Regular Article

Preliminary Evaluation of Three-Dimensional Primary Human Hepatocyte Culture System for Assay of Drug-Metabolizing Enzyme-Inducing Potential

Hiroshi Arakawa,[a,b] Hiroki Kamioka,[a] Tomoko Jomura,[c] Satoshi Koyama,[a] Yoko Idota,[a] Kentaro Yano,[a] Hajime Kojima,[d] and Takuo Ogihara*[,a,e]

[a] *Laboratory of Biopharmaceutics, Department of Pharmacology, Faculty of Pharmacy, Takasaki University of Health and Welfare; 60 Nakaorui-machi Takasaki, Gunma 370–0033, Japan:* [b] *Faculty of Pharmacy, Institute of Medical, Pharmaceutical and Health Sciences, Kanazawa University; Kakuma-machi, Kanazawa, Ishikawa 920–1192, Japan:* [c] *Biotech Application Group Research and Development, Toyo Gosei Co., Ltd.; 4–2–1 Wakahagi, Inzai, Chiba 270–1609, Japan:* [d] *Division of Risk Assessment, Biological Safety Research Center, National Institute of Health Sciences; 1–18–1 Kamiyoga, Setagaya-ku, Tokyo 158–8501, Japan: and* [e] *Laboratory of Clinical Pharmacokinetics, Graduate School of Pharmaceutical Sciences, Takasaki University of Health and Welfare; 60 Nakaorui-machi Takasaki, Gunma 370–0033, Japan.*

Received November 15, 2016; accepted April 12, 2017

Drug-induced liver injury (DILI) is a common reason for withdrawal of candidate drugs from clinical trials, or of approved drugs from the market. DILI may be induced not only by intact parental drugs, but also by metabolites or intermediates, and therefore should be evaluated in the enzyme-induced state. Here, we present a protocol for assay of drug-metabolizing enzyme-inducing potential using three-dimensional (3D) primary cultures of human hepatocytes (hepatocyte spheroids). Hepatocyte spheroids could be used up to 21 d after seeding (pre-culture for 7 d and exposure to inducer for up to 14 d), based on preliminary evaluation of basal activities of CYP subtypes and mRNA expression of the corresponding transcription factor and xenobiotic receptors (aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and pregnane X receptor (PXR)). After 2 d exposure of hepatocyte spheroids to omeprazole, phenobarbital and rifampicin (typical inducers of CYP1A2, 2B6 and 3A4, respectively), CYP1A2, 2B6 and 3A4 mRNA expression levels were significantly increased. The mRNA induction of CYP2B6 remained reasonably stable between days 2 and 14 of exposure to inducers, while induction of both CYP1A2 and 3A4 continued to increase up to day 14. These enzyme activities were all significantly increased compared with the control until day 14. Our findings indicate that our 3D hepatocyte spheroids system would be especially suitable for long-term testing of enzyme activity induction by drugs, either to predict or to verify clinical events.

Key words three-dimensional culture; primary human hepatocyte; drug-metabolizing enzyme; induction; spheroid; drug-induced liver injury

Drug-induced liver injury (DILI) is a common reason for withdrawal of candidate drugs from clinical trial or of approved drugs from the market.[1)] Thus, it is extremely important to detect DILI at as early a stage as possible in the drug development process. On the other hand, induction of drug-metabolizing enzymes may lead to therapeutic failure due to decreased blood concentrations of the concomitant drugs, and is a potentially serious clinical problem.[2–4)] Because DILI may be induced not only by intact parental drugs, but also by metabolites or intermediates,[5,6)] the risk of DILI increases when metabolic enzymes are induced by concomitant drugs, and by drugs with self-inducing potential. Therefore, as a part of the testing process, it is necessary to examine DILI under enzyme-induced conditions.

The enzyme-inducing potential of test compounds is often evaluated using human hepatocytes, such as primary cryopreserved hepatocytes or human-derived HepaRG cells, because the induction of CYP subtypes shows species differences.[7–10)] In addition, two-dimensional (2D) monoculture of hepatocytes can be performed to evaluate CYP induction. Recently, the European Center for the Validation of Alternative Methods (ECVAM) submitted its test draft guidelines for CYP induction assays of chemical materials using 2D monoculture of HepaRG cells (human primary cryopreserved hepatocytes are also permitted) to the Organisation for Economic Co-operation and Development (OECD).[11,12)]

The method described in the ECVAM report is intended as a step towards international standardization. However, this method has several disadvantages. Firstly, 2D cultures of hepatocytes have significant limitations in terms of the expression profiles of drug-metabolizing enzymes and the maximum culture period,[13)] and they are not suitable for long-term exposure studies of drug candidates. On the other hand, all guidelines by health authorities in Europe,[14)] the U.S.[15)] and Japan[16)] for drug-metabolizing enzyme induction by medicines currently require measurement of mRNA expression changes of CYP1A2, 2B6 and 3A4 in *in vitro* assays. Such guidelines are inconsistent with ECVAM's test draft guideline, which requires only enzyme activity measurements. Moreover, CYP induction is caused by activation of the corresponding transcription factor and xenobiotic receptors; for example, CYP1A2, 2B6 and 3A4 are induced by activation of aryl hydrocarbon receptor (AhR, *AHR*),[17)] constitutive androstane receptor (CAR, *NR1I3*)[18)] and pregnane X receptor (PXR, *NR1I2*),[7,19)] respectively. Thus, mRNA expression assays of these genes should preferably be included as part of a CYP

* To whom correspondence should be addressed. e-mail: togihara@takasaki-u.ac.jp

induction study.

