4. Discussion

DILI is an important issue for drug development and clinical drug therapy; however, prediction of DILI in preclinical studies is difficult because the underlying mechanisms are not fully understood. In this study, using data from comprehensive analyses of the gene expression levels of drug-induced hepatotoxicity *in vivo* in mice and considering metabolic activation reactions and immune- and inflammatory-related factors in humans, we aimed to establish an *in vitro* cell-based assay system with which to identify drugs with hepatotoxic potential.

To establish a novel *in vitro* cell-based screening system to identify drugs with hepatotoxic potential, we first thought that comprehensive analyses of the gene expression in the DILI mouse models are essential. In addition, it is also important to separately consider the changes in gene expression due to pharmacological effects and toxicological effects. Therefore, we selected 4 sets of hepatotoxic and non-hepatotoxic drugs for *in vivo* hepatic gene expression analyses. The hepatotoxic drugs HAL, DIC, FLU, and DIX are known to rarely cause severe hepatotoxicity in humans. Recently, the mouse DILI models of these drugs were well established by our group (Kobayashi *et al.*, 2009; Higuchi *et al.*, 2011; Yano *et al.*, 2012). The chosen non-hepatotoxic drugs, ISO, IBU, BIC, and AMP, possess pharmacological properties and chemical structures similar to HAL, DIC, FLU, and DIX, respectively, and are known to be much less hepatotoxic. These drugs showed no hepatotoxic effect with the same dosing conditions as the hepatotoxic drugs (Figs. 1A and 1B), suggesting that the pharmacological effects may not be involved in DILI under the present experimental conditions. There is no negative control drug for APAP that has similar pharmacological effects and chemical structure; therefore, APAP was used as a positive control drug.

We also reported that immune- and inflammatory-related factors are involved in the pathogenesis of DILI in HAL-, DIC-, FLU-, and DIX-treated mice (Kobayashi *et al.*, 2009; Higuchi *et al.*, 2011; Yano *et al.*, 2012). Taking the information from these DILI models and

several related reports into consideration, as shown in Table 3, 29 immune- and inflammatory-related factors, such as Th cell-related factors, cytokines, chemokines, DAMPs and their related receptors, CD8-positive cell-related factors, and TIM family members, were selected and comprehensively analyzed (Fig. 2A and 2B). In some cases, as shown in Fig. 2A, the non-hepatotoxic drugs induced the expression of inflammatory factors. For example, IBU elevated the mRNA expression of hepatic IL-1β, TNFα, IL6, and MIP-2, and AMP elevated IL-6 and MIP-2 expression even though no significant increases in plasma ALT and AST were observed. Therefore, it is necessary to subtract the gene expression changes due to the pharmacological effect. Based on the *in vivo* DILI model studies, we focused on the 5 factors, S100A8, S100A9, RAGE, IL-1β, and NALP3, for the following reasons: (1) marked increases of the mRNA expression were observed after the administration of hepatotoxic-positive drugs compared with the non-hepatotoxic drugs and (2) increased mRNA expression was observed in 3 out of the 4 hepatotoxic drugs. Based on our previous studies and other reports of DILI mouse models (Higuchi *et al.*, 2011; Yano *et al.*, 2012; Yao and Brownlee, 2010), HMGB1 was suggested to be a predictive biomarker of DILI (Fig. 4A). However, HMGB1 is actively secreted from activated immune cells and is also passively released from necrotic cells (Wang *et al.*, 2004). Thus, the release of HMGB1 measured by ELISA does not correlate with the increased expression of hepatic HMGB1 mRNA. The present cell-based screening system is designed to be an easy assay system for measuring mRNA expression changes; thus, HMGB1 was not used in the present screening system.

