Human Hepatocarcinoma Functional Liver Cell-4 Cell Line Exhibits High Expression of Drug-Metabolizing Enzymes in Three-Dimensional Culture

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The expression levels of CYP and uridine diphosphate-glucuronosyl transferase (UGT) are lower in hepatocellular carcinoma cell lines than in human primary hepatocytes. However, a functional liver cell (FLC)-4 cell line that has a greater capacity to secrete liver-specific proteins than other hepatocellular carcinoma cells has recently been established. A three-dimensional culture using Engelbreth–Holm–Swan (EHS) gel induces the secretion of liver-specific proteins via the induction of hepatocyte nuclear factor-4α (HNF-4α). The aim of this study was to evaluate the mRNA expression of the enzymes CYP and UGT in FLC-4 and HepG2 cells in monolayer and three-dimensional cultures using EHS gel. The mRNA levels of HNF-4α, albumin, pregnane X receptor (PXR), constitutive androstane receptor (CAR), CYPs (1A2, 2E1, 2C9, 2C19, 2D6, and 3A4) and UGTs (1A1, 1A6, 1A9, and 2B7) were determined using real-time reverse transcription (RT) PCR. In a monolayer culture, the mRNA expression levels of HNF-4α, albumin, PXR, CAR, CYPs (2E1, 2C9, 2C19, 2D6, and 3A4) and UGTs (1A1, 1A6, and 1A9) were higher in FLC-4 cells than in HepG2 cells. In FLC-4 cells, the mRNA expression levels of HNF-4α, albumin, PXR, CAR, CYPs (2E1, 2C8, 2C19, and 3A4) and UGTs (1A1, 1A6, 1A9, and 2B7) significantly increased in three-dimensional culture. FLC-4 cells cultured in EHS gel showed significantly increased expression levels of CYPs and UGTs. The results of this study suggest that human hepatocellular carcinoma FLC-4 cells cultured in EHS gel show potential for use in studying in vitro drug metabolism.

Key words CYP; uridine diphosphate-glucuronosyl transferase; expression; functional liver cell; three-dimensional culture

Human hepatocellular carcinoma cell lines (e.g. HepG2, Hep3B, and HuH7) are widely used to study drug metabolism and toxicology in vitro. The expression levels of CYP and uridine diphosphate-glucuronosyl transferase (UGT) in hepatocellular carcinoma cell lines are known to be lower than those in human primary hepatocytes. However, under typical culture conditions, human primary hepatocytes are unable to sustain stable hepatic functions, such as production of drug-metabolizing enzymes. Therefore, hepatic microsomes are usually used in in vitro experiments related to drug metabolism. Functional liver cell (FLC) lines have been established from resected liver tumors of Japanese patients. These cells synthesized and secreted human albumin, alpha-fetoprotein, and other proteins in vitro.

Many reports have shown that the differentiation status of primary culture hepatocytes could be enhanced by three-dimensional culture. Engelbreth–Holm–Swan (EHS) gel, derived from mouse EHS sarcoma, induces the expression of hepatocyte nuclear factor-4α (HNF-4α). HNF-4α has been reported to play a significant role in the regulation of many genes expressed in the liver and is trans-activate the human pregnane X receptor (PXR) gene. Human PXR is known to be a transcriptional regulator of genes encoding drug-metabolizing enzymes, including CYP2B6, CYP2C9, CYP3A4, and UGT1A1. Therefore, the expression levels of CYPs and UGTs in FLC-4 cells grown in three-dimensional culture using EHS gel might be higher than those in monolayer culture. However, the expression levels of drug-metabolizing enzymes under the above conditions are unknown.

The authors declare no conflict of interest.

