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Letter

Utility of human hepatocyte spheroids without feeder cells for evaluation of hepatotoxicity

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ABSTRACT — We investigated the utility of three-dimensionally cultured hepatocytes (spheroids) without feeder cells (Sph(f-)) for the prediction of drug-induced liver injury (DILI) in humans. Sph(f-) and spheroids cultured on feeder cells (Sph(f+)) were exposed to the hepatotoxic drugs flutamide, diclofenac, isoniazid and chlorpromazine at various concentrations for 14 days, and albumin secretion and cumulative leakages of toxicity marker enzymes, aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and γ-glutamyl transpeptidase (γ-GTP), were measured. The cumulative AST, LDH or γ-GTP leakages from Sph(f-) were similar to or greater than those from Sph(f+) for all drugs tested, although ALT leakages showed no consistent difference between Sph(f+) and Sph(f-). In the case of Sph(f-), significant correlations among all the toxicity markers except for γ-GTP were observed. As regards the drug concentrations causing 1.2-fold elevation of enzyme leakage ($F_{1.2}$), no consistent difference between Sph(f+) and Sph(f-) was found, although several $F_{1.2}$ values were undetermined, especially in Sph(f+). The IC_{50} of albumin secretion and $F_{1.2}$ of AST leakage from Sph(f-) were equal to or lower than those of Sph(f+) for all the tested drugs. These results indicate that feeder cells might contribute to resistance to hepatotoxicity, suggesting DILI could be evaluated more accurately by using Sph(f-). We suggest that long-term exposure of Sph(f-) to drugs might be a versatile method to predict and reproduce clinical chronic toxicity, especially in response to repeated drug administration.

Key words: Hepatotoxicity, Human hepatocytes, Spheroid, Three-dimensional culture, Feeder cells

INTRODUCTION

Drug-induced liver injury (DILI) is a primary reason for withdrawal of candidate drugs at the clinical trial stage or of approved drugs from the market (Lasser *et al*., 2002). Thus, methods to predict DILI with high accuracy and sensitivity are required. Conventionally, *in vivo* studies using animals are performed, but this is not reliable, because DILI may be induced not only by intact parental drugs, but also by species-specific metabolites or intermediates (Olson *et al*., 2000). *In vitro* studies using primary-cultured human hepatocytes are also employed for evaluation of DILI as an alternative to *in vivo* animal studies. However, expression of several drug-metabolizing enzymes decreases during culture, leading to reduced metabolic potential (Guillouzo and Guguen-Guillouzo, 2008; Hewitt *et al*., 2007). Therefore, the applicability of conventional primary cultures of hepatocytes to evaluate long-term hepatotoxicity may be limited (Gómez-Lechón *et al*., 2001; Meng, 2010).

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We previously established that three-dimensionally (3D) cultured hepatocytes (hepatocyte spheroids) are suitable for long-term metabolic assays (Ohkura *et al*., 2014; Arakawa *et al*., 2017). These spheroids remained active for at least 21 days, exhibiting well-maintained secretion of albumin, a stable leakage level of aspartate aminotransferase (AST) and unchanged morphology. Using these hepatocyte spheroids, we examined sequential metabolic reactions of several drugs by Phase I and Phase II enzymes, and identified human-specific metabolites that had not previously been found in conventional hepatocyte culture systems. In addition, we suggested a protocol for assay of drug-metabolizing enzyme induction using these hepatocyte spheroids. Moreover, we evaluated the utility of these hepatocyte spheroids as an assay system for predicting DILI (Ogihara *et al*., 2015). Long-term exposure of spheroids to hepatotoxic drugs resulted in concentration-dependent reduction of albumin secretion and elevation of AST leakage. The estimated 50% inhibitory concentration (IC_{50}) values for decrease of albumin secretion changed from 7 days to 14 days, but similar values were obtained at 14 and 21 days. Since the values of drug concentration inducing 1.2-fold elevation ($F_{1.2}$) of AST leakage were also similar at 14 and 21 days, an incubation period of 14 days was considered sufficient. All the tested compounds, except for drugs inducing mitochondrial dysfunction (Crompton *et al*., 1988; McKenzie *et al*., 1995; Tujios and Fontana, 2011), showed a good correlation between IC_{50} for albumin secretion and $F_{1.2}$ for AST leakage.

