be adapted from existing gene therapy and antisense technology. For miRNAs whose expression is reduced in diseases, re-introduction of miRNA into the proper tissue could provide a therapeutic benefit by restoring the regulation of target gene(s). An RNA-interference-based method using chemically modified antagomirs or locked nucleic acid (LNA)-modified oligonucleotides is already being developed. The therapeutic potency has been demonstrated in vivo using experimental animals such as mouse and monkey (Krützfeldt et al., 2005; Lanford et al., 2010). Furthermore, there are several ongoing clinical trials (Seto, 2010; Wahid et al., 2010). The most advanced miRNA-based therapeutics is targeted to the liver-specific miR-122, which is involved in hepatitis C replication and cholesterol metabolism. The company reported that there have been no apparent adverse reactions so far. In addition, some pioneering pharmaceutical companies have initiated studies on creating therapeutic candidates with miRNA mimics or miRNA inhibitors for cancer, cardiovascular diseases, neurological disorders, and viral infections. It is possible that the miRNA-based therapy could be used to regulate drug sensitivity. For example, the expression changes of miRNA in cancer cells are associated with resistance to anti-cancer drugs (Sarkar et al., 2010), and there is accumulating evidence that many drug transporters such as MDR1/P-glycoprotein, breast cancer resistance protein (BCRP/ABCG2), and multi-drug resistance-associated protein 1 (MRP1) are regulated by miRNAs (Zhu et al., 2008; Pan et al., 2009b; Liang et al., 2010). It is also possible that miRNAs could be utilized to selectively modulate drug metabolizing enzymes, drug transporters, and nuclear receptors toward optimal drug responses in pharmacotherapy. Targeting miRNAs for therapy could be an emerging field, although there are many hurdles to be overcome: stability, appropriate in vivo delivery systems, and selectivity. An individual miRNA could regulate several genes and pathways simultaneously suggesting that miRNA modulation could be powerful. However, attention must be paid to the possibility that miRNA manipulation may cause unanticipated effects, because the targets of the each miRNA have not been thoroughly clarified.

7. miRNA-related polymorphisms

Single nucleotide polymorphisms (SNPs) are the most common human genetic variants. The SNPs may affect either the expression or activities of various enzymes, and therefore may be associated with differences in the physiological or pharmacological outcomes. Polymorphisms can be present not only in the mRNA but also in mature miRNA sequences. In addition, polymorphisms in pri-miRNA and pre-miRNAs affect the expression level of mature miRNAs (Iwai & Naraba, 2005; Duan et al., 2007) and might lead to modification of the expression level of target genes. A polymorphism in an mRNA target site would be target-specific, whereas a polymorphism in an miRNA may affect the expressions of multiple genes and have serious consequences. A polymorphism in the genes encoding miRNA-processing enzymes may have a large impact (Horikawa et al., 2008).

Thousands of miRNA-related polymorphisms have been identified and are cataloged in databases such as Patrocles (http://www.patrocles.org/Patrocles.htm) and PolymiRTS (http://compbio.uthsc.edu/miRSNP/). Many miRNA-related polymorphisms have been shown to be associated with diseases (Sethupathy & Collins, 2008), because a gain-of function or loss-of-function of miRNA polymorphisms would result in changes in the expression of target mRNAs that are related to diseases: e.g., *SLITRK1* (Slit and Trk-like 1) gene/miR-189/Tourette's syndrome, *AGTR1* (angiotensin receptor 1) gene/miR-155/hypertension, and *FGF20* (fibroblast growth factor 20) gene/miR-433/Parkinson disease.

Pharmacogenetics research has matured considerably during the past few decades. The importance and implications of genetic polymorphisms in genes encoding drug metabolizing enzymes are largely recognized. Representative polymorphisms that can be used clinically are those of UDP-glucuronosyltransferase/irinotecan and thiopurine methyltransferase/thiopurines. In contrast to the accumulating evidence of polymorphisms on coding genes that affect drug responses and adverse effects, there are few examples regarding miRNA-related polymorphisms that can affect drug responses. A SNP in the binding site of miR-24 in the 3′-UTR of human dihydrofolate reductase gene leads to the overexpression of dihydrofolate reductase and methotrexate resistance (Mishra et al., 2007). Interestingly, it has been reported that SNPs in the pri-miR26a-1 or pri-miR-100 genes were significantly associated with the tumor response or time to progression in patients treated with 5-fluorouracil and CPT-11 (Boni et al., in press), although the molecular mechanism by which these polymorphisms act is not yet understood.