Antoine *et al.* (2009) reported that HMGB1 protein, an inflammatory indicator, in serum was linked to the mechanisms and pathological changes induced by APAP in mouse. Changes of the expression levels of S100A8 and S100A9 mRNA and HMGB1 protein were correlated in carbamazepine-induced liver injury in mouse (Higuchi *et al.*, 2012b). Reactive metabolite was suggested to generate the reactive oxygen species (ROS) in rat (Zou *et al.*, 2010), mouse (Higuchi *et al.*, 2012b) and human (Lu and Uetrecht, 2008). In human, it was

reported that ROS increased the expression of the TLR and the RAGE, as well as their ligands, such as S100 proteins and HMGB1 (Yao and Brownlee, 2010). The activation of TLR or RAGE results in the induction of inflammatory cytokines and chemokines in human (Lotze et al., 2007). Cytokines and chemokines, followed by inflammation or the infiltration of lymphocytes to hepatocytes, are involved in immune-mediated hepatotoxicity, and they are predominantly secreted from immune cells such as T lymphocytes and macrophages (Kita et al., 2001 ; Oo and Adams, 2010), suggesting that they might be involved in pathogenesis of DILI in human. Plasma ALT level and HMGB1 level was correlated in APAP-induced liver injury patients (Craig *et al.*, 2011). In the present study, the increased mRNA expression of S100A8 and S100A9 and the increased plasma protein level of HMGB1 were observed in the livers of mice administered hepatotoxic drugs (Figs. 2 and 4A), and the inhibition of TLR4 using eritoran and the neutralization of RAGE by anti-RAGE antibody attenuated drug-induced liver injury (Figs. 4B and 4C), suggesting that RAGE have an important role in the pathogenesis of DILI. Taking these results and information into consideration, the changes of the expression level of S100A8, S100A9 and RAGE would be appropriate biomarkers for prediction of DILI in human.

As reviewed by Latz (2010) and Scaffidi *et al.* (2002), the NALP3 inflammasome is activated by DAMPs that are released from injured cells. The NALP3 inflammasome generates mature IL-1β via proteolytic pathways. Recently, oxidative stress has been shown to play a principal role in the activation of the NALP3 inflammasome in cells or hepatocytes of mouse and human (Martinon *et al.*, 2009, Bryant and Fitzgerald, 2009; Zhou *et al.*, 2010). The NALP3 inflammasome plays a crucial role in the proinflammatory cytokine activation following APAP-induced liver injury in mouse (Maher, 2009; Imaeda *et al.*, 2009). In addition, we previously reported that IL-1β is involved in the early phase of DIC-induced liver injury (Yano *et al.*, 2012). Taking together, the NALP3-IL-1β signaling pathway is strongly suggested involving in DILI, however, there is no direct evidence about the relationship with

DILI severity in human. In this study, the mRNA expression of IL-1βwas also increased in hepatotoxic drug-treated mouse livers (Fig. 2); therefore, the NALP3-IL-1β signaling pathway also appears to be involved in DILI. Thus, those two factors were also thought to be useful for cell-based screening assay.

Next, we intended to apply the 5 biomarkers to an *in vitro* assay system to determine the risk of DILI of various drugs. Direct immunostimulatory drugs, MBZ and LPS, were used to screen the pro-inflammatory response of human monocytic leukemia cells (Fig. 5), according to our previous study (Mizuno *et al.*, 2011). Then, K562 and HL-60 cells were selected to detect the changes in the pro-inflammatory responses. In addition, CYP-mediated bioactivation is known to be involved in the onset of hepatotoxicity for many drugs (Hess and Rieder, 1997). Considering the metabolic activation of drugs, we performed the assay in the presence of HLM and NADPH. Thus, the anticipated mechanism of the present *in vitro* system is as follows: (1) hepatotoxic drugs are metabolized to an active metabolite by HLM; (2) the active metabolite stimulates the human monocytic leukemia cells; and (3) immune- and inflammatory-related responses are detected via changes in mRNA expression levels.

Concerning the concentration of drugs used in the cell-based assay, up to 30 times the clinically efficacious concentration or 100 μM was recommended by O'Brien *et al.* (2006). In the early stages of drug development, the clinically efficacious concentration is unavailable; thus, the use of 100 μM was adapted for the present study. However, according to many reports, the concentration of APAP was 1,000 μM.