On the other hand, other toxicity markers, such as alanine aminotransferase (ALT), lactate dehydrogenase (LDH) and γ-glutamyl transpeptidase (γ-GTP), were not measured in the previous study (Ogihara *et al*., 2015), because the feeder cells used as a support to enable the hepatocytes to adhere to the substrate plate interfered with the measurement of these enzymes. Therefore, we re-investigated the utility of the spheroids for prediction of hepatotoxicity in humans using a hepatocyte lot that showed high adhesive potential even in the absence of feeder cells. Spheroids without feeder cells (Sph(f-)) and those on feeder cells (Sph(f+)) were exposed to a panel of well-known hepatotoxic drugs, i.e., flutamide, diclofenac, isoniazid and chlorpromazine, at various concentrations for 14 days (the same period as in the previous study). Albumin secretion and leakages of AST, ALT, LDH and γ-GTP from Sph(f-) were compared with those from Sph(f+) and from feeder cells alone. The correlations between albumin secretion and cumulative leakages of the toxicity markers were also evaluated, and the IC_{50} values for albumin secretion and $F_{1.2}$ values for the toxicity markers in Sph(f+) and Sph(f-) were compared.

MATERIALS AND METHODS

Materials

Chlorpromazine, flutamide, isoniazid, Dulbecco's modified Eagle's medium (D-MEM) and penicillin (10,000 unit/mL)-streptomycin (10,000 μg/mL) were purchased from Sigma Aldrich (St. Louis, MO, USA). Cryopreserved human hepatocytes (Lot No. 228), Matrigel® (Cat. No.356237) and ISOM's medium were purchased from Becton Dickinson (Tokyo, Japan). A 3D culture system, Cell-able® with 96-well plates, and RM101 medium were purchased from Toyo Gosei (Tokyo, Japan). 3T3 Swiss Albino cells were purchased from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). All other reagents and solvents were commercial products of analytical grade.

3D culture of human hepatocytes

Sph(f+) and Sph(f-) were cultured for a maximum of 21 days after starting assays to assess their characteristics. The time schedules of the 3D cultures are shown in Fig. 1. In the case of Sph(f+), feeder cells (3T3 Swiss Albino cells) were cultured in D-MEM with penicillin (100 unit/mL)-streptomycin (100 μg/mL) and 10% fetal bovine serum (FBS) in humidified air containing 5% CO_2 at 37°C. Feeder cells were plated at a density of 8×10^3 cells/well on a 96-well Cell-able® at 5 days before starting the assay (Day -5). After 3 days, cryopreserved human hepatocytes were seeded. They were stored in liquid nitrogen until use, then immediately immersed in a water bath pre-warmed to 37°C. After dissolution, they were decanted into ISOM's medium, centrifuged at 50 × g for 5 min, and re-suspended in ISOM's medium containing 10% FBS. Cell viability was assessed by trypan blue exclusion, and only suspensions with a viability of over 80% were used. These were seeded at a density of 2.0×10^4 cells/well on the Cell-able® (Day -2). The next day, 80 μL/well of the culture medium was replaced with 80 μL/well of RM-101, and the hepatocytes were maintained for a day for spheroid formation before starting assays (Day -1). Medium was replaced with fresh RM-101 every 2 to 3 days (see Fig. 1). In the case of Sph(f-), human hepatocytes were seeded at a density of 2.0×10^4 cells/well on the Cell-able® (Day -2). The next day (Day -1), 80 μL/well of the culture medium was replaced with 80 μL/well of RM-101 and incubation was continued for a day. All media were replaced with fresh RM-101 containing 2.5% Matrigel® 2 days after hepatocyte seeding (Day 0). All media were further replaced with fresh RM-101 on the designated day, as shown in Fig. 1. RM-101 containing 2.5% Matrigel® was used at

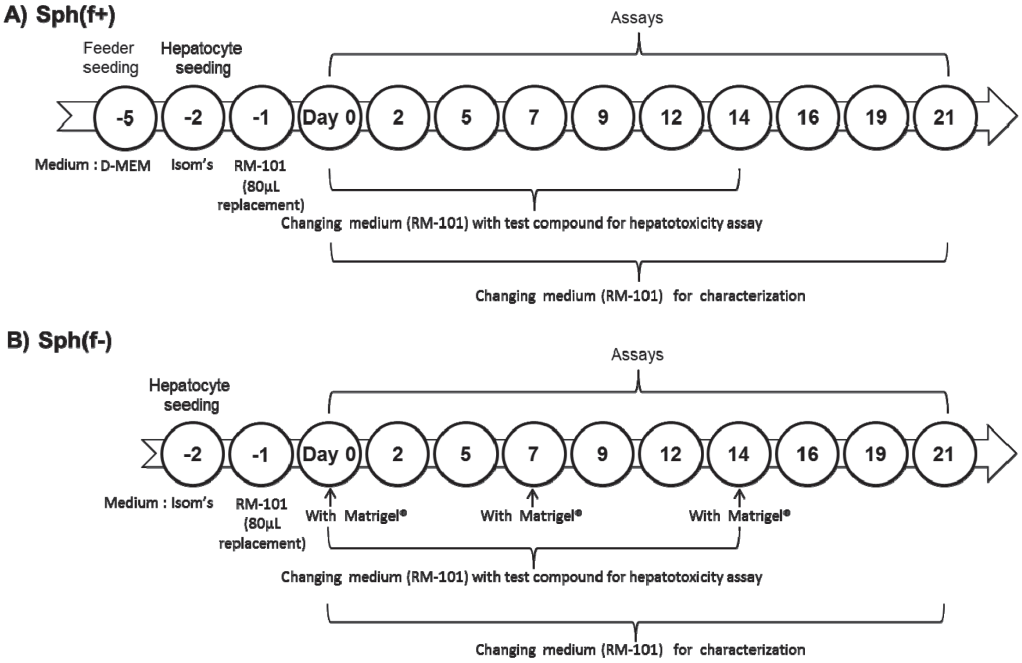

Fig. 1. Time schedules of toxicity evaluation using human hepatocyte spheroids without and with feeder cells, Sph(f-) and Sph(f+), respectively.