The use of three-dimensional (3D) cultures of human hepatocytes (hepatocyte spheroids), in which liver-specific functions are better maintained, has been proposed[20,21] instead of 2D culture systems. We recently showed that hepatocyte spheroids are suitable for long-term metabolic assays,[22] where they remained active for at least 21 d. They exhibited consistent 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, identifying human-specific metabolites previously unseen in conventional hepatocyte culture systems. This 3D culture system could detect DILI with high accuracy and sensitivity compared with conventional methods, including 2D culture systems.[23] Spheroids exposed to hepatotoxic drugs over longer periods result in concentration-dependent albumin secretion reduction and an increase in cumulative AST leakage. The estimated 50% effective concentration (IC_{50}) values for decrease of albumin secretion and 1.2-fold elevation ($F_{1.2}$) of AST leakage changed from 7 to 14 d, but similar values were obtained at 14 and 21 d. All tested compounds, except for those drugs which induced mitochondrial dysfunction, showed a strong correlation between IC_{50} for albumin secretion and $F_{1.2}$ for AST leakage. Further, investigation of both toxicity and metabolic enzyme activities in the same system has rarely been reported. Therefore, we believe that hepatocyte spheroids would be more effective than conventional culture systems for tracing of metabolic processes and predicting DILI under enzyme-induced conditions.

In the present study, we aimed to establish the utility of the 3D primary hepatocyte culture system for *in vitro* assay of drug-metabolizing enzyme-inducing potential, using our previously established method with minor modifications. Firstly, we compared the property of hepatocytes in 2D and 3D cultures by measuring the activities of CYP1A2, 2B6 and 3A4, and the mRNA expression levels of AhR, CAR and PXR. Then, we compared the utility of these systems for predicting CYP-inducing potential by measuring the mRNA expression and activities of the three CYPs within the survival period of the hepatocytes in the presence of typical inducers.

MATERIALS AND METHODS

Materials Acetaminophen, bupropion and midazolam were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Omeprazole, phenobarbital and phenacetin were purchased from Tokyo Chemical Industry (Tokyo, Japan). Hydroxybupropion and 1′-hydroxymidazolam were purchased from Cayman Chemical Company (Ann Arbor, MI, U.S.A.) and Cerilliant Corporation (Round Rock, TX, U.S.A.), respectively. Cryopreserved human hepatocytes (lot No. Hu8110), cryopreserved Hepatocytes Recovery Medium (CHRM®) and Hepatocyte Plating Supplement Pack (CM3000) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). William's Medium E was purchased from Invitrogen (Durham, NC, U.S.A.). A 3D culture system, Cell-able™ 96-well and 24-well plates, and RM101 medium were donated by Toyo Gosei Co., Ltd. (Chiba, 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.

Culture of Human Hepatocytes For 3D culture of hepatocytes, feeder cells (3T3-Swiss Albino cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Ltd.) with penicillin (100 μg/mL, Nacalai Tesque, Kyoto, Japan), streptomycin (100 μg/mL, Wako Pure Chemical Industries, Ltd.) and 10% fetal bovine serum (Invitrogen). The cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. Feeder cells were plated at a density of 8.0×10^3 cells/well on 96-well format Cell-able™. After 2 d, cryopreserved human hepatocytes were seeded. They were stored in liquid nitrogen until use, and immediately immersed in water pre-warmed to 37°C. After dissolution of the hepatocytes, they were decanted into CHRM® and centrifuged at 100×***g*** for 10 min, then re-suspended in William's Medium E containing CM3000. Viability was assessed by trypan blue exclusion, and suspensions with viability of over 90% were used. They were seeded at a density of 2.0×10^4 cells/well on the feeder cells and maintained using RM101 medium before the assays. The medium was changed three times per week. For 2D culture, hepatocytes were seeded at 5.0×10^4 cells/well on a collagen I-coated 96-well plate and maintained using RM101, which was changed three times per week before the assays.

Total RNA was extracted from human hepatocytes at 9, 14 and 21 d in 3D culture, and at 2 and 7 d after seeding in 2D culture, as well as from non-cultured hepatocytes. The mRNA expression levels of AhR, CAR and PXR were measured by quantitative RT-PCR (qRT-PCR), using THUNDERBIRD SYBR qPCR Mix (Toyobo, Osaka, Japan). Since hypoxanthine guanine phosphoribosyl-transferase 1 (HPRT1) mRNA exhibited stable expression in human hepatocytes, the mRNA expression of target genes was normalized to HPRT1, and was calculated relative to that of the cryopreserved hepatocytes (set to 100%) using the 2-ΔΔ threshold cycles (Ct) method.[24] The gene-specific primer sequences are listed in Table 1.

To measure metabolic activities of cultured hepatocytes,

Table 1. Specific Primers for Xenobiotic Factors and Metabolic Enzymes

Gene	Forward (5′–3′)	Reverse (5′–3′)
AhR	TCATCTGGTTTTCTGG	ATGGATGGTGGCTGAA
CAR	CCTCTGGTCACACACT	TCAATGGGCAGGGAAC
PXR	CAGTGGGAATCTCGGC	CTCTTGGACTGCTTGG
CYP1A2	GAATGGCTTCTACATCCCCA	TCATCTTCTCACTCAAGGGCT
CYP2B6	ACATCATCCCCAAGGACACAGA	GCATCCAGAAAGTGGTCAGG
CYP3A4	TATGGAAAAGTGTGGGGCTT	TCCGGTTTGTGAAGACAGAAT
HPRT1	AAGATGGTCAAGGTCGCAAG	TCAAATCCAACAAAGTCTGGC

