J Pharmacol Sci **115**, 516 – 524 (2011)

Journal of Pharmacological Sciences

Full Paper

Comparative Study of Culture Conditions for Maintaining CYP3A4 and ATP-Binding Cassette Transporters Activity in Primary Cultured Human Hepatocytes

Yuko Takeba[1,*], Naoki Matsumoto[1], Sachiko Takenoshita-Nakaya[1], Yoshie Harimoto[1], Toshio Kumai[2], Yuichi Kinoshita[1], Hiroshi Nakano[3], Takehito Ohtsubo[3], and Shinichi Kobayashi[1]

[1]*Department of Pharmacology,* [2]*Department of Pharmacogenomics, and* [3]*Division of Gastroenterological Surgery, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki, Kanagawa 216-8511, Japan*

Received August 18, 2010; Accepted February 16, 2011

Abstract. The aim of this study was to determine suitable culture conditions for maintaining the activity of cytochrome p450 (CYP) 3A4 and drug transporters in primary cultured human hepatocytes. Human hepatocytes were isolated using the two-step collagenase perfusion technique and were cultured with four different media, serum-free William's E medium (serum-free WEM), WEM containing fetal calf serum (FCS-WEM), WEM with human serum (HS-WEM), and Lanford's medium. The albumin levels were maintained for 7 days in hepatocytes. Although *CYP3A4* mRNA levels gradually decreased from 3 days, CYP3A4 and hepatocyte nuclear factor-4α alpha protein levels and activities were maintained for 7 days in hepatocytes cultured with serum-free WEM and Lanford's but not in those with FCS-WEM and HS-WEM. Furthermore, CYP3A4 protein levels were significantly increased by the addition of rifampicin and dexamethasone to the culture media, indicating that the induction potential was maintained. The protein levels of P-glycoprotein, multi-drug-resistance-2, and breast cancer-resistance protein were maintained for 7 days in all media. Serum-free WEM and Lanford's also maintained protein levels of CYP2C19, CYP2D6, and organic anion transporter polypeptide in the hepatocytes. Serum-free WEM and Lanford's may be appropriate culture media for maintaining CYP3A4 and drug transporter protein levels in primary cultured hepatocytes.

Keywords: CYP3A4, HNF-4α, primary hepatocyte, ATP-binding cassette transporter, Lanford's medium

Introduction

Primary cultures of human hepatocytes are important tools to evaluate specific liver functions, especially of drug-metabolizing enzymes and transporters. Primary metabolic studies have been performed with primary cultured rat hepatocyte cultures, but interspecies differences have been reported, especially with regard to cytochrome P450 (CYP) induction (1 – 3). Therefore, it is important to establish appropriate experimental systems for the primary culture of human hepatocytes for the evaluation of drug metabolism. The aim of this study is to find appropriate conditions for culturing human primary hepatocytes that enable them to sustain near-normal hepatocellular morphology and allow induction of the expression of liver-specific genes such as albumin and CYP enzymes in vitro in a manner that reflects the in vivo conditions (4, 5).

CYP3A4 is a major subfamily of human CYPs involved in the metabolism of many drugs. CYP3A4 enzyme activity has been evaluated using model inducers such as rifampicin in primary cultured hepatocytes (3). The expression of CYP3A can be analyzed based on the mRNA and protein levels. Testosterone 6β-hydroxylation activity is accepted as measurement of CYP3A activity. However, the measurement of CYP3A4 activity using testosterone 6β-hydroxylation activity in primary hepatocytes requires highly sensitive analytical equipment

*Corresponding author. takebay@marianna-u.ac.jp
Published online in J-STAGE on March 24, 2011 (in advance)
doi: 10.1254/jphs.10215FP

such as high performance liquid chromatography-mass spectrometry/mass spectrometry (6). In addition, CYP3A4 levels in primary cultures of human hepatocytes decline during 2 – 4 days in culture to about 15% – 30% compared with levels in freshly isolated human hepatocytes (7). It is thus important to establish culture conditions for maintaining spontaneous CYP3A4 expression and convenient methods to measure CYP3A4 activity in human primary hepatocytes.