Day 7 and Day 14. Morphology was examined at each assay point. Moreover, the concentration of human albumin in the collected culture medium was measured using a Human Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., Montgomery, TX, USA) according to the manufacturer's protocol.

Evaluation of hepatotoxicity

Sph(f+) and Sph(f-) were cultured in RM-101 medium containing the test compounds for 14 days after starting the assays (the same period as in the previous study) (Ogihara *et al*., 2015). The concentration of the compound was set based on our previous paper using primary 3D-cultured hepatocytes with feeder cells (Ogihara *et al*., 2015). Therefore, the highest exposure dose of each test compound was set at a concentration 100 times higher than the maximum blood concentration at the clinical dose, and five exposure doses differing by a factor of three were used. The resulting test concentrations were: flutamide (1, 3, 10, 30, 100 μM) (Regenthal *et al*., 1999), diclofenac (3, 10, 30, 100, 300 μM) (Xu *et al*., 2008), isoniazid (10, 30, 100, 300, 1000 μM) (Xu *et al*., 2008), and chlorpromazine (0.3, 1, 3, 10, 30 μM) (Xu *et al*., 2008). Medium containing test compounds with 0.1% Dimethyl sulfoxide was changed periodically up to Day 12, as shown in Fig. 1. Supernatant was collected at each replacement time and kept at -80°C until measurement. Human albumin concentration in the collected culture medium was measured as mentioned above. The activities of AST, ALT, LDH and γ-GTP in the collected medium were measured using a Hitachi 7180 Automatic Analyzer system (Hitachi High-Technologies Corporation, Tokyo, Japan).

Data analysis

Albumin secretion levels from Sph(f+) and Sph(f-) were measured in triplicate or quadruplicate, and the results are presented as the mean ± S.E. All measurements were performed in duplicate in the case of hepatotoxicity evaluation, and data are shown as the mean or as individual points. The IC_{50} of drugs for reduction of albumin secretion was calculated using JMP® 10 software (SAS Institute Inc., Cary, NC, USA). The relationship between drug concentration and cumulative enzyme leakage was approximated by use of the exponent formula shown in equation 1, using EXCEL ® 2010 (Microsoft, WA, USA). The $F_{1.2}$ values of enzyme leakage were calculated according to equation 2:

$$L = L_0 \times \exp(E \times C) \quad (1)$$

$$F_{1.2} = \ln 1.2 / E \quad (2)$$

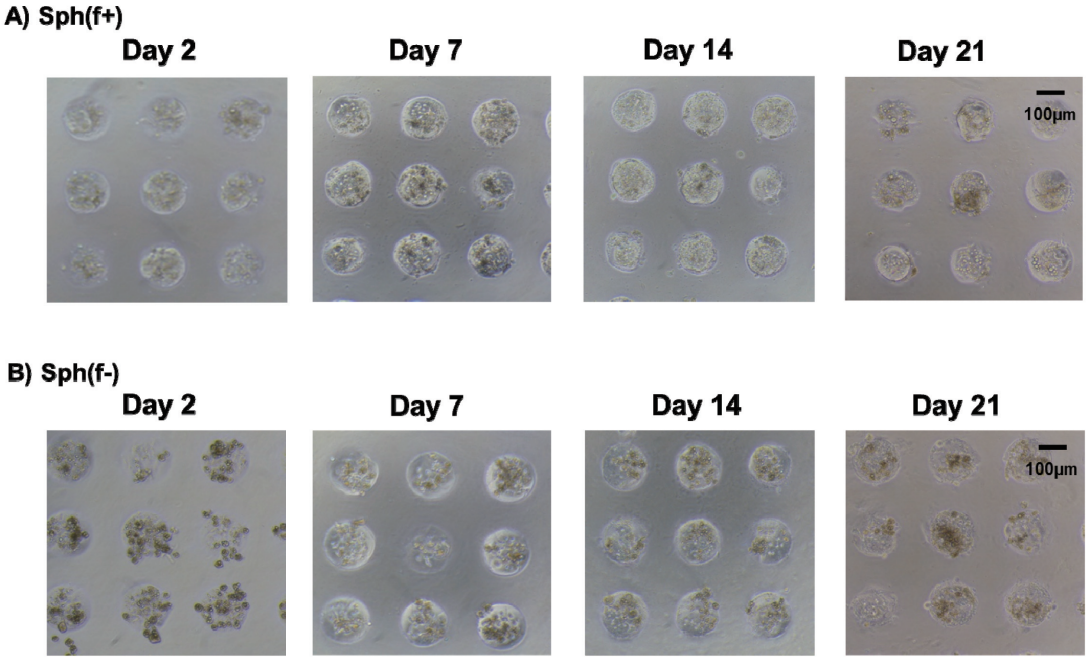

Fig. 2. Changes in the morphology of human hepatocyte spheroids during culture for 21 days.