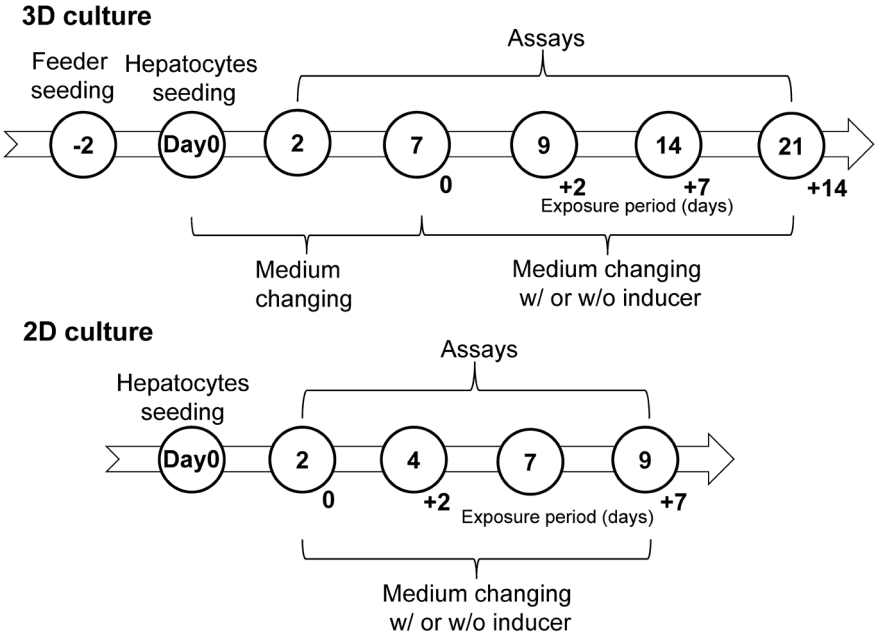

Fig. 1. Time Schedules of CYP Induction Assay Using 2D and 3D Hepatocytes

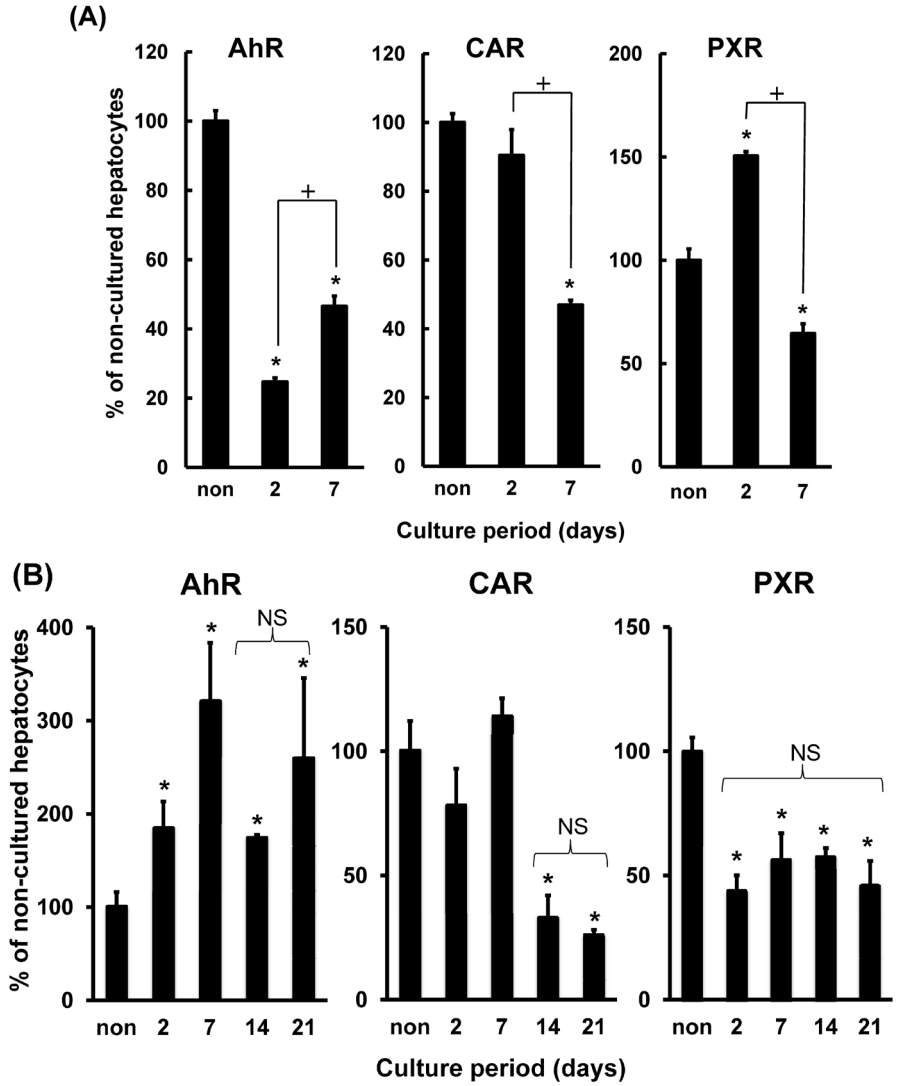

Fig. 2. mRNA Expression Levels of AhR, CAR and PXR in 2D- and 3D-Cultured Human Hepatocytes

The expression levels of AhR, CAR, PXR mRNAs in 2D-cultured (A) and 3D-cultured (B) human hepatocytes were measured by the qRT-PCR method. Data represent the mean±S.E.M. (n=4) as % of the values in non-cultured human hepatocytes. * or $^{+}$ indicates significant differences from non-cultured or 2 d-cultured hepatocytes (p<0.05 by Tukey's test), respectively. NS: not significant.

the medium was aspirated and replaced with William's medium E (pH 7.4) containing phenacetin (40 μM), bupropion (40 μM) or midazolam (10 μM) at 9, 14 and 21 d after seeding in 3D culture (2, 7, 14 d after formation of spheroids) and at 2 and 7 d after seeding in 2D culture. Metabolic activities of non-cultured hepatocytes, which were suspended at a density of 2×10^4 cells in William's medium E at pH 7.4 containing phenacetin, bupropion or midazolam, were also measured. After 60 min, the drug-containing medium was collected, and acetonitrile was added. For quantitation, each mixture was centrifuged at 16000×***g*** and 4°C for 5 min and filtered through a 0.45-μm membrane filter. The supernatant was analyzed with a liquid chromatograph mass spectrometer (LC-MS/MS).