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MATERIALS AND METHODS

Cell Cultures Monolayer Culture FLC-4 cells (previous names, JHH-4 (JCRB0435) established by Nagamori) were obtained from Health Science Research Resources Bank (Osaka, Japan). HepG2 cells (JCRB1054) were obtained from Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). FLC-4 and HepG2 cells (passage 20–60) were grown and maintained in Dulbecco’s modified Eagle’s medium (DMEM, 4.5 g glucose/L, Invitrogen Co., CA, U.S.A.), containing 10% fetal bovine serum (BioSource International Inc., CA, U.S.A.), penicillin (50IU/mL) and streptomycin (50IU/mL) (PS, Life Technologies Corporation, Carlsbad, CA, U.S.A.) at 37°C with 5% CO2 in 25-cm² tissue culture flasks (Becton-Dickinson Ind., NJ, U.S.A.). The FLC-4 and HepG2 cell lines were subcultured by treatment with 0.25% trypsin-1 mM ethylenediaminetetraacetic acid (EDTA) in Ca²⁺ and Mg²⁺ free phosphate-buffered saline (PBS) solution every 3d. The cells were then detached from the stock cultures by trypsin digestion, washed once by centrifugation, resuspended and subcultured in 10mL of medium in culture flasks at a concentration of 5×10⁵ cells/mL. The cultures were observed on a regular basis under an inverted light microscope to monitor growth and contamination.

Three-Dimensional Culture FLC-4 cells (1.5×10⁶ cells/150μL) and growth factor-reduced Matrigel (Becton Dickinson, Lincoln Park, NJ, U.S.A.) were mixed in equal amount
and placed on a 24-well multiplate for 30 min at 37°C with 5% CO₂. One milliliter of DMEM with 10% FBS and 1% PS was added each well and treated with medium (control) for 3, 7, and 14 d.

**RNA Isolation and RNA Analysis** Total RNA was extracted from FLC-4 and HepG2 using TRI reagent™ (Molecular Research Center, Inc., Cincinnati, OH, U.S.A.). cDNA was synthesized by reversing transcription using total RNA solution (Transcriptor Universal cDNA Master, Roche Diagnostics, Basel, Switzerland). The primers and probe were designed according to the nucleotide sequence of the human CYP1A2, 2E1, 2C8, 2C9, 2C19, 2D6, 3A4, UGT1A1, 1A6, 1A9, 2B7, HNF-4α, albumin, and 18S ribosomal RNA (rRNA) using ProbeFinder v2.45 (http://qpcr.probefinder.com/roche3.html). This online software specifies a set of specific primers plus the TaqMan locked nucleic acid probe from the Roche Universal Probe Library collection. The promoters used in this study is shown in Table 1. Universal Probe #11 for PXR and constitutive androstane receptor (CAR), #22 for UGT2B7, #27 for albumin, #63 for CYP2D6, #67 for CYP2E1, #77 for CYP3A4, HNF-4α, HNF-4, albumin, and 18S rRNA, #78 for UGT1A1, UGT1A6 and UGT1A9, #79 for CYP2C9 and CYP2C19, and #80 for CYP2E1 were purchased from Roche Diagnostics. mRNA measurement was performed using the LightCycler® (Roche Diagnostics). The results were adjusted using 18S rRNA, a housekeeping gene, as the internal standard substance.

**Data Analysis** Results are expressed as means±S.D. Statistical analyses were performed using the Tukey multiple comparison tests as parametric test and Scheffe multiple comparison tests as non-parametric test. A p<0.05 was statistically significant.

**RESULTS**

After 72h in culture, FLC-4 cells showed a spread-out monolayer morphology when cultured on uncoated dish. On the other hand, when cells were cultured on EHS gel, cell shape became cuboidal (Fig. 1).

**mRNA Expression of HNF-4α and Albumin** In the monolayer culture, the mRNA expression of HNF-4α in FLC-4 cells is significantly higher than that in HepG2 cells (p<0.05, Fig. 2). The albumin mRNA expression in FLC-4 cells is 27-fold higher than that in HepG2 cells. In FLC-4 cells, the mRNA expression of HNF-4α is significantly increased in three-dimensional culture on 7d (p<0.05) and that of albumin is significantly increased in three-dimensional culture on 7 and 14d (p<0.05, 7d; p<0.01, 14d).

**mRNA Expression of PXR and CAR** The PXR and CAR mRNA expressions in FLC-4 cells are 2.3- and 1.5-fold higher than those in HepG2 cells, respectively. In FLC-4 cells, the mRNA expressions of PXR and CAR are significantly increased in three-dimensional culture on 7d (Fig. 3, p<0.05).