Furthermore, drug transporters as well as drug-metabolizing enzymes are important factors involved in drug–drug interactions. Multi drug resistance-1 gene product, P-glycoprotein, breast cancer-resistance protein (BCRP/ABCG2), and multidrug-resistant protein (MRP) are ATP-binding cassette transporters and drug efflux transporters (8 – 10). The substrates of these transporters including topotecan, SN-38, and doxorubicin have been associated with multidrug resistance in cancer (11 – 13). To our knowledge, few studies have examined the expression of cytochrome P450 enzymes and drug transporters together in primary human hepatocytes by monolayer culture methods, although several studies have investigated only the CYP enzymes induction capacity change according to the culture conditions.

Sunouchi et al have reported the isolation procedure of the hepatocytes from Japanese liver tissues and the culture methods using a new media, Lanford's (14). Lanford's media was suitable for long-term culture in primary human hepatocytes (15). However, it is unknown whether the Lanford's media can maintain CYP3A and drug transporters. In this study, we investigated the effects of various culture media including Lanford's to determine the most appropriate culture methods for maintaining the production of CYP3A4, P-glycoprotein, BCRP, and MRP-2 in primary cultured human hepatocytes.

Materials and Methods

Antibodies

The antibodies used in the present study were anti-CYP3A rabbit polyclonal IgG antibody (Ab), anti-CYP2C19 rabbit polyclonal IgG Ab, and anti-CYP2D6 mouse monoclonal IgG Ab (BD Biosciences, Woburn, MA, USA); anti-BCRP mouse monoclonal IgG Ab (MONSAN, Uden, The Netherlands); anti-albumin mouse monoclonal IgG Ab (MP Biomedicals, Santa Ana, CA, USA); anti-MRP-2 rabbit polyclonal IgG Ab (Abcam, Cambridge, UK); anti-hepatocyte nuclear factor (HNF)-4α goat polyclonal IgG Ab; anti-P-glycoprotein rabbit polyclonal IgG Ab; and anti-organic anion transporter polypeptide (OATP) goat polyclonal IgG Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA).

Human liver tissues

Human liver tissues were obtained from 13 patients undergoing partial hepatectomy for tumors in our institution. Written informed consent was obtained from each patient prior to surgery. Six patients had liver metastases of colon cancer. Four patients had hepatocellular carcinoma. One patient had a hepatic abscess, and 2 had cholangiocarcinoma. No patients showed evidence of concurrent hepatic viral infections. In addition, 13 liver samples were obtained from organs of Caucasian and Hispanic transplantation donors. These 13 samples were supplied by the National Disease Research Interchange (Philadelphia, PA, USA), through the Biomedical Research Institute, Human and Animal Bridging Research Organization (Chiba). The study was approved by the St. Marianna University School of Medicine Ethics Committee.

Isolation and culture of human hepatocytes

Hepatocytes were isolated and cultured based on a modification of a two-step collagenase digestion method (16, 17). The isolated hepatocytes were plated onto 30-mm collagen I–coated dishes or 24-well plates. Hepatocytes were cultured with William's E medium (WEM) for 24 h at 37°C under an atmosphere of 5% CO_2 and 95% O_2. After 24-h plating in WEM, the medium was changed to WEM containing 500 μg/ml of insulin (Wako Pure Chemical, Osaka), 10 ng/ml of epithelial growth factor (R&D, Minneapolis, MN, USA), 10 μM of nicotinic acid (Wako), 1 μM of ascorbic acid (Wako), 100 units/ml of penicillin, and 100 μg/ml of streptomycin. For the next step, we cultured the cells in four different media, serum-free WEM, WEM containing 10% fetal calf serum (FCS-WEM), WEM containing 10% human serum (HS-WEM), and Lanford's medium (Nissui Pharmaceutical, Tokyo), at 37°C under an atmosphere of 5% CO_2 and 95% O_2. The medium in all cultures was changed every 48 h thereafter. The time after primary culture for 24 h was designated as day 0.

Induction of CYP3A4

To examine the ability to induce CYP3A4 protein expression, the primary hepatocytes were treated with 20 and 50 μM of rifampicin or dexamethasone for 48 h. All inducers were dissolved in DMSO, which was added to the serum free culture media or Lanford's at a final concentration of 0.1% (13.4 mM).