Induction of Metabolic Enzymes The culture schedules are shown in Fig. 1. Seven days after seeding of cryopreserved human hepatocytes, the formatted spheroids were exposed to omeprazole (100 μM), phenobarbital (1 mM) or rifampicin (10 μM) as CYP inducers in RM101 medium containing 0.1% dimethyl sulfoxide (DMSO) for 2, 7 and 14 d. For 2D culture, 2 d after seeding of the hepatocytes, the cells were exposed to the same conditions as the 3D culture. The drug-containing medium was changed three times per week for both 2D and 3D cultures. On the designated day, total RNA was extracted, and the mRNA expression levels of CYP1A2, CYP2B6 and CYP3A4 were measured by qRT-PCR as outlined above (Table 1). We confirmed in advance that mRNA expression of CYP1A2, CYP2B6 or CYP3A4 and activities of acetaminophen deethylation, bupropion hydroxylation or midazolam hydroxylation were not detected in feeder cells. To calculate metabolic activities, the metabolites of phenacetin *O*-demethylation by CYP1A2, bupropion hydroxylation by CYP2B6, and midazolam hydroxylation by CYP3A4/5 were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). Since time linearity of metabolite production was confirmed for 60 min, specific metabolic potential (pmol/min/10^6) was calculated by dividing the amount of metabolite (pmol) by incubation time (min) and the number of viable hepatocytes (10^6 cells). Moreover, 2D and 3D human

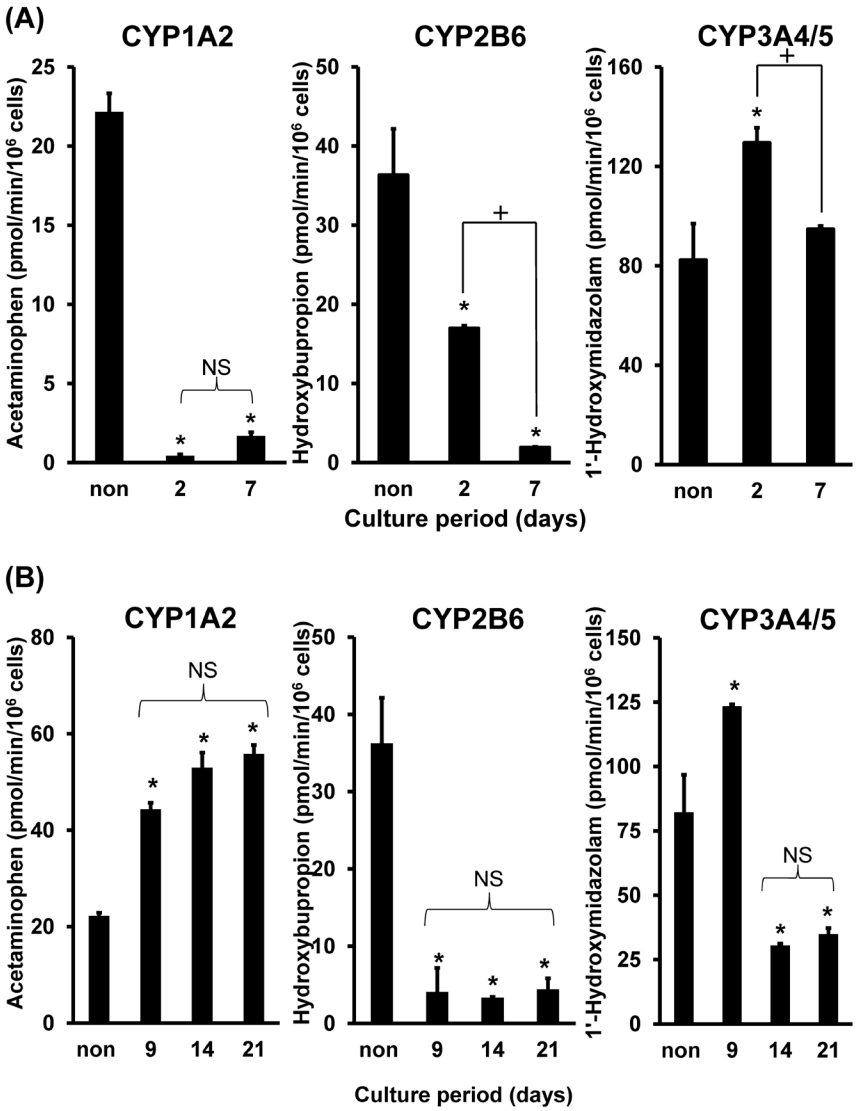

Fig. 3. Basal Activities of CYP1A2, 2B6 and 3A4/5 in 2D- and 3D-Cultured Human Hepatocytes

The activities of CYP1A2, 2B6 and 3A4/5 in non-cultured, 2D-cultured (A) and 3D-cultured (B) human hepatocytes were measured in terms of formation of acetaminophen, hydroxybupropion and 1′-hydroxymidazolam, respectively. 2D- and 3D-cultured human hepatocytes were cultivated for 2 and 7 d, and for 9, 14 and 21 d, respectively. Data represents the mean±S.E.M. (n=4). * indicates significant differences from non-cultured hepatocytes ($p<0.05$ by Dunnett's test). $^+$ indicates significant differences between 2 and 7 d of culture period ($p<0.05$ by Tukey's test). NS: not significant.

hepatocytes were cultured in RM101 medium containing 0.1% DMSO without CYP inducers for identical periods, and the mRNA expression levels and metabolic activities of CYPs were measured by the methods mentioned above.

LC-MS/MS Analysis The amounts of metabolites were determined with a LCMS-8040 triple quadrupole LC-MS/MS (Shimadzu, Kyoto, Japan) coupled to an LC-20A system (Shimadzu). The mass numbers of the molecular and product ions for each compound were as follows: acetaminophen (152.0→110.1), hydroxybupropion (256.0→238.0), 1′-hydroxymidazolam (342.0→324.0). Labsolutions software version 5.75 SP2 (Shimadzu) was used for data manipulation. The detection limit was 10 nM for each compound.