Actually, the SNPs exist on the 3′-UTR of many genes encoding P450s and other drug metabolizing enzymes. So far, most of them might be overlooked, because they are unlikely to affect the enzyme property or expression levels. Further studies are warranted to investigate whether the SNP may lose or gain the recognition site of miRNA, causing differences in the clinical outcome. Pharmacogenomics research on miRNAs should provide clues to understanding the cause of interindividual variability in drug responses and adverse reactions.

8. Conclusions

We now recognize the critical roles of miRNAs in numerous physiological processes and their involvement in human diseases. There is increasing interest in understanding the contribution of miRNAs to pharmacological and toxicological outcomes. How is miRNA expression changed under physiological conditions (diet, alcohol, smoking, environmental chemicals, stress, supplement, medication, or diseases)? Do the differences in the miRNA expression affect the drug response or susceptibility to xenobiotic toxicity? How do the miRNA-related polymorphisms affect pharmacokinetics and pharmacodynamics? Clarifying such issues will provide us better understanding of the intra- and interindividual variability in drug responses. miRNAs have clearly opened a new field in DMPK and toxicology.

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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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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.

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.

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 postmarketing 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; Higuchi *et al.*, 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 and 2008; Dugan *et al.*, 2010 and 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 5,700 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; Higuchi *et al.*, 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 instructions. The expression levels of mouse chemokine (C-X-C motif) ligand (CXCL) 1, Fas ligand (FasL), GATA-binding domain (GATA)-3, granzyme B, HMGB1, heat shock protein (HSP) 70, interferon (IFN)-γ, IL-1β, IL-6, IL-12p35, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein (MIP)-2, "NATCH, LRR, and pyrin domain-containing protein 3" (NALP3), perforin, receptor for advanced glycation endproducts (RAGE), retinoid-related orphan receptor

(ROR)-γt, signal transducer and activators of transcription factor (STAT) 1, STAT3, STAT6, S100A8, S100A9, T-box expressed in T cells (T-bet), T cell immunoglobulin and the mucin domain (TIM) family (TIM1, TIM2, TIM3, TIM4), TNFα toll-like receptor (TLR) 4, vascular cell adhesion molecule (VCAM) 1, and GAPDH were quantified by real-time RT-PCR. The proinflammatory calgranulins S100A8 and S100A9 are cytoplasmic proteins that are expressed by neutrophils, monocytes and activated macrophages under physiological conditions. The primer sequences are shown in Table 1. The expression levels of human NALP3, RAGE, S100A8, S100A9, IL-1β,and GAPDH were also quantified by real-time RT-PCR. The primer sequences are shown in Table 2. For the RT step, total RNA (10 μg) and 150 ng of random hexamer 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 resulting reaction mixture was incubated at 30°C for 10 min and 42°C for 1 h and then heated at 98°C for 10 min to inactivate the enzyme. Real-time RT-PCR was performed using an Mx3000 P instrument (Stratagene, La Jolla, CA). The PCR mixture contained 1 μL of template cDNA, SYBR Premix Ex Taq solution and 8 pmol of forward and reverse primers. The amplified products were monitored directly by measuring the increase of the SYBR Green I (Molecular Probes, Eugene, OR) dye intensity.

2.4 Administration of a TLR4 antagonist and an anti-mouse RAGE antibody.

Mice were intravenously treated with eritoran, a TLR4 antagonist (50 μg/mouse in 0.2 mL sterile saline), or an anti-mouse/rat RAGE antibody (100 μg/mouse in 0.2 mL sterile PBS) simultaneously with the drug administration, as previously described (Higuchi *et al.*, 2012b). Rat IgG2a was administered as a control for the RAGE neutralization study (100 μg rat IgG2a in 0.2 mL sterile saline). Blood was collected from the inferior vena cava at 6 h after DIC and DIX administration, and 24 h after the HAL administration.