We selected 17 positive (WDN, BBW, and WNG) and 13 negative (SAFE) representative compounds with regard to DILI (Table 4). As shown in Figs. 6A and 6C, there was a large overlap in the distribution of the mRNA expression levels between hepatotoxic and non-hepatotoxic drugs. Based on retrospective findings, it was reported that the daily dose is one of the most important factors for DILI, and very low-dose drugs (< 10 mg/day) seem to be devoid of any DILI induction potential (Uetrecht, 2000). In addition, 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 useful factors for determining hepatotoxicity. However, in this study, discrimination between hepatotoxic and non-hepatotoxic drugs was still insufficient even when considering the maximum daily dose (Fig. 6B and 6D). We found that the use of "total sum score of gene expression level", an integrated score of the relative expression levels of S100A8, S100A9, RAGE, NALP3 and IL-1β mRNA in HL-60 or K562 cells, could help identify drugs with a high risk of hepatotoxicity. The top 10 drugs included hepatotoxic drugs with a high risk of DILI (WDN and BBW) (Fig. 7). This assay system may be one of the useful methods for assessing the metabolic activation reaction and may be beneficial for predicting the risks of liver injury by drugs in preclinical development, without considering the maximum daily dose.

In this study, we focused on metabolic activation by CYPs by addition of HLM and NADPH; however, hydrolase and/or UDP-glucuronosyltransferase enzymes should be considered because these enzymes have also been suggested to be involved in metabolic activation (Spahn-Langguth and Benet, 1992; Kobayashi *et al.*, 2012). For example, acyl-glucuronides formed from carboxylic acid-containing metabolites can bind covalently to proteins and other macromolecules because of their electrophilicity, which suggests that they are linked to drug toxicity. In addition, valproic acid, which has a high risk for hepatotoxicity, exceptionally showed the lowest total sum score of gene expression level. This might imply the mechanism of DILI caused by valproic acid could not be explained by the inflammatory- or immune-related factors. Indeed, a metabolite of valproic acid is responsible for the mitochondrial damage, impairment of fatty acid mitochondrial β-oxidation, and lipid accumulation (Baillie, 1988), which may be associated with hepatotoxicity. A predictive method that takes mitochondrial toxicity into consideration might improve predictive accuracy. On the other hand, some SAFE drugs, pravastatin, levofloxacin, and ibuprofen, showed high scores. Taking together, the predictive potential for clinical DILI of this study is

not conclusive and it should be improved in the near future.

In conclusion, using the *in vivo* mouse DILI models with 4 sets of hepatotoxic and non-hepatotoxic drugs, we found that the hepatic mRNA levels of S100A8, S100A9, RAGE, NALP3, and IL-1β could be biomarkers of DILI. These 5 biomarkers were investigated for their utility in a cell-based screening system that considers metabolic activation reactions and immune- and inflammatory-related factors. Then, we proposed the use of the total sum score of gene expression level, that is, an integrated score of the relative mRNA expression levels of biomarkers for predicting the risk of DILI in preclinical drug development.

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Conflict of interest

Authors have no conflicts to declare.

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Figure legends

Fig. 1. Time-dependent changes of plasma ALT and AST levels in mice administered hepatotoxic drugs.

Hepatotoxic drugs (APAP: 300 mg/kg, *i.p.*; HAL: 30 mmol/kg, *i.p.*; DIC: 150 mg/kg, *i.p.*; FLU: 1500 mg/kg, *p.o.*; DIX: 600 mg/kg, *i.p.*) and non-hepatotoxic control drugs (ISO: 30 mmol/kg, *i.p.*; IBU: 150 mg/kg, *i.p.*; BIC: 1500 mg/kg, *p.o.*; AMP: 1000 mg/kg, *i.p.*) were administered. Each drug was administered once to mice, and blood samples were collected for the assessment of ALT and AST levels 1, 3, 6, and 24 h after drug administration. Data are shown as the mean ± SEM of results from 5 mice. Differences compared to the control group were considered significant at $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

Fig. 2. Time-dependent changes of the mRNA expression profiles of inflammatory- and immune-related factors in drug-induced liver injury.