Data Analysis All data were presented as mean±standard error of the mean (S.E.M.) (n=4), except mRNA expression of AhR, CAR and PXR in non-cultured hepatocytes (n=1). The mRNA expression levels of them in cultured hepatocytes were presented as a percentage of those in non-cultured hepatocytes. The mRNA expression levels and metabolic activities of CYPs under CYP-induced conditions were presented as the fold induction *versus* non-CYP-induced conditions. Statistical analysis was undertaken using a two-sided Student's t-test or Tukey's test. Values of $p<0.05$ were considered significant.

RESULTS

Basal Activities of 2D- and 3D-Cultured Human Hepatocytes Expression of AhR mRNA in 3D-cultured hepatocytes tended to be higher than that in non-cultured hepatocytes, while expression of CAR and PXR mRNAs in 3D-cultured hepatocytes tended to be lower than in non-cultured hepatocytes (Fig. 2). Expressions of these genes in spheroids were stable at least after 14 d after seeding, while those in 2D culture were unstable between days 2 and 7. Moreover, as shown in Fig. 3, basal activities of CYP1A2, 2B6 and 3A4/5 in 2D and 3D cultured hepatocytes were measured in comparison with those of non-cultured hepatocytes. CYP1A2-mediated phenacetin O-deethylation activity was

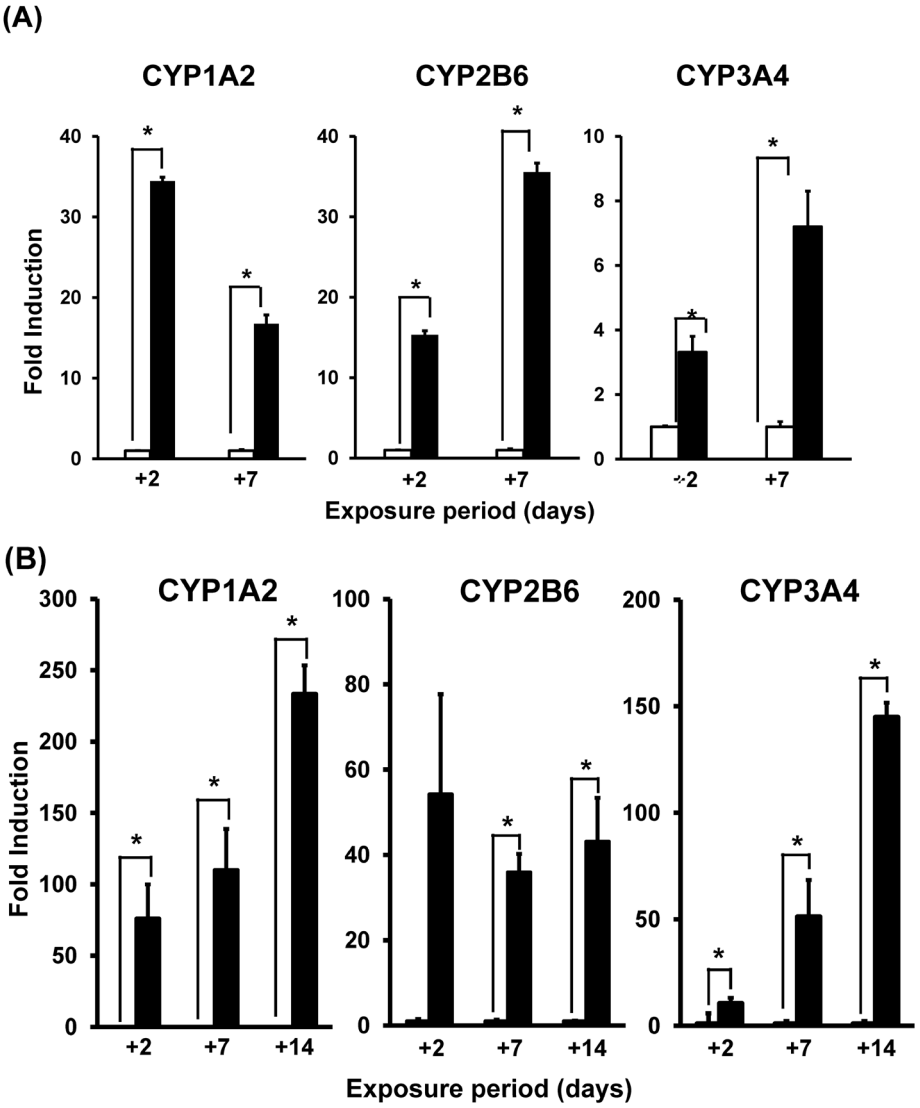

Fig. 4. Fold Induction of mRNA of CYP1A2, 2B6 and 3A4 in 2D- and 3D-Cultured Hepatocytes

(A) In the case of 2D culture, two days after seeding, human hepatocytes were exposed to 100 μM omeprazole (CYP1A2), 1 mM phenobarbital (CYP2B6) or 10 μM rifampicin (CYP3A4/5), or DMSO (0.1%) as the vehicle control. (B) After formation of 3D structure at day 7, 3D-cultured hepatocytes were exposed to 100 μM omeprazole (CYP1A2), 1 mM phenobarbital (CYP2B6) or 10 μM rifampicin (CYP3A4/5), or DMSO (0.1%) as the vehicle control. The bars represent mRNA expression of each CYP in cells exposed to 0.1% DMSO or drug (omeprazole, phenobarbital or rifampicin), respectively. Data represents the mean±S.E.M. (n=4) as fold induction *vs.* the control. * indicates significant differences from the control ($p<0.05$ by Student's t-test).