RNA extraction and real-time reverse-transcription polymerase chain reaction

Total RNA of primary hepatocytes was extracted using the RNAgent total RNA isolation system (Promega, Madison, WI, USA). cDNA was synthesized from 1 μg

of total RNA using a RETROscript kit (Ambion Inc., Austin, TX, USA) in a final volume of 20 μl and stored at −20°C. Quantitative analysis of cDNA was performed with an 3520S Light Cycler (Roche Diagnostics, Basel, Switzerland) using methods described previously (18). Briefly, PCR was performed in 20 μl of total reaction volume containing 2 μl of cDNA, 10 pmol of specific primers for *CYP3A4* and *GAPDH*, 3 mM of $MgCl_2$, and 2 μl of SYBER Green according to the manufacturer's protocol using a Light Cycler FastStart DNA Master SYBER Green kit (Roche Diagnostics). The cycling protocol for *CYP3A4* and *GAPDH* consisted of one cycle of 10 min at 95°C, followed by 40 cycles of denaturation for 15 s at 95°C, annealing for 10 s at 56°C, and extension for 21 s at 72°C. The primers for *CYP3A4* were 5′-GTG TGG GGC TTT TAT GAT G-3′ (sense) and 5′-GGC GAC TTT CTT TCA TCC T-3′ (antisense). The primers for *GAPDH* were 5′-GAC AAC TTT GGT ATC CGT GGA-3′ (sense) and 5′-TAC CAG GAA TGA GCT TGA C-3′ (antisense). The threshold cycles (Ct) were calculated with Light Cycler software (ver. 5.32). Standard curves were plotted with Ct versus log cDNA quantities, and the quantities of samples were analyzed using the multiplex comparative threshold method, where the amount of the gene was normalized to the housekeeping gene *GAPDH* in each sample.

Western blot analysis

The nuclear and cytoplasm proteins in primary hepatocytes were prepared using a commercial kit (NE-PER, nuclear and cytoplasmic extraction kit; Pierce Biotechnology, Rockford, IL, USA) (19). The protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). After heating at 100°C for 3 min, equivalent amounts of proteins (50 μg) were resolved by 10% SDS-polyacrylamide gel electrophoresis. Thereafter, proteins were transferred to Hybond-HCL membranes (Amersham Bioscience, Buckinghamshire, UK) for 70 min at 10 V and 300 mA. The membrane was blocked with 5% skim milk for anti-CYP3A4 Ab, anti-P-gp Ab, anti-MRP-2 Ab, and anti-α-Tubulin Ab and with 2% BSA for anti-BCRP Ab in Tris-buffered saline containing 0.05% Tween 20 (TTBS: NaCl 150 mM, Tris-HCl 100 mM, pH 7.5, Tween 20 0.05%). Incubation with the specific primary Ab was carried out for 2 h at the appropriate dilution. After washing five times for 5 min each with TTBS, the blot was reacted with a horseradish peroxidase (HRP)-conjugated second Ab for 1 h at room temperature. Reactive proteins were detected in the chemiluminescence assay (ECL Plus kit, Amersham Bioscience). The intensity of the detected bands was analyzed by the ATTO densitograph software CS analyzer ver. 3.0 (Tokyo).

CYP3A4 enzyme activity

CYP3A activity in primary cultured hepatocytes was measured using a P450-Gro assay kit (Promega) according to the manufacturer's instructions (20, 21). This assay is performed by incubating the cytochrome P450 and a luminogenic cytochrome P450 substrate, a derivative of beetle luciferin. Then, An NADPH regenerating system is required to initiate and sustain the CYP3A4 reaction. The luciferin substrate is converted by cytochrome P450 to luciferin, which in turn reacts with luciferase to produce light. The amount of light produced is directly proportional to the CYP3A4 activity. Briefly, after incubation with luciferin 5 μM for 20 min at 37°C, the hepatocytes were incubated with an NADPH regenerating system [1.3 mM β-nicotinamide-adenine dinucleotide phosphate, oxidized form and monosodium salt ($NADP^+$); 3.3 mM D-glucose 6-phosphate; 3.3 mM $MgCl_2$; 200 mM KPO_4, pH 7.4; 0.2 unit/ml glucose-6-phosphate dehydrogenase (Oriental Yeast, Osaka)] for 30 min, Finally, CYP3A luciferase activity in the hepatocytes was measured using a luminocounter (Dainihon Sumitomo Pharma, Osaka).

Statistical analysis

Data are expressed as the mean ± S.E.M. The statistical analysis used a Steel multiple comparison method of the Dwass type (KyPlot software, ver. 5.0; KyensLab, Tokyo) to confirm the difference between two groups, followed by comparison with the Mann-Whitney U-test. A *P*-value of less than 0.05 was considered to represent a statistically significant difference.