Each drug was administered to the mice. At 1, 3, 6, and 24 h after administration, the hepatic mRNA expression level of 29 different types of inflammatory- and immune-related factors listed in Table 3 were measured by real-time RT-PCR. (A) Results of S100A8, S100A9, RAGE, TLR4, IL-1β, TNFα IL-6, NALP3, MIP-2 and TIM1 are shown. The expression level of hepatic mRNA was normalized to that of Gapdh mRNA. The change in the expression level is indicated by the fold-change compared to the vehicle-administered control group. Data are shown as the mean from 5 independent experiments. (B) Graphical expression of the data for S100A8, S100A9, RAGE, NALP3, and IL-1β. Data are presented as the log2 value ± SEM of the results from 5 mice.

Fig. 3. Time-dependent changes of the plasma ALT level and the hepatic mRNA expression of S100A8 and S100A9 in HAL-administered mice.

The plasma ALT level and hepatic S100A8 or S100A9 mRNA levels were measured 1, 3, 6, and 24 h after the HAL administration (30 mmol/kg, *i.p.*). The expression level of hepatic mRNA was normalized to that of Gapdh mRNA. Data are shown as the mean ± SEM of results from 5 mice. Differences of ALT levels compared to the control group were considered significant at $*p < 0.05$ and $***p < 0.001$. Differences of mRNA levels compared to the control group were considered significant at $\#p < 0.05$ and $\#\#p < 0.01$.

Fig. 4. Time-dependent changes of plasma HMGB1 levels and the effect of the inhibition of TLR4 and RAGE on plasma ALT levels during drug-induced liver injury.

(A) The plasma HMGB1 level was measured by ELISA 1, 3, 6, and 24 h after the administration of hepatotoxic (HAL, DIC, FLU, and DIX) and non-hepatotoxic control (ISO, IBU, BIC and AMP) drugs. (B) Eritoran (50 μg/mouse, *i.v.*), a TLR4 antagonist, was administered to the mice simultaneously with HAL (30 mmol/kg, *i.p.*), DIC (150 mg/kg, *i.p.*), or DIX (600 mg/kg, *i.p.*). The plasma ALT level was measured 6 h after DIC or DIX administration, or 24 h after HAL administration. (C) Anti-mouse RAGE antibody (0.1 mg/mouse, *i.p.*) or IgG2a (control) was simultaneously administered with DIX (600 mg/kg, *i.p.*). The plasma ALT level was measured 6 h after DIX administration. Data are shown as the mean ± SEM of results from 5 mice. Differences compared to the control mice (A), the drug alone treatment group (B) or the IgG2a-administered mice (C) were considered significant at $*P < 0.05$ and $**P < 0.01$.

Fig. 5. Effects of LPS and MBZ on the mRNA expression levels of NALP3, RAGE, S100A8, and S100A9 in human monocytic leukemia cells.

(A) THP-1, (B) K562, (C) KG-1, (D) HL-60, and (E) differentiated THP-1 cells were treated with 10 μg/mL LPS or 10 μM MBZ for 3 and 6 hours. The mRNA expression level was measured by real-time RT-PCR analysis. The mRNA expression level was normalized to

that of GAPDH mRNA. Data are shown as the mean from 2 independent experiments. ND; not detectable.

Fig. 6. Effect of the incubation of hepatotoxic and control SAFE drugs with HLMs on the expression levels of NALP3 and IL-1β mRNA and those expression levels multiplied by the maximum daily dose of drugs.

The comparison of the expression levels of NALP3 mRNA in K562 (A) and IL-1β mRNA in HL-60 (C) cells exposed to the hepatotoxic and control SAFE drugs incubated with HLMs are shown. The relative expression levels of NALP3 or IL-1β mRNA multiplied with the maximum daily dose of the hepatotoxic and control SAFE drugs are shown in Figs. 6B and 6D, respectively. Differences between two groups were considered significant at $*p < 0.05$ and $**p < 0.01$

Fig. 7. Total sum score of gene expression level of 30 drugs with different levels of risk for hepatotoxicity (WDN, withdrawn; BBW, black box warning; WNG, warning; SAFE, no warning, Table 4).

The "total sum score of gene expression level" was defined as an integrated score of the relative expression levels of NALP3 mRNA in K562 cells, and RAGE, S100A8, S100A9 and IL-1β mRNA in HL-60 cells incubated with and without HLMs. The expression level of hepatic mRNA was normalized to that of GAPDH mRNA. Drugs are ranked in descending order of total sum score of gene expression level. Red indicates WDN drugs, orange indicates BBW drugs, purple indicates WNG drugs, and black indicates SAFE drugs, as shown in Table 4.