where L is the amount of observed cumulative enzyme leakage, L_0 is the extrapolated enzyme leakage at 0 μM of test drug, E is an estimated exponential constant, and C is the drug concentration.

Pearson's correlation coefficient test was used for correlation analysis among five toxicity parameters, and values of $P < 0.05$ or $P < 0.01$ were considered significant.

RESULTS

Characteristics of human hepatocyte spheroids

The morphology of Sph(f-) was maintained for 21 days, as was that of Sph(f+), although adhesion of Sph(f-) in the early stages was weak (Fig. 2). Albumin secretion by Sph(f-) was lower than that of Sph(f+), though the pattern of change was similar for both. The secretion by both Sph(f-) and Sph(f+) increased significantly from 2 to 7 days after the start of assays, but was reduced at 14 days, and then remained stable from Day 14 to Day 21 (Fig. 3). No secretion of albumin from feeder cells alone was observed.

Detection of hepatotoxicity markers in spheroids

Spheroids were exposed to flutamide, diclofenac, isoniazid or chlorpromazine for 14 days. Typical morphology of spheroids exposed to flutamide is shown in

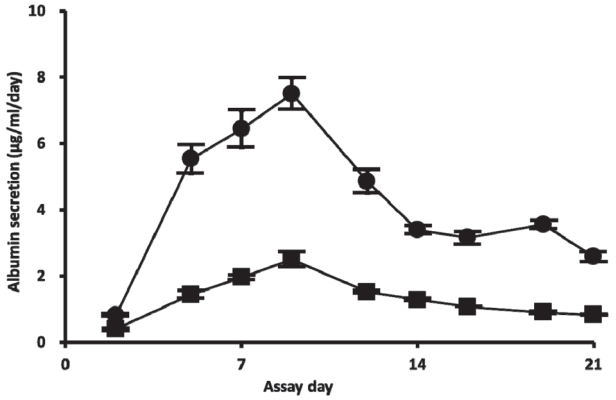

Fig. 3. Albumin secretion from human hepatocyte spheroids, Sph(f-) and Sph(f+). Closed circles and closed squares represent Sph(f+) and Sph(f-), respectively. Each plot indicates the mean ± S.D. (n = 3-4).

Fig. 4. Morphological changes such as cell death were observed at higher concentrations of flutamide, especially with Sph(f-). Cumulative enzyme leakages from Sph(f+), Sph(f-) and feeder cells exposed to hepatotoxic drugs were measured, and representative data for the second highest concentration of each compound are shown in Fig. 5. Cumulative AST leakage from Sph(f+) general-

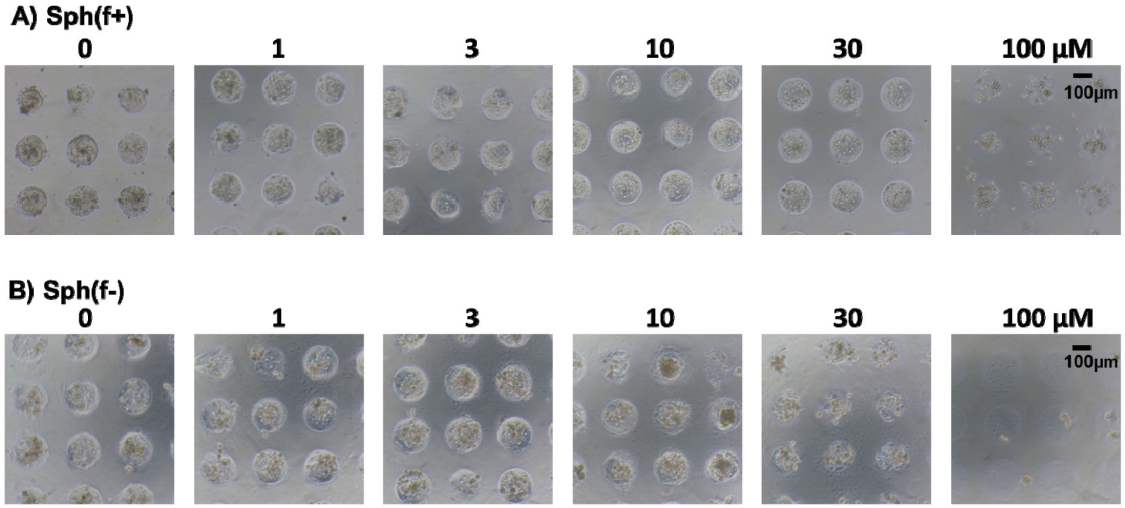

Fig. 4. Changes in the morphology of human hepatocyte spheroids, Sph(f-) and Sph(f+), during exposure to flutamide. Spheroids were treated with flutamide for 14 days.