**CYP mRNA Expressions** The CYP1A2 mRNA expression in monolayer culture is not changed between HepG2 and FLC-4 cells and not changed on 3, 7, and 14d in three-dimensional culture (Fig. 4). The CYP2E1 mRNA expression in FLC-4 monolayer culture is 6.5-fold higher than that in HepG2 monolayer culture. In FLC-4 cells, the mRNA expression of CYP2E1 is significantly increased in three-dimensional culture on 3, 7, and 14d (p<0.01). The CYP2C8 mRNA expression in monolayer culture is not changed between HepG2 and FLC-4 cells and in FLC-4 cells the mRNA expression is significantly increased in three-dimensional culture on 7 and 14d (p<0.05). The CYP2C9 mRNA expression in FLC-4 monolayer culture is 29-fold higher than that in HepG2 cells. In FLC-4 cells, the mRNA expression of CYP2C9 is 2.1-2.4-fold increased in three-dimensional culture on 3, 7, and 14d, compared with monolayer culture. The CYP2C19 mRNA expression in FLC-4 monolayer culture is not detected in HepG2.

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**Table 1. Primer Sequences Used for Real-Time RT-PCR Analyses**

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Fig. 1. Morphology of Cells of the Functional Liver Cell (FLC)-4 Line Cultured on Uncoated (Monolayer) or Engelbreth–Holm–Swan (EHS) Gel-Coated (EHS, Three-Dimensional Culture) Plastic Dishes

Scale bar=100μm.
In FLC-4 cells, the mRNA expression of CYP2C19 is significantly increased in three-dimensional culture on 14 d \( (p < 0.05) \). The CYP2D6 mRNA expression in FLC-4 monolayer culture is 6.5-fold higher than that in HepG2 cells. In FLC-4 cells, the mRNA expression of CYP2D6 is tended to increase in three-dimensional culture. The CYP3A4 mRNA expression in FLC-4 monolayer culture is 2.4-fold higher than that in HepG2 cells. In FLC-4 cells, the mRNA expression of CYP3A4 is significantly increased in three-dimensional culture on 3 and 7 d \( (p < 0.05, 3 \text{ d}; p < 0.01, 7 \text{ and } 14 \text{ d}) \). The CYP3A7 mRNA is not changed between HepG2 and FLC-4 cells. In FLC-4 cells, the mRNA expression of CYP3A7 is significantly increased in three-dimensional culture on 7 and 14 d \( (p < 0.05) \), compared with monolayer culture.

**UGT mRNA Expressions** In the monolayer culture, the mRNA expression of UGT1A1 in FLC-4 cells is significantly higher than that in HepG2 cells (Fig. 5, \( p < 0.05 \)). In FLC-4 cells, the mRNA expression of UGT1A1 is significantly increased in three-dimensional culture on 3, 7, and 14 d \( (p < 0.05, 3 \text{ d}; p < 0.01, 7 \text{ and } 14 \text{ d}) \). The UGT1A6 mRNA expression in FLC-4 monolayer culture is 5.7-fold higher than that in HepG2 monolayer culture. In FLC-4 cells, the mRNA expression of UGT1A6 is significantly increased in three-dimensional culture on 7 and 14 d \( (p < 0.05) \), compared with monolayer culture. The UGT1A9 mRNA expression in FLC-4 monolayer culture is 22.7-fold higher than that in HepG2 monolayer culture. In FLC-4 cells, the mRNA expression of UGT1A9 is significantly increased in three-dimensional culture on 14 d \( (p < 0.05) \), compared with monolayer culture. The UGT2B7 mRNA is not changed between HepG2 and FLC-4 cells. In FLC-4 cells, the mRNA expression of UGT2B7 is significantly increased in three-dimensional culture on 7 and 14 d \( (p < 0.05) \), compared with monolayer culture.

**DISCUSSION**

In a previous study, it was reported that the mRNA expression levels of CYP1A2, 2C9, 3A4, and UGT1A1 increased in FLC-4 cells that were grown in a three-dimensional culture system using micro-space cell culture plates. However, the mRNA expression levels of other CYPs and UGTs in these cells were not clearly determined. In this study, we examined the mRNA expressions of representative drug-metabolizing enzymes.

Although many human hepatoma cell lines have been established, cells of these lines are very low-functioning compared to human liver cells. It has also been reported that these cell lines differ from human hepatocytes in terms of cell morphology, protein synthesis, and enzymes involved in drug metabolism. In the present study, the expression levels of CYPs and
UGTs in FLC-4 cells were higher than those in HepG2 cells. The expression levels of CYPs and UGTs in FLC-4 cells were significantly increased by three-dimensional culture, compared with monolayer culture. In this study, we only measured the mRNA expression levels of drug-metabolizing enzymes; we did not evaluate the activity of these enzymes. It has been previously reported that good correlations exist between mRNA expression levels and enzyme activities of CYPs and UGTs. Therefore, the three-dimensional culture of FLC-4 cells using EHS gel potentially increases the enzyme activities of CYPs and UGTs. The results of our study suggest that this three-dimensional culture system increases the mRNA expression levels of drug-metabolizing enzymes.