Results

Morphological changes in primary cultured human hepatocytes

We first compared the morphological changes in the human hepatocytes grown in the four different culture media by consecutive observations of morphology and survival. The hepatocytes cultured with serum-free WEM and Lanford's maintained their polygonal shape until day 7. After day 7, small numbers of hepatocytes were transformed into fibroblastic cells. On the other hand, the hepatocytes cultured with FCS-WEM and HS-WEM maintained their polygonal shape for only about 3 days, and thereafter fibroblastic cells gradually proliferated with a concomitant decrease in the number of hepatocytes. Eventually, fibroblastic cells predominated on day 14. The hepatocytes cultured with FCS-WEM and HS-WEM changed from the polygonal shape to fibroblastic cells more rapidly than those cultured with serum-free WEM and Lanford's (Fig. 1).

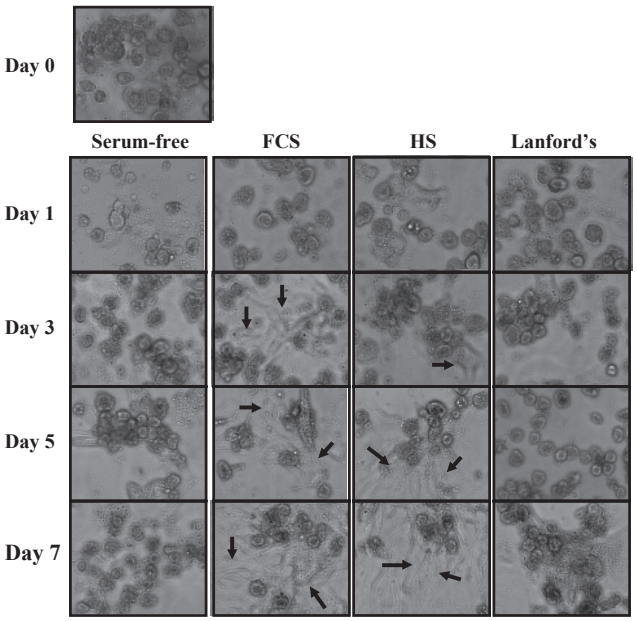

Fig. 1. Phase-contrast photographs of cultured primary human hepatocytes. Consecutive morphological observations were performed. All photos had the same original magnification (×200). Hepatocytes cultured with FCS-WEM and HS-WEM were transformed into fibroblastic-like cells from day 3. The data are representative of six independent experiments. The arrows indicate fibroblastic-cells transformed from the normal hepatocytes.

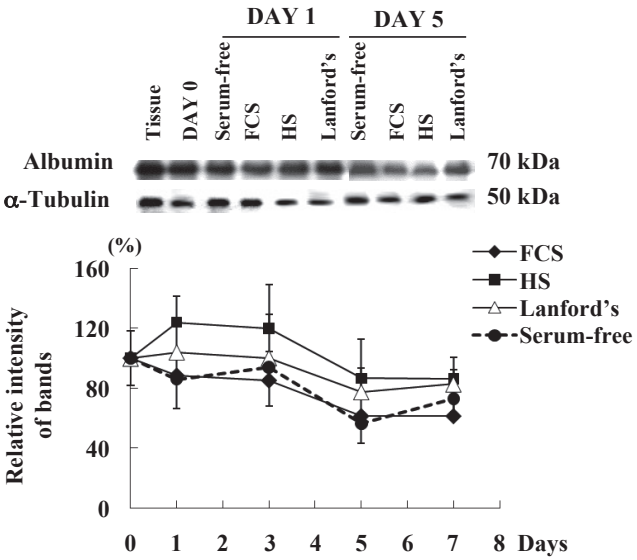

Fig. 2. Albumin protein levels analyzed using Western blotting. Quantitative results of detected proteins determined by densitometric analysis are shown. Protein levels are expressed as the percentage of the values in the day 0 group. Data are expressed as the mean ± S.E.M. and are representative of six independent experiments.

Albumin levels

The albumin protein levels as an indicator of liver function in the hepatocytes were investigated. The albumin levels were maintained at more than 80% of the baseline level (on day 0) until day 7 in the hepatocytes cultured with all media (Fig. 2).