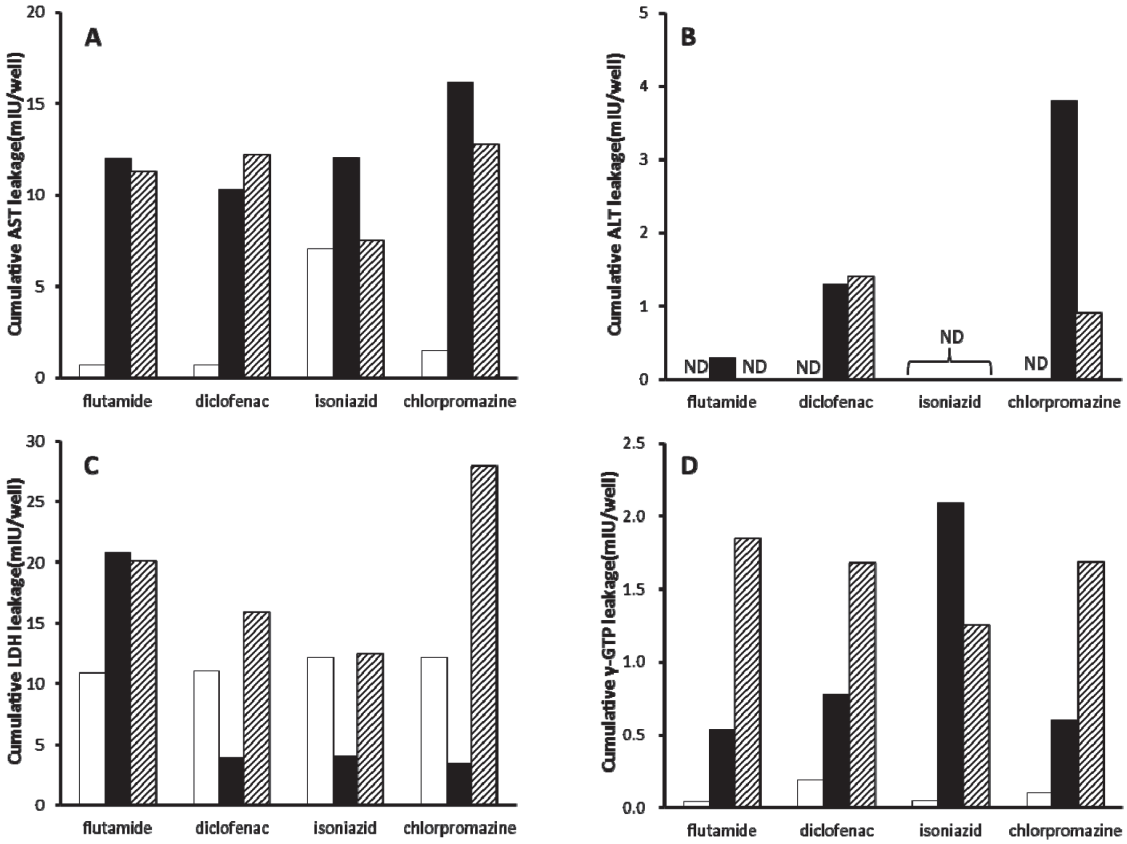

Fig. 5. Cumulative leakage of toxicity markers from human hepatocyte spheroids, Sph(f-) and Sph(f+), during exposure to test compounds. Spheroids were treated for 14 days with flutamide (30 μM), diclofenac (100 μM), isoniazid (300 μM), or chlorpromazine (10 μM). Representative results obtained at the second highest concentration of each compound are shown. Cumulative leakages of AST (A), ALT (B), LDH (C) and γ-GTP (D) as toxic markers are measured in feeder cells only (□), in Sph(f+) (■) and in Sph(f-) (▨). Each plot indicates the mean of two independent experiments.

ly corresponded well to the sum of that from Sph(f-) and that from feeder cells alone. However, cumulative ALT leakage from Sph(f-) showed no apparent relationship to that from Sph(f+); no ALT leakage from feeder cells was observed under these conditions. Cumulative LDH leakage from Sph(f-) was equal to or greater than that from Sph(f+), although leakage from feeder cells was also observed. Cumulative γ-GTP leakage from Sph(f-) was greater than that from Sph(f+), except in the case of isoniazid; slight γ-GTP leakage from feeder cells was seen. In addition, these phenomena were generally observed in other lower drug concentrations (Supplementary Fig. 1).