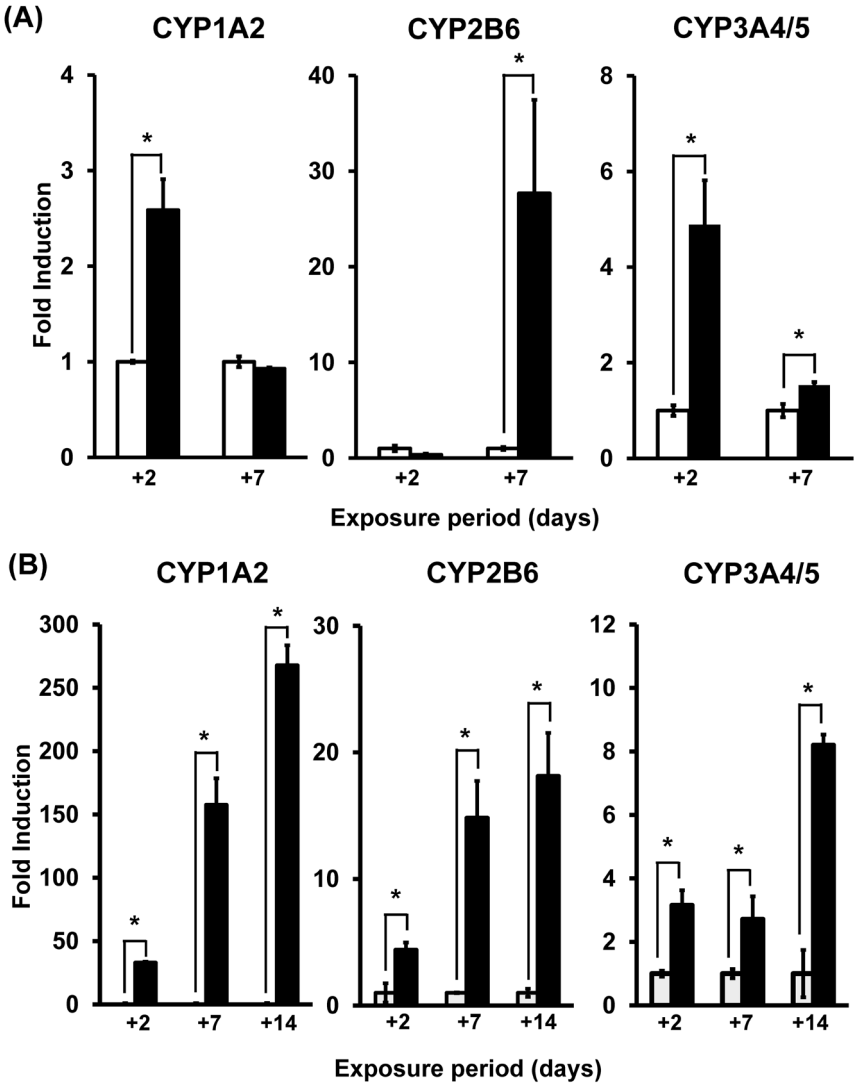

Fig. 5. Fold Induction of Metabolic Activity of CYP1A2, 2B6 and 3A4/5 in 2D- and 3D-Cultured Hepatocytes

(A) In the case of 2D culture, two days after seeding, human hepatocytes were exposed to 100 μM omeprazole (CYP1A2), 1 mM phenobarbital (CYP2B6) or 10 μM rifampicin (CYP3A4/5), or DMSO (0.1%) as the vehicle control. (B) After formation of 3D structure at day 7, 3D-cultured hepatocytes were exposed to 100 μM omeprazole (CYP1A2), 1 mM phenobarbital (CYP2B6) or 10 μM rifampicin (CYP3A4/5), or DMSO (0.1%) as the vehicle control. The activities of CYP1A2, 2B6 and 3A4/5 in non-cultured, 2D- and 3D-cultured human hepatocytes were measured in terms of formation of acetaminophen, hydroxybupropion and 1′-hydroxymidazolam, respectively. Closed and open bars represent metabolic activity of each CYP when cells were exposed to DMSO (0.1%) or drug (omeprazole, phenobarbital or rifampicin), respectively. Data represents the mean±S.E.M. (n=3 or 4) as fold induction *vs.* DMSO control. * indicates significant differences from the control ($p<0.05$ by Student's t-test).

increased and was stable after day 9 in 3D culture, whereas that in 2D culture was markedly decreased compared with that of non-cultured hepatocytes. CYP2B6-mediated bupropion hydroxylation activity was decreased in both cultures compared with that of non-cultured hepatocytes, though the activity in the 3D culture remained stable from days 14 to 21. CYP3A4/5-mediated midazolam hydroxylation activity tended to decrease in 3D-culture, but remained stable from 14 to 21 d after seeding, whereas the activity in 2D-cultured hepatocytes was maintained for at least 7 d after seeding. Based on these results and previous report,[23] subsequent studies using 2D-cultured hepatocytes were performed within 9 d after seeding (pre-culture for 2 d plus inducer exposure for a maximum of 7 d), and those using hepatocyte spheroids were performed within 21 d (pre-culture for 7 d plus inducer exposure for a maximum of 14 d).

Induction of CYPs In case of 3D culture, after 2 d's exposure of hepatocyte spheroids to omeprazole, phenobarbital and rifampicin, mRNA expression levels of CYP1A2, 2B6 and 3A4 were significantly higher than the control levels without inducers (Fig. 4B). The induction of CYP2B6 mRNA remained reasonably stable between days 2 and 14 after exposure to inducer, while the induction of both CYP1A2 and 3A4 continued to increase until day 14 as exposure period. In case of 2D culture, fold inductions of mRNA expression levels of all tested enzyme were reduced compared with 3D culture. Moreover fold induction of CYP1A2 after 7 d's exposure of 2D hepatocyte to omeprazole was lower than that after 2 d exposure.