CYP3A4 expression and activity in primary human hepatocytes

We first studied the spontaneous *CYP3A4* mRNA and protein expression in the primary human hepatocytes. Spontaneous *CYP3A4* mRNA expression was maintained at more than 80% of the level on day 0 for 3 days in the hepatocytes cultured with Lanford's, while in other mediums, it decreased to 50% of the day-0 level on day 3 or thereafter (Fig. 3A). CYP3A4 protein levels in the hepatocytes cultured with FCS-WEM and HS-WEM also gradually decreased from day 3.

On the other hand, CYP3A4 protein levels in the hepatocytes cultured with serum-free WEM and Lanford's maintained 80% of the day-0 level for 3 or 5 days, respectively (Fig. 3B). CYP3A4 activity was maintained up to 7 days in the hepatocytes cultured with serum-free WEM, Lanford's, and FCS-WEM, but its activity in hepatocytes cultured with HS-WEM tended to be lower than those in the other three media (Fig. 3C).

HNF-4α protein levels

HNF-4α is an important regulator for promoting *CYP3A* gene expression in the human liver (22, 23). Although a transient increase in the HNF-4α protein level was observed in the hepatocytes cultured with serum-free-WEM, Lanford's, and HS-WEM on day 1, these increases were gradually attenuated from day 3. HNF-4α levels were maintained for 7 days in the hepatocytes cultured with Lanford's. HNF-4α levels were significantly decreased on day 7 in the hepatocytes cultured with FCS-WEM compared with those cultured with Lanford's (Fig. 4).

Induction of CYP3A4 protein

The hepatocytes cultured with serum-free WEM and Lanford's for 2 days were incubated for 48 h in the presence of 20 and 50 μM of rifampicin or dexamethasone as typical CYP3A4 inducers. Rifampicin and dexamethasone increased CYP3A4 protein levels in the hepatocytes cultured with serum-free WEM. CYP3A4 protein levels in the hepatocytes were also increased by these inducers when cultured with Lanford's (Fig. 5).

Drug transporter protein levels in human hepatocytes

P-gp, MRP-2, and BCRP proteins were all expressed in human hepatocytes cultured with the four media. On day 3, P-gp protein levels were maintained at approxi-