The correlations among the parameters obtained with Sph(f+) and Sph(f-) were examined. Typical examples and a summary of the correlation coefficients among toxicity parameters are shown in Fig. 6 and Table 1. In the case of Sph(f-), a high correlation was seen between leakages of AST and ALT or LDH (Fig. 6), while there was a moderate correlation between albumin secretion and leakage of AST or LDH. The correlations between γ-GTP and other markers were poor. On the other hand, in the case of Sph(f+), almost all correlations were weaker than those obtained with Sph(f-).

To calculate the IC_{50} values of albumin secretion and $F_{1.2}$ values of enzyme leakage, we examined the concentration dependence of cytotoxicity. The leakage from Sph(f+) was calculated as the apparent leakage from Sph(f+) minus the leakage from feeder cells. The case of flutamide is shown as an example in Fig. 7. The IC_{50} values of albumin secretion were calculated as 50.4 μM and 18.5 μM for Sph(f+) and Sph(f-), respectively. The $F_{1.2}$ values of AST leakage were calculated as 47.7 μM and 17.7 μM for Sph(f+) and Sph(f-), respectively. The results for all tested drugs are summarized in Table 2. The IC_{50} values of albumin secretion and $F_{1.2}$ values of AST for Sph(f-) were equal to or lower than those of Sph(f+) for

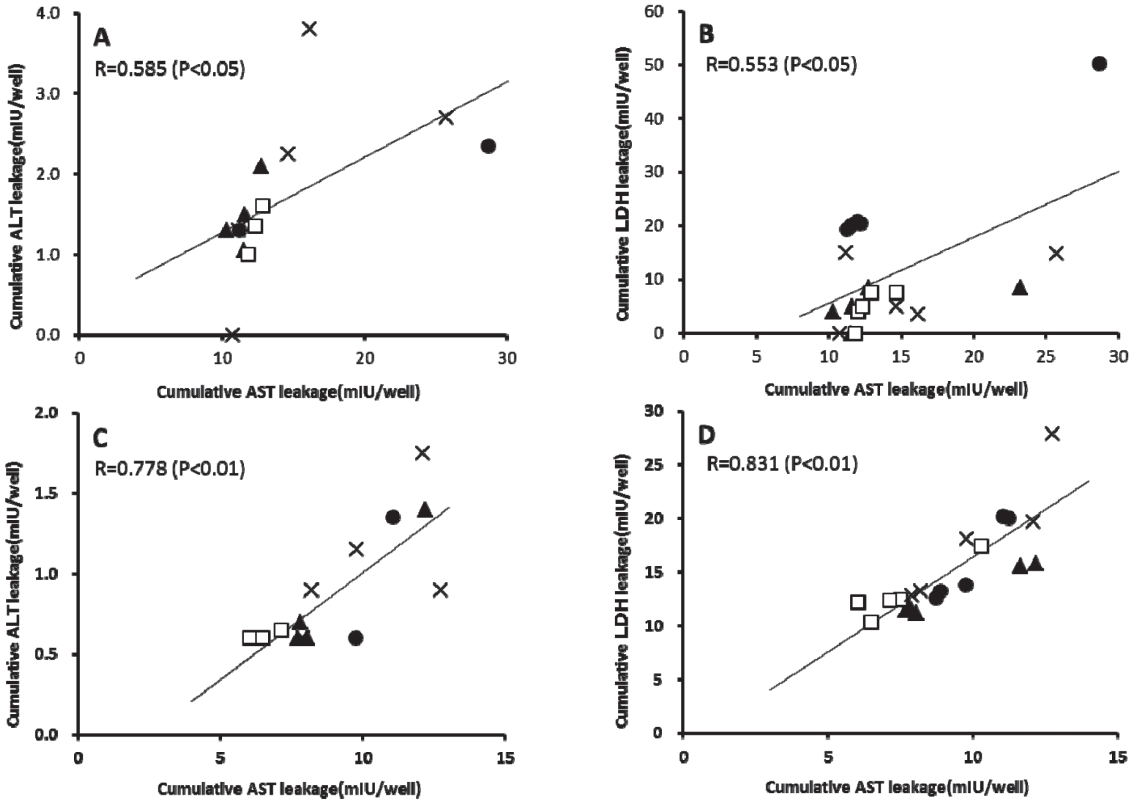

Fig. 6. Correlations between AST and ALT or LDH toxicity markers for the four test compounds in human hepatocyte spheroid assays. Correlation of cumulative leakages between AST and ALT (A and C) or AST and LDH (B and D) obtained with Sph(f+) (A and B), and Sph(f-) (C and D) are plotted for flutamide (●), diclofenac (▲), isoniazid (□) and chlorpromazine (×). The results obtained at all tested concentrations of each compound are shown. Each plot indicates the mean of two independent experiments.