We used EHS gel to culture the FLC-4 cells in a three-dimensional configuration (Fig. 1). In monolayer culture, cells of the FLC-4 line showed enhanced expression of the HNF-4α and albumin genes, but expression of these genes were low in HepG2 cells (Fig. 2). HNF-4α is a highly conserved gene, suggesting that its integrity is necessary to maintain liver function. It has been reported that a three-dimensional spherical cell shape is important in liver-specific gene expression. Our results indicate that FLC-4 cells retained their spherical cell shape-dependent induction of hepatocyte functions. In monolayer culture, the mRNA expression levels of CYP2E1, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP3A4 in HepG2 cells were very low, compared with those of FLC-4 cells. In FLC-4 cells, the mRNA expression levels of CYP2E1, CYP2C8, CYP2C9,
and CYP3A4 were significantly increased by three-dimensional culture. Furthermore, the mRNA expression levels of UGT1A1, UGT1A6, UGT1A9, and UGT2B7 were also significantly increased by growth in three-dimensional culture. The level of HNF-4α mRNA expression was also significantly increased. HNF-4α is required for the development of the liver and it is known as a “master regulator” that regulates the expression of many genes. HNF-4α has been reported to modulate CYP expression in the liver by interacting with xenosensor receptors (e.g. PXR and CAR), glucocorticoid receptors, feeding-fasting cycle target PGC-1α, sexual-dimorphism factor Stat5b, and other liver-enriched factors, such as C/EBPs. The expression levels of CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP3A4, UGT1A1, UGT1A6, UGT1A9, and UGT2B7 have been reported to be regulated by HNF-4α. However, HNF-4α induction did not alter the expression of CYP2D6. This phenomenon may indicate the existence of other regulators of CYP2D6.

In this study, the expressions of PXR and CAR mRNA were significantly increased by three-dimensional culture. PXR and CAR are known to influence the expression of CYP2B, CYP2C, CYP3A, UGT1A, and UGT2B genes, and HNF-4α has been reported to be important for the PXR and CAR-mediated transcriptional activation. Therefore, the increased expression of PXR and CAR might be involved in the up regulation of CYPs and UGTs. The expression of CYP2E1 has been reported to be regulated by nuclear factor erythroid 2-related factor 2 (Nrf2). The induction of CYP2E1 may be related to the pathway via Nrf2 mRNA expression. Further studies are required to investigate the induction mechanism of CYP2E1. CYP2E1 has been reported to metabolize and activate many toxicologically important substrates to more toxic products indicating that this method for three-dimensional culture using FLC-4 cells could be applied for in vitro toxicological experiments.

In conclusion, FLC-4 cells cultured in EHS gel significantly increased the expression levels of CYPs and UGTs. The results of this study might suggest that human hepatocellular carcinoma FLC-4 cells cultured in EHS gel are useful for studying in vitro drug metabolism.

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**Fig. 5. Levels of UGT1A1, UGT1A6, UGT1A9, and UGT2B7 mRNA Expression Were Observed in HepG2 and FLC-4 Cells**

Levels of mRNA expression were determined using real-time PCR. HepG2 and FLC-4 cells in monolayer culture were seeded at a density of 1.5×10⁵ cells/well on conventional cell culture plates and cultured for 3 d. FLC-4 cells in three-dimensional culture were seeded at a density of 1.5×10⁵ cells/well on conventional cell culture plates and cultured for 3, 7, and 14 d. The expression levels of UGTs mRNA were normalized to those of 18S ribosomal RNA (18S rRNA). 2D, FLC-4 cells in monolayer culture for 3 d; 3D, FLC-4 cells in three-dimensional culture; 18S, 18S rRNA. Data are shown as mean±S.D. (n=3–4): *p<0.05, **p<0.01, compared to HepG2 cells in monolayer culture. †p<0.05, ††p<0.01, compared to FLC-4 cells in monolayer culture. ‡‡p<0.01, compared to FLC-4 cells in three-dimensional culture for 3 d.


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