2.5. Cell culture.

Human monocytic leukemia THP-1 cells were obtained from Riken Gene Bank (Tsukuba, Japan). Human promyelocytic leukemia HL-60 cells and human acute myeloid leukemia K562 cells and KG-1 cells were obtained from American Type Culture Collection (Manassas, VA). THP-1 and K562 cells were cultured in RPMI 1640 medium (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA). HL-60 and KG-1 cells were cultured in RPMI 1640 medium supplemented with 20% FBS. These cells were maintained at 37°C under an atmosphere of 5% CO_2. In the experiment using differentiated THP-1 cells, THP-1 cells were treated with PMA (phorbol 12-myristate 13-acetate) at 20 ng/mL for 48 h to trigger THP-1 cells to undergo differentiation into macrophages.

2.6. Drug treatment of human monocytic leukemia cells and the effects of HLM-dependent drug metabolism.

In LPS or MBZ exposure experiments, human monocytic leukemia cells, THP-1, HL-60, K562 and KG-1 cells were seeded at a density of 1×10^6 cells/well in 24-well plates with culture medium containing 10 μg/mL LPS or 10 μM MBZ and then incubated at 37°C for 3 and 6 h. In the experiments conducted to investigate the effect of the HLM-dependent drug metabolism of 30 drugs, HL-60 or K562 cells were seeded at a density of 1×10^6 cells/well in 24-well plates with medium containing the indicated concentration of the drugs (shown in Table 4) and 5% FBS, 1 mM β-NADPH and 1 mg/mL HLM. The cells were then incubated at 37°C for 6 h. Heat-inactivated (treated at 56°C for 30 min) HLMs were used as a control. The final concentration of dimethyl sulfoxide (DMSO) in the culture medium was 0.1%. We confirmed that cell viability was above 80% under the condition for any drug treatment.

2.7. Statistical analysis.

The data are shown as the means ± SEM. Comparison of multiple groups was made with Kruskal-Wallis ANOVA followed by Dunn's test. Comparison of two groups was made using Welch's *t*-test. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Time-dependent changes of plasma transaminase levels in hepatotoxic drug-administered mice.

To conduct a comprehensive analyses of the time-dependent changes of hepatic gene expression in mice with drug-induced hepatotoxicity, drugs known to cause DILI, including APAP (300 mg/kg, *i.p.*), HAL (30 mmol/kg, *i.p.*), DIC (150 mg/kg, *i.p.*), FLU (1500 mg/kg, *p.o.*), and DIX (600 mg/kg, *i.p.*) were administered to female BALB/c mice. Plasma ALT and AST levels were significantly increased in hepatotoxic drug-administered mice (Fig. 1). Slight increases in the plasma ALT and AST levels were observed 1 h after these drugs were administered, and increases in the ALT and AST levels were observed 6 h after DIC, FLU, and DIX administration. Increased levels were also observed 24 h after APAP and HAL administration. ISO, IBU, BIC, and AMP were used as the non-hepatotoxic control drugs; these control drugs have pharmacological properties and chemical structures that are similar to HAL, DIC, FLU, and DIX, respectively. The control drugs showed no hepatotoxic or much less hepatotoxic effects when administered with the same dosing program as the hepatotoxic drugs.

3.2. Time-dependent mRNA expression profiles in mouse models of drug-induced liver injury.

Time-dependent expression profiles of hepatic mRNAs in the mouse models of drug-induced liver injury were investigated by real-time RT-PCR analysis focusing on inflammation- and immune-related factors (Table 3). The hepatic mRNA expression levels of Th cell stimulation-related factors (T-bet, GATA-3, ROR-γt, STAT1, STAT3, STAT6), cytokines (TNFα, IL-1β, IL-6, IFN-γ, IL-12), chemokines (MIP-2, MCP-1, CXCL1, VCAM1), damage-associated molecular pattern molecules (DAMPs: S100A8, S100A9, HSP70, HMGB1), receptors (TLR4, NALP3, RAGE), CD8-positive cell-related factors