Table 1. Correlation coefficients among toxicity markers in human hepatocyte spheroid assay of the 4 test compounds.

	AST	ALT	LDH	γ-GTP
Sph(f+)				
Albumin	0.823**	0.635*	0.279	0.201
AST		0.585*	0.553*	0.462*
ALT			0.285	0.353
LDH				0.437
Sph(f-)				
Albumin	0.712**	0.470*	0.672**	0.501*
AST		0.778**	0.831**	0.476*
ALT			0.593*	0.312
LDH				0.561*

Significant correlation (**P < 0.01, *P < 0.05)

Values are based on a corresponding data set made up of 20 samples (5 concentrations of each of the 4 compounds).

Table 2. IC_{50} values for albumin secretion and $F_{1.2}$ values for enzyme leakage of the 4 test compounds in human hepatocyte spheroid assays.

Compound	Flutamide		Diclofenac		Isoniazid		Chlorpromazine	
Marker	Sph(f+)	Sph(f-)	Sph(f+)	Sph(f-)	Sph(f+)	Sph(f-)	Sph(f+)	Sph(f-)
Albumin (IC_{50} μM)	50.4	18.5	56.7	24.9	140.2	122.5	16.9	7.7
AST ($F_{1.2}$ μM)	47.7	17.7	138.5	115.3	-	378.4	5.2	8.9
ALT ($F_{1.2}$ μM)	29.4	38.8	130.9	12.2	-	-	1.3	0.5
LDH ($F_{1.2}$ μM)	17.7	11.6	213.8	179.8	-	472.5	3.2	11.8
γ-GTP ($F_{1.2}$ μM)	-	10.5	21.1	32.9	-	1322	-	7.7

Values are based on a corresponding data set made up of 10 samples (duplicate of each of 5 concentrations).

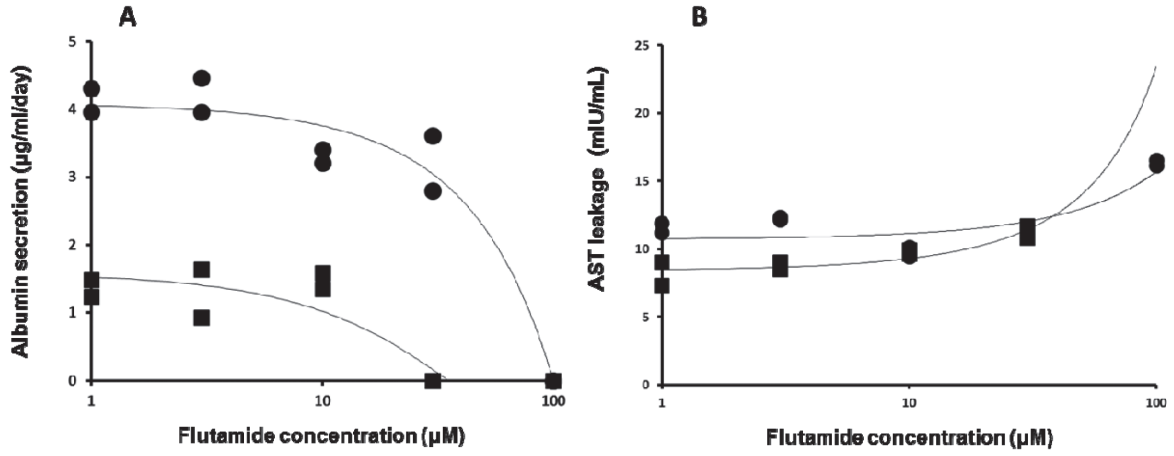

Fig. 7. Albumin secretion (A) and cumulative leakage of AST (B) from human hepatocyte spheroids, Sph(f-) and Sph(f+), during exposure to flutamide for 14 days. Closed circles and closed squares represent Sph(f+) and Sph(f-), respectively. Each plot indicates the individual data (n = 2).

all tested drugs. The $F_{1.2}$ values of leakage enzymes other than AST did not show any consistent difference between Sph(f+) and Sph(f-), although several parameters were not determined, especially for Sph(f+).

DISCUSSION

In this study, we evaluated the utility of Sph(f-) as an assay system for predicting DILI. Sph(f-) remained active for at least 21 days as indicated by the unchanged

morphology and continued secretion of albumin, which showed similar changes in Sph(f+) and Sph(f-) (Figs. 2 and 3), in accordance with our previous findings in Sph(f+) (Ogihara *et al*., 2015). In addition, albumin secretion from Sph(f+) without inducers were always higher than that of Sph(f-) as shown in Fig. 2. This phenomenon might be indicated that feeder cells involved maintaining hepatocyte cell viability.