Table 1. Primer sequences used for real-time RT-PCR analyses in the *in vivo* study.

Target		Sequence
CXCL1	F (5'-3')	GATTCACCTCAAGAACATCCAGAG
	R (5'-3')	GAAGCCAGCGTTCACCAGAC
FasL	F (5'-3')	AGAAGGAACTGGCAGAACTC
	R (5'-3')	GCGGTTCCATATGTGTCTTC
GATA-3	F (5'-3')	GGAGGACTTCCCCAAGAGCA
	R (5'-3')	CATGCTGGAAGGGTGGTGA
Granzyme B	F (5'-3')	TCGAGAGGACTTTGTGCTG
	R (5'-3')	CCTCTTGGCCTTACTCTTC
HMGB1	F (5'-3')	GGAGATCCTAAAAAGCCGAG
	R (5'-3')	ATAACGAGCCTTGTCAGCCT
HSP70	F (5'-3')	GGAGTTCAAGAGGAAGCACA
	R (5'-3')	TGGATGTGTAGAAGTCGATG
IFN-γ	F (5'-3')	TCAAGTGGCATAGATGTGGAAGAA
	R (5'-3')	TGGCTCTGCAGGATTTTCATG
IL-1β	F (5'-3')	GTTGACGGACCCCAAAAGAT
	R (5'-3')	CACACACCAGCAGGTTATCA
IL-6	F (5'-3')	CCATAGCTACCTGGAGTACA
	R (5'-3')	GGAAATTGGGGTAGGAAGGA
IL-12p35	F (5'-3')	TGCTGAAGACCACAGATGAC
	R (5'-3')	GAAGTCTCTCTAGTAGCCAG
MCP-1	F (5'-3')	TGTCATGCTTCTGGGCCTG
	R (5'-3')	CCTCTCTCTTGAGCTTGGTG
MIP-2	F (5'-3')	AAGTTTGCCTTGACCCTGAAG
	R (5'-3')	ATCAGGTACGATCCAGGCTTC
NALP3	F (5'-3')	AGCCTTCCAGGATCCTCTTC
	R (5'-3')	CTTGGGCAGCAGTTTCTTTC
Perforin	F (5'-3')	ACAGTAGAGTGTCGCATG
	R (5'-3')	ACAGCCGTGATAAAGTGC
RAGE	F (5'-3')	GAAACTTCTGATTCCCGATGG
	R (5'-3')	GCTCAACCAACAGCTGAATG
ROR-γt	F (5'-3')	ACCTCCACTGCCAGCTGTGTGCTGTC
	R (5'-3')	TCATTTCTGCACTTCTGCATGTAGACTGTCCC
STAT1	F (5'-3')	GTTTCAGCTCTGCTCCATAC
	R (5'-3')	CTGCTGAAGCTCGAACCAC
STAT3	F (5'-3')	TGCAGAGCAGGTATCTTGAG
	R (5'-3')	TGCTGCTTCTCTGTCACTAC

STAT6	F (5'-3')	ATCTTCAACGACAACAGCCTCA
	R (5'-3')	GGAGAAGGCTAGTGACATATTG
S100A8	F (5'-3')	GAGTGTCCTCAGTTTGTGCAG
	R (5'-3')	TAGACATATCCAGGGACCCAG
S100A9	F (5'-3')	GATGGCCAACAAAGCACCTT
	R (5'-3')	CCTCAAAGCTCAGCTGATTG
T-bet	F (5'-3')	TGCCCGAACTACAGTCACGAAC
	R (5'-3')	AGTGACCTCGCCTGGTGAAATG
TIM1	F (5'-3')	AGATTCCCACACGTCCTCCAA
	R (5'-3')	TGTCACCTCAGCTGTTGTCTC
TIM2	F (5'-3')	TACAAACCAGAGGCCACTAC
	R (5'-3')	AGATGCCAACATAGAAGCCC
TIM3	F (5'-3')	TTACCCTCAACTGTGTCCTG
	R (5'-3')	CATCAGTTCTGAGCAACTCG
TIM4	F (5'-3')	AAGGTCCAGTTTGGTGAAGTG
	R (5'-3')	GTCATGACTGTTGTTGGAAGC
TNFα	F (5'-3')	TGTCTCAGCCTCTTCTCATTCC
	R (5'-3')	TGAGGGTCTGGGCCATAGAAC
TLR4	F (5'-3')	TTCTTCTCCTGCCTGACACC
	R (5'-3')	CCATGCCATGCCTTGTCTTC
VCAM1	F (5'-3')	AGCCTCAACGGTACTTTGGA
	R (5'-3')	GCGTTTAGTGGGCTGTCTAT
Gapdh	F (5'-3')	AAA TGG GGT GAG GCC GGT
	R (5'-3')	ATT GCT GAC AAT CTT GAG TGA