(granzyme B, perforin, FasL), and the TIM family members (TIM1, TIM2, TIM3, TIM4) were measured (Table 3, Figs. 2A and 2B). In our previous studies (Higuchi *et al.*, 2011; Kobayashi *et al.*, 2009), we confirmed that the expression profiles of mRNA and protein were similar for the interleukins, chemokines and receptors listed in Table 3, except in the case of HMGB1. Thus, changes in the mRNA expression levels were mainly investigated in the present study. Time-dependent changes in the expression levels of the hepatic mRNAs were shown in the hepatotoxic or non-hepatotoxic drug-administered groups compared with the vehicle-administered control groups (Fig. 2A). For example, the expression level of RAGE was increased after APAP, DIC, FLU, and DIX were administered to mice compared with the levels in mice who received vehicle. Increased MIP-2 mRNA expression was also observed after APAP, HAL, DIC, IBU, FLU, DIX, and AMP administration (Figs. 2A). In particular, 6 h after HAL administration, S100A8 and S100A9 expression were markedly increased, whereas the plasma ALT levels were not markedly increased (Fig. 3). In addition, the increase of S100A8 and S100A9 expression is likely specific for the hepatotoxic drug-administered groups, suggesting that those genes might be useful biomarkers for hepatotoxicity induced by drugs. However, S100A8 and S100A9 are not suitable biomarkers for FLU-induced hepatotoxicity, suggesting that several factors are necessary for developing a panel of biomarkers to determine the risk for drug hepatotoxicity. In Fig. 2B, the time-dependent changes of the expression levels of the S100A8, S100A9, RAGE, NALP3, and IL-1β were demonstrated in detail. Taking those expression profiles into consideration, changes in the mRNA expression of these 5 factors were relatively specific in the groups that received hepatotoxic drugs compared with those that received non-hepatotoxic drugs (Fig. 2A and 2B). Therefore, we focused on these 5 factors as candidate biomarkers to determine the risk of hepatotoxicity for the drugs in the *in vitro* studies. The change of the expression level of MIP-2 was the highest among the immune- and inflammatory-related factors in all the DILI-positive drugs. However, the results of MIP-2 showed high variability among the *in vivo*

experiments, which considerably affect the changes total sum score of gene expression level. To avoid low reproducibility, MIP-2 was not selected.

3.3. Role of TLR4 and RAGE in the pathogenesis of drug-induced liver injury

S100A8 and S100A9 are ligands of TLR4 and RAGE; HMGB1 is also a well-known ligand. HMGB1 is secreted from activated immune cells and is also passively released from necrotic cells (Wang *et al.*, 2004). It has been demonstrated that the release of HMGB1 is not correlated with changes in hepatic HMGB1 mRNA expression (Higuchi *et al.*, 2012b). Thus, the plasma HMGB1 level was measured using ELISA, which demonstrated a significant increase in expression in the hepatotoxic drug-administered groups compared with the non-hepatotoxic drug-administered groups (Fig. 4A). To investigate whether the TLR4 and RAGE signaling pathways are involved in DILI, eritoran, a TLR4 antagonist, or a monoclonal anti-RAGE antibody was administered to mice according to our previously described methods (Higuchi *et al.*, 2012b). Eritoran treatment significantly suppressed the plasma ALT levels in HAL-, DIC-, and DIX-administered mice (Fig. 4B), and anti-RAGE antibody administration also ameliorated DIX-induced liver injury (Fig. 4C). These results suggested that the TLR4 and RAGE signaling pathways might be involved in DILI.

3.4. Effect of immunostimulatory drugs on the mRNA level of the 5 biomarkers in human monocytic cell lines

To evaluate the 5 biomarkers for their utility in an *in vitro* assay system to distinguish the risk of the hepatotoxicity of drugs, we investigated the changes of the expression levels of NALP3, RAGE, S100A8, and S100A9 mRNA in human monocytic leukemia cells, such as THP-1, K562, KG-1, and HL-60, and differentiated THP-1 cells. Before considering the metabolic activation reaction in the cell-based assay, the effect of a parent drug (before being metabolized) on the expression of 4 biomarkers should be considered. The hepatotoxic drugs

in Table 4 are not guaranteed to undergo metabolism in the monocyte/macrophage-related cells. Thus, the direct immunostimulatory drugs, MBZ and LPS, were first investigated. As we previously reported (Mizuno *et al.*, 2011), MBZ strongly stimulated the expression of IL-8 and TNFα in THP-1, HL-60 and KG-1 cells. In the present study, direct immunostimulatory drugs, LPS (10 μg/mL) and MBZ (10 μM), were used to treat the cells for 3 and 6 h, and then the expression levels of these 4 biomarker's mRNAs were measured by real-time RT-PCR (Fig. 5). LPS and MBZ increased the expression level of NALP3 mRNA in K562 cells, and the expression levels of S100A8, S100A9, and RAGE mRNA were increased in HL-60 cells. In contrast, the expression levels of NALP3, RAGE, S100A8, and S100A9 in THP-1, KG-1 and differentiated THP-1 cells were not changed by their treatment with these immunostimulatory drugs. The expression level of IL-1β was not investigated in this experiment (Fig. 5) because we have previously reported that LPS and MBZ are unable to stimulate IL-1β expression in these cell lines (Mizuno *et al.*, 2011), however other drugs, such as aminopyrine and diclofenac, stimulate IL-1β expression in HL-60 cells (Supplementary Fig. 2D). For subsequent analyses, HL-60 and K562 cells were used to evaluate the immunostimulatory potency of hepatotoxic drugs.