When Sph(f+) and Sph(f-) were exposed to several hepatotoxic drugs for 14 days, cumulative AST, LDH or γ-GTP leakage from Sph(f-) was equal to or greater than that from Sph(f+) in general. However, leakage of these markers from feeder cells was also observed, and therefore these results suggest that the feeder cells might contribute to resistance to hepatocyte toxicity, at least in some cases; this in turn implies that DILI could not be evaluated accurately with Sph(f+). Amounts of cumulative leakage of marker enzymes except γ-GTP in Sph(f-) showed good or moderate correlations with each other. However, the correlations among markers in Sph(f+) were poor, indicating that Sph(f+) would be less reliable than Sph(f-) for toxicity evaluation. The reason why γ-GTP shows a poor correlation with other hepatocyte toxicity markers may be that commercially available hepatocytes contain bile duct cells, and γ-GTP is also a toxicity marker for the bile duct. Therefore, it seems possible that this system using Sph(f-) could simultaneously evaluate bile duct toxicity, independently of hepatotoxicity.

We previously suggested that albumin secretion and AST leakage were available as toxicity markers for evaluation of DILI using Sph(f+) (Ogihara *et al*., 2015), and that the $F_{1.2}$ value of AST leakage as drug concentration would be a suitable criterion of toxicity. Most of the tested compounds, except drugs that cause mitochondrial dysfunction (Crompton *et al*., 1988; McKenzie *et al*., 1995; Tujios and Fontana, 2011), showed good correlations between the IC_{50} value of albumin secretion and $F_{1.2}$ value of AST leakage. The findings of the present study confirmed a good correlation between these parameters in Sph(f+), and in addition, we found that the $F_{1.2}$ values of other leakage marker enzymes from Sph(f-) were also available, except for γ-GTP (Table 1). Our results also indicate that the Sph(f-) system is more sensitive, based on the morphological changes observed in the presence of flutamide (Fig. 4).

Several *in vitro* studies using primary-cultured human hepatocytes or human immortalized cells have been reported for evaluating DILI, using flutamide (Gerets *et al*., 2012), diclofenac (Bort *et al*., 1999), isoniazid (Wang *et al*., 2002) or chlorpromazine (Gerets *et al*., 2012) as model drugs. These studies all utilized the IC_{50} value of albumin secretion as a toxicity parameter, and all of them yielded values similar to or greater than our IC_{50} value. It is particularly noteworthy that the IC_{50} of isoniazid was 122.5 μM in our present study, whereas it was reported to be more than 10 mM using human immortalized cells, HepG2 (Wang *et al*., 2002). The maximum plasma concentration of isoniazid in the clinical context is reported to be 76.6 μM (Xu *et al*., 2008), and the protein binding rate of the drug is relatively low (0.08 mol/mol of protein, according to the drug information in the package insert) suggesting that the effective unbound fraction might be 70 μM at maximum, which is close to our estimated IC_{50} value. Therefore, our system might be more effective than conventional *in vitro* studies for predicting DILI in the clinical context (Gómez-Lechón *et al*., 2001; Meng, 2010).

The toxic markers were selected based on actual clinical use in this study, that is, IC_{50} value of albumin secretion and $F_{1.2}$ value of enzyme leakages were performed. Although MTT assay and IC_{50} values of enzyme leakages are commonly used to evaluate cytotoxicity, these assays cannot be measured continuously during the clinical phase. One of the advantages of this system is that it can measure continuously and simultaneously the toxic markers using live cells over a period of time.

Various studies using 3D cultured hepatocytes or cell lines have been reported. Hepatocyte sandwich culture, which helps to develop the polarity of hepatocytes, is used to detect hepatobiliary transport and cholestatic injury (Xu *et al*., 2008). Organ-on-a-chip platforms can imitate the hemodynamics of the *in vivo* liver by perfusion, and generate efficient nutrient exchange and shear stress at the *in vitro* setting (Domansky *et al*., 2010). On the other hand, such 3D-culture methods are difficult to operate, leading to their being unsuitable for large-scale screening of new chemical compounds (Lauschke *et al*., 2016). Manipulating hepatocytes in the Cell-able® is rather simple and fulfills the terms of high throughput screening for exclusion of hepatotoxins. In the present study, we established the Sph(f-) which can evaluate a wide range of hepatotoxic markers compared with Sph(f+), and could expand the versatility of the Cell-able® system. Although feeder cells could involve maintaining hepatocyte cell viability, the ability of Sph(f-) to perform multiple studies in one system make it potentially superior to that of other methods.

In conclusion, our results indicate that the present Sph(f-) system with long-term drug exposure could be superior to other available methods, including the use of Sph(f+), for versatile and sensitive prediction and reproduction of clinical chronic hepatotoxicity, especially as

observed in the repeated administration of drugs.

However, it should be noted that this study was performed using a single lot of hepatocytes; since validation requires the use of at least three different lots of hepatocytes, we are currently conducting additional studies.

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Conflict of interest---- Tomoko Jomura is an employee of Toyo Gosei Co., Ltd. The other authors have no potential conflict of interest.

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