As shown in Fig. 5, these metabolic activities were significantly increased compared with the control until day 14 in the 3D culture. The metabolic activities of these enzymes had good correlation for those mRNA expressions as shown in Fig. 4B. In contrast, in the case of 2D culture, the increase of CYP1A2 activity was maintained for only 2 d. In addition, CYP3A4 activity after inducer exposure for 7 d was decreased compared with that after 2 d, while CYP2B6 activity after 7 d was notably higher than that after 2 d.

DISCUSSION

The CYP activities of 2D-cultured hepatocytes were not stable; in particular, the CYP1A2 and CYP2B6 activities were reduced within 2 d (Fig. 3A). This is consistent with a previous report that the metabolic activities of several CYP isoforms in 2D culture generally decrease within a few hours after seeding, and the survival time of hepatocytes is just a few days.[13] On the other hand, the CYP activities in 3D culture were relatively stable for at least 21 d after seeding (Fig. 3B). Further, the mRNA expression of AhR, CAR and PXR maintained for 21 d in the 3D culture (Fig. 2). Therefore, we adopted the schedules shown in Fig. 1 for evaluation of metabolic enzyme induction using cultured hepatocytes.

In the case of 3D-cultured hepatocytes, the activities of CYPs increased time-dependently for up to 14 d upon exposure to inducers (Figs. 4B, 5B). On the other hand, induction of the enzymes in 2D culture showed little time-dependency. Therefore, CYP1A2 induction activity was found for 2 d exposure to omeprazole, but not for 7 d exposure, while the reverse is true in the case of CYP2B6. Moreover, morphology of human hepatocytes in 2D cultures could not be maintained after 9 d (2 d pre-culture and 7 d inducer exposure) as mentioned previously.[22,23] These findings indicate that 3D culture would be far superior to 2D culture, especially for long-term induction experiments. In addition, we found a relatively poor correlation between CYP2B6 induction in terms of mRNA and enzyme activity as supported by other studies.[25,26] These results suggest that CYP2B6 induction in the clinical context should be predicted on the basis of activity changes, not mRNA changes. At present, the guideline of the European Medicines Agency (EMA)[14] and guidance by the United States Food and Drug Administration (FDA)[15] and the Japanese Ministry of Health, Labour and Welfare (MHLW)[16] for drug-metabolizing enzyme induction require measurement of mRNA expression changes of CYP1A2, 2B6 and 3A4 in *in vitro* assays. It has been reported that assays of mRNA were sensitive to inducers and gave few false-negative results, at least for CYP3A4, whereas assays of metabolic activity gave a much higher rate of false-negatives.[27] However, in the clinical situation, changes in drug clearance depend upon changes in the metabolic enzyme activities.

As a long-term exposure model, human hepatoma cell lines such as HepaRG cells have recently been used to evaluate DILI, drug metabolism *via* Phases I and II enzymes, and induction of CYP1A2, 2B6 and 3A4.[28] Such models have the advantages of unlimited supply and low inter-lot fluctuation. However, HepaRG cells are a cancer cell line, and do not accurately reflect the normal human genetic background. Also, they cannot be applied to examine inter-individual variations. Data from HepaRG is only described as "supportive" in the guideline from EMA and guidance of the FDA and MHLW. Therefore, we believe 3D culture of primary human hepatocytes would be preferable for long-term induction assays.

In conclusion, we found that CYP1A2, 2B6 and 3A4 were induced by long-term exposure to typical inducers in a hepatocyte spheroid system with time dependency. We propose that this 3D system would be useful for long-term testing of enzyme activity induction by drugs to predict and verify clinical events. We have previously shown that hepatocyte spheroids are suitable for long-term metabolic activity assays,[22] and that hepatotoxic potential can be predicted with high accuracy and sensitivity by the same protocol as this study applying the 3D culture system.[23] Overall, our results indicate that our 3D cultures of human hepatocytes would be more efficient than conventional culture systems for tracing of metabolic processes, predicting hepatotoxicity, and evaluating enzyme induction, since all these assessments can be made simultaneously. However, it should be noted that this study was performed using a single lot of hepatocytes. Validation tests require the use of at least three different lots of hepatocytes (in three individual facilities if possible). We are currently conducting additional studies aimed at validating this method using the hepatocyte spheroid system, and we hope to propose it to OECD as a new test guideline in the near future.

Acknowledgment A part of this research is supported by a Grant for Research on Regulatory Science of Pharmaceuticals and Medical Devices (No. 16mk0101051h0201) from the Japan Agency for Medical Research and Development, AMED.

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

REFERENCES

1) Lasser KE, Allen PD, Woolhandler SJ, Himmelstein DU, Wolfe SM, Bor DH. Timing of new black box warnings and withdrawals for prescription medications. *JAMA*, **287**, 2215–2220 (2002).
2) Hebert MF, Roberts JP, Prueksaritanont T, Benet LZ. Bioavailability of cyclosporine with concomitant rifampin administration is markedly less than predicted by hepatic enzyme induction. *Clin. Pharmacol. Ther.*, **52**, 453–457 (1992).
3) Bhaloo S, Prasad GV. Severe reduction in tacrolimus levels with rifampin despite multiple cytochrome P450 inhibitors: a case report. *Transplant. Proc.*, **35**, 2449–2451 (2003).
4) Backman JT, Olkkola KT, Neuvonen PJ. Rifampin drastically reduces plasma concentrations and effects of oral midazolam. *Clin. Pharmacol. Ther.*, **59**, 7–13 (1996).
5) Wang YM, Chai SC, Brewer CT, Chen T. Pregnane X receptor and drug-induced liver injury. *Expert Opin. Drug Metab. Toxicol.*, **10**, 1521–1532 (2014).
6) Li F, Lu J, Cheng J, Wang L, Matsubara T, Csanaky IL, Klaassen CD, Gonzalez FJ, Ma X. Human PXR modulates hepatotoxicity associated with rifampicin and isoniazid co-therapy. *Nat. Med.*, **19**, 418–420 (2013).
7) Lehmann JM, McKee DD, Watson MA, Willson TM, Moore JT, Kliewer SA. The human orphan nuclear receptor PXR is activated by compounds that regulate CYP3A4 gene expression and cause drug interactions. *J. Clin. Invest.*, **102**, 1016–1023 (1998).
8) LeCluyse EL. Pregnane X receptor: molecular basis for species differences in CYP3A induction by xenobiotics. *Chem. Biol. Interact.*, **134**, 283–289 (2001).
9) Kocarek TA, Schuetz EG, Strom SC, Fisher RA, Guzelian PS. Comparative analysis of cytochrome P4503A induction in primary cultures of rat, rabbit, and human hepatocytes. *Drug Metab. Dispos.*, **23**, 415–421 (1995).
10) Poland A, Mak I, Glover E. Species differences in responsiveness to 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene, a potent phenobarbital-like inducer of microsomal monooxygenase activity. *Mol. Pharmacol.*, **20**, 442–450 (1981).
11) OECD guideline for the testing of chemicals 1. "Draft proposal for a new performance based test guideline 2. Human cytochrome P450