3.5. An in vitro cell-based assay assessing the mRNA expression level of NALP3, RAGE, S100A8, S100A9, and IL-1β as biomarkers to identify hepatotoxic drugs

To investigate whether the risk of hepatotoxicity can be estimated by using these 5 biomarkers in HL-60 and K562 cells, we performed gene expression analysis of NALP3, RAGE, S100A8, S100A9, and IL-1β mRNA after exposure to 30 different drugs as listed in Table 4. The 30 representative drugs each have a different level of risk for hepatotoxicity (WDN, withdrawn; BBW, black box warning; WNG, warning; SAFE, no warning; the Physicians' Desk Reference (1995, 2000, 2004, and 2008). These drugs were used to treat HL-60 and K562 cells for 6 h in the presence or absence of HLMs. The sample of "the

absence of HLMs" was incubated with the heat-inactivated (treated at 56°C for 30 min) HLMs as mentioned in Materials and Methods. Changes of the relative expression level for each of the 5 biomarkers are shown in Supplemental Figures 1 and 2. The mRNA expression level was normalized to that of GAPDH mRNA in Supplemental Figures 1 and 2. NALP3 mRNA expression levels were not different between SAFE drugs- and hepatotoxic (WDN, BBW, and WNG) drugs-treated cells. Treatment of the cells with HLMs did not affect NALP3 mRNA expression levels (Fig. 6A). IL-1β mRNA expression levels were also not different between SAFE control drugs- and hepatotoxic drugs-treated cells without HLMs treatment, whereas under HLMs treatment, IL-1β mRNA levels were significantly higher in hepatotoxic drugs-treated cells than in SAFE control drugs-treated cells. Increase of IL-1β levels by the HLMs treatment was observed only in hepatotoxic drugs-treated cells, which might indicate that P450-mediated bioactivation of hepatotoxic drugs play a role in hepatotoxicity. However, the relative mRNA expression levels in the most hepatotoxic drugs-treated cells overlapped with those in the SAFE control drugs-treated cells despite of HLMs treatment (Fig. 6C). Taking together, these levels were unable to distinguish between the hepatotoxic and the SAFE control drugs clearly. Studies by Usui *et al.* (2009) and Nakayama *et al.* (2009) demonstrated that the maximum daily dose of a drug could be one of the factors that can determine hepatotoxicity. To investigate whether the maximum daily dose could be used to distinguish between the hepatotoxic drugs and the SAFE drugs, the relative mRNA expression levels were multiplied by the maximum daily dose (Figs. 6B and 6D). The daily doses of the drugs from the Physician's Desk Reference (1995, 2000, 2004, and 2008) are shown in Table 4. The NALP3 and IL-1β mRNA expression levels multiplied by the maximum daily dose tended to be significantly higher for hepatotoxic drugs than for the SAFE drugs; however, the distinction was still insufficient (Figs. 6B and 6D). Furthermore, we conducted the analyses of S100A8, S100A9, and RAGE in the same *in vitro* cell-based assay as in Fig. 6, and no significant difference was observed between the hepatotoxic and the SAFE control drugs

(data not shown). We found that an integrated score of the relative expression levels of S100A8, S100A9, RAGE, and IL-1β mRNA in HL-60 cells and NALP3 mRNA in K562 cells in the presence and absence of HLMs cells is likely to distinguish between the hepatotoxic WDN and BBW drugs from the SAFE control drugs (Fig. 7). This simple added score of the relative expression levels was termed the "total sum score of gene expression level". No added or external factors were processed. In the descending order of total sum score of gene expression level shown in Fig. 7, the top 10 drugs included all of the WDN and BBW drugs in Table 4 except valproic acid. These data indicate that the present cell-based assay system using the "total sum score of gene expression level" can be used to evaluate the high risk of hepatotoxicity resulting from the parent drug and/or its metabolite concurrently, without considering the maximum daily dose.