F: Forward primer, R: Reverse primer.

Table 2. Primer sequences used for real-time RT–PCR analyses for the *in vitro* study.

Target		Sequence
Human NALP3	F (5'-3')	CCA CGC TAA TGA TCG ACT TC
	R (5'-3')	GTA AAC CCA TCC ACT CCT CT
Human RAGE	F (5'-3')	GGC TGG AAT GGA AAC TGA AC
	R (5'-3')	TGG TCT CCT TTC CAT TCC TG
Human S100A8	F (5'-3')	CTG GAG AAA GCC TTG AAC TC
	R (5'-3')	GAA TGA GGA ACT CCT GGA AG
Human S100A9	F (5'-3')	ACA GAG TGC AAG ACG ATG AC
	R (5'-3')	AGG TCC TCC ATG ATG TGT TC
Human IL-1β	F (5'-3')	GCTGATGGCCCTAAACAGATG
	R (5'-3')	TTCTCCTGGAAGGTCTGTGG
Human GAPDH	F (5'-3')	CCA TGA GGT CCA CCA CCC TGT T
	R (5'-3')	TGG GTG GCA GTG ATG GCA TGG A

F: Forward primer, R: Reverse primer.

Table 3. Inflammatory- and immune-related factors.

Type	Factors
Th cells-related factor	RORγt, T-bet, GATA3, STAT1, STAT3, STAT6
Cytokine	TNFα, IFN-γ, IL-1β, IL-6, IL-12
Chemokine	MIP-2, MCP-1, CXCL1, VCAM1
DAMP	S100A8, S100A9, HSP70, HMGB1
Receptor	TLR4, NALP3, RAGE
CD8 positive cells-related factor	Granzyme B, Perforin, FasL
TIM family	TIM1, TIM2, TIM3, TIM4

Table 4. Safety profiles of study drugs regarding drug-induced hepatotoxicity in humans.

	No.	Drug	Concentration (μM)	Daily dose (mg/day)
WDN	1	Aminopyrine	100	3,000
	2	Troglitazone	100	600
	3	Zomepirac	100	600
BBW	4	Carbamazepine	100	1,200
	5	Flutamide	100	750
	6	Ticlopidine	100	500
	7	Valproic acid	100	4,200
WNG	8	Acetaminophen	1,000	4,000
	9	Clopidogrel	100	75
	10	Diclofenac	100	200
	11	Erythromycin	100	1,000
	12	Furosemide	100	80
	13	Indomethacin	100	200
	14	Phenytoin	100	600
	15	Procainamide	100	4,000
	16	Sulfamethoxazole	100	1,600
	17	Tacrine	100	160
SAFE	18	Acetylsalicylic acid	100	300
	19	Caffeine	100	900
	20	Dexamethasone	100	20
	21	Ibuprofen	100	600
	22	Levofloxacin	100	750
	23	Losartan	100	100
	24	Olanzapine	100	20
	25	Warfarin	100	45
	26	Pioglitazone	100	80
	27	Pravastatin	100	8
	28	Rosiglitazone	100	400
	29	Theophylline	100	320
	30	Valsartan	100	10

WDN, withdrawn; BBW, black box warning; WNG, warning; SAFE, no warning.

Figure 1