(CYP) n-fold induction in vitro test method.": ‹https://www.oecd.org/chemicalsafety/testing/CYP-induction-PBTG-final-for-WNT-comments.pdf›, cited 11 November, 2016.
12) European Union Reference Laboratory for Alternatives to Animal Testing (EURL ECVAM). "Toxicokinetics.": ‹https://eurl-ecvam.jrc.ec.europa.eu/validation-regulatory-acceptance/toxicokinetics›, cited 11 November, 2016.
13) Smith CM, Nolan CK, Edwards MA, Hatfield JB, Stewart TW, Ferguson SS, Lecluyse EL, Sahi J. A comprehensive evaluation of metabolic activity and intrinsic clearance in suspensions and monolayer cultures of cryopreserved primary human hepatocytes. *J. Pharm. Sci.*, **101**, 3989–4002 (2012).
14) European Medicines Agency. "Guideline on the investigation of drug interactions.": ‹http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/07/WC500129606.pdf%3E›, cited 11 November, 2016.
15) US Food and Drug Administration. "Drug Interaction Studies—Study Design, Data Analysis, Implications for Dosing, and Labeling Recommendations.": ‹http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/UCM292362.pdf%3E›, cited 11 November, 2016.
16) Japanese Ministry of Health, Labour and Welfare. "Drug interaction guideline for drug development and labeling recommendations.": ‹http://www.nihs.go.jp/mhlw/20131488.pdf%3E›, cited 11 November, 2016.
17) Zhou SF, Yang LP, Zhou ZW, Liu YH, Chan E. Insights into the substrate specificity, inhibitors, regulation, and polymorphisms and the clinical impact of human cytochrome P450 1A2. *AAPS J.*, **11**, 481–494 (2009).
18) Sueyoshi T, Kawamoto T, Zelko I, Honkakoski P, Negishi M. The repressed nuclear receptor CAR responds to phenobarbital in activating the human CYP2B6 gene. *J. Biol. Chem.*, **274**, 6043–6046 (1999).
19) Bertilsson G, Heidrich J, Svensson K, Asman M, Jendeberg L, Sydow-Backman M, Ohlsson R, Postlind H, Blomquist P, Berkenstam A. Identification of a human nuclear receptor defines a new signaling pathway for CYP3A induction. *Proc. Natl. Acad. Sci. U.S.A.*, **95**, 12208–12213 (1998).
20) Khetani SR, Bhatia SN. Microscale culture of human liver cells for drug development. *Nat. Biotechnol.*, **26**, 120–126 (2008).
21) Meng Q. Three-dimensional culture of hepatocytes for prediction of drug-induced hepatotoxicity. *Expert Opin. Drug Metab. Toxicol.*, **6**, 733–746 (2010).
22) Ohkura T, Ohta K, Nagao T, Kusumoto K, Koeda A, Ueda T, Jomura T, Ikeya T, Ozeki E, Wada K, Naitoh K, Inoue Y, Takahashi N, Iwai H, Arakawa H, Ogihara T. Evaluation of human hepatocytes cultured by three-dimensional spheroid systems for drug metabolism. *Drug Metab. Pharmacokinet.*, **29**, 373–378 (2014).
23) Ogihara T, Iwai H, Inoue Y, Katagi J, Matsumoto N, Motoi-Ohtsuji M, Kakiki M, Kaneda S, Nagao T, Kusumoto K, Ozeki E, Jomura T, Tanaka S, Ueda T, Ohta K, Ohkura T, Arakawa H, Nagai D. Utility of human hepatocyte spheroids for evaluation of hepatotoxicity. *Fund Toxicol. Sci.*, **2**, 41–48 (2015). https://www.jstage.jst.go.jp/article/fts/2/1/2_41/_pdf
24) Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods*, **25**, 402–408 (2001).
25) Yajima K, Uno Y, Murayama N, Uehara S, Shimizu M, Nakamura C, Iwasaki K, Utoh M, Yamazaki H. Evaluation of 23 lots of commercially available cryopreserved hepatocytes for induction assays of human cytochromes P450. *Drug Metab. Dispos.*, **42**, 867–871 (2014).
26) Rodriguez-Antona C, Donato MT, Pareja E, Gomez-Lechon MJ, Castell JV. Cytochrome P-450 mRNA expression in human liver and its relationship with enzyme activity. *Arch. Biochem. Biophys.*, **393**, 308–315 (2001).
27) Fahmi OA, Kish M, Boldt S, Obach RS. Cytochrome P450 3A4 mRNA is a more reliable marker than CYP3A4 activity for detecting pregnane X receptor-activated induction of drug-metabolizing enzymes. *Drug Metab. Dispos.*, **38**, 1605–1611 (2010).
28) Andersson TB, Kanebratt KP, Kenna JG. The HepaRG cell line: a unique *in vitro* tool for understanding drug metabolism and toxicology in human. *Expert Opin. Drug Metab. Toxicol.*, **8**, 909–920 (2012).