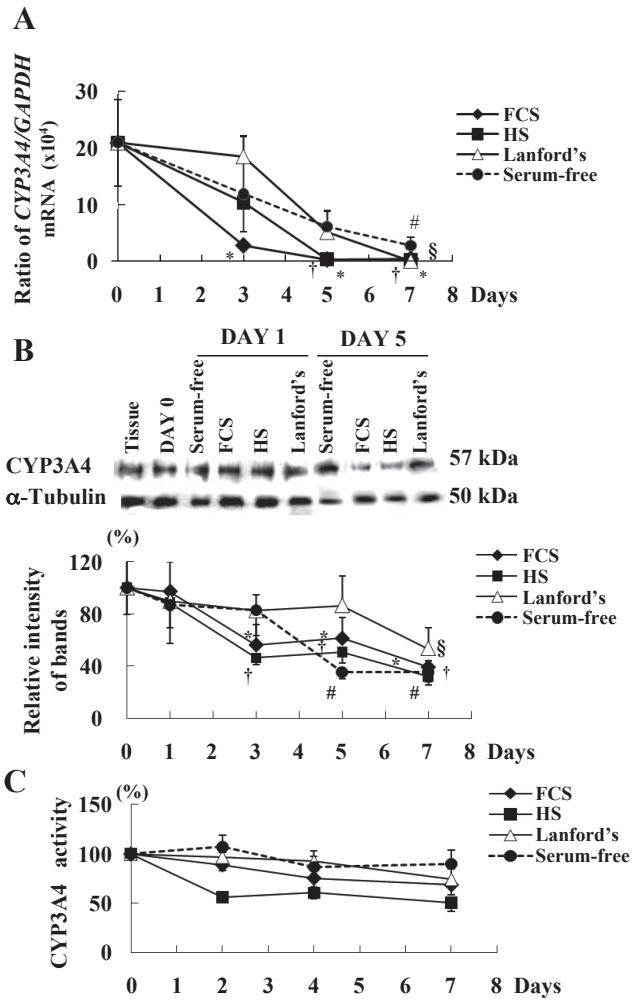

Fig. 3. Levels of *CYP3A4* mRNA, protein, and CYP3A activity in primary human hepatocytes. A: *CYP3A4* mRNA levels evaluated in hepatocytes cultured in four media using real-time RT-PCR. The expression of *CYP3A4* mRNA relative to *GAPDH* mRNA is shown as the mean ± S.E.M. Data are representative of six independent experiments. B: CYP3A4 protein levels analyzed by Western blotting. Quantitative results of detected proteins determined by densitometric analysis are shown. Protein levels are expressed as the percentage of the values in the day-0 group. Data are expressed as the mean ± S.E.M and are representative of seven independent experiments. CYP3A protein levels were significantly decreased on day 3 in hepatocytes cultured with FCM-WEM and HS-WEM, while those of hepatocytes cultured with Lanford's and serum-free WEM were maintained. α-Tubulin was used as the control. C: CYP3A activity measured in hepatocytes using the luciferase assay. CYP assay as new a method. CYP3A4 activity levels are expressed as a percentage of the values in the day-0 group. Data are expressed as the mean ± S.E.M. and are representative of six independent experiments. $^{*}P < 0.05$ vs. day 0 of FCS-WEM, $^{\dagger}P < 0.05$ vs. day 0 of HS-WEM, $^{\S}P < 0.05$ vs. day 0 of Lanford's, $^{\#}P < 0.05$ vs. day 0 of serum-free WEM.

mately 60% of those on day 0 in all culture media. On day 1, MRP-2 protein levels were maintained at 90% of those on day 0 in the hepatocytes cultured with FCS-

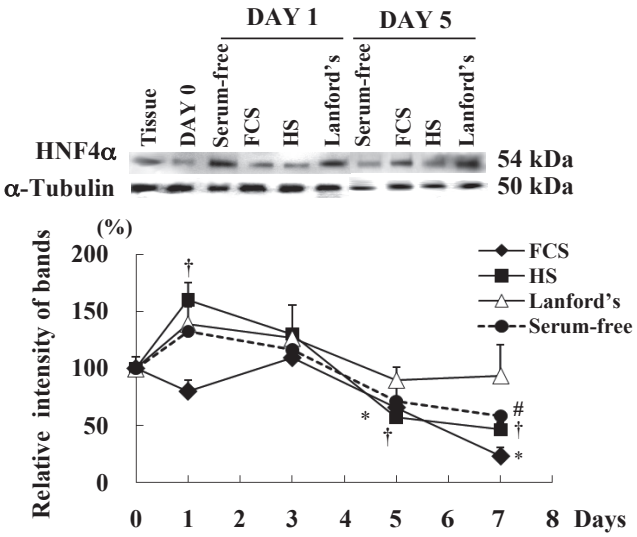

Fig. 4. HNF- 4α protein levels analyzed by Western blotting. Quantitative results of detected proteins determined by densitometric analysis are shown. Protein levels are expressed as the percentage of the values in the day-0 group. Data are expressed as the mean ± S.E.M. and are representative of six independent experiments. $^{*}P < 0.05$ vs. day 0 of FCS-WEM, $^{\dagger}P < 0.05$ vs. day 0 of HS-WEM, $^{\#}P < 0.05$ vs. day 0 of serum-free WEM.

WEM, HS-WEM, and Lanford's and then gradually decreased from day 3. MRP-2 protein levels were maintained at 70% or greater compared with those on day 0 for 7 days in the hepatocytes cultured with serum-free WEM. BCRP protein levels were maintained at more than 80% compared with those on day 0 in all culture conditions for 7 days (Fig. 6).

Other CYPs and OATP protein levels in human hepatocytes cultured with serum-free WEM and Lanford's

We also investigated the expression of CYP2C, CYP2D, and OATP in primary hepatocytes with serum-free WEM and Lanford's. Because only the antibodies of CYP2C19, CYP2D6, and OATP2 could be obtained, those protein levels were investigated in the hepatocytes with serum-free WEM and Lanford's. The protein levels of CYP2C19, CYP2D6, and OATP2 were well maintained at day 3 and 5, being similar to those observed on day 0, in the hepatocytes (Fig. 7).

Discussion

In the present study, we investigated the optimal culture conditions for maintaining spontaneous CYP3A4 expression and drug transporters activity in primary cultured human hepatocytes. Furthermore, we studied whether the hepatocytes can induce CYP3A expression with the addition of specific substrates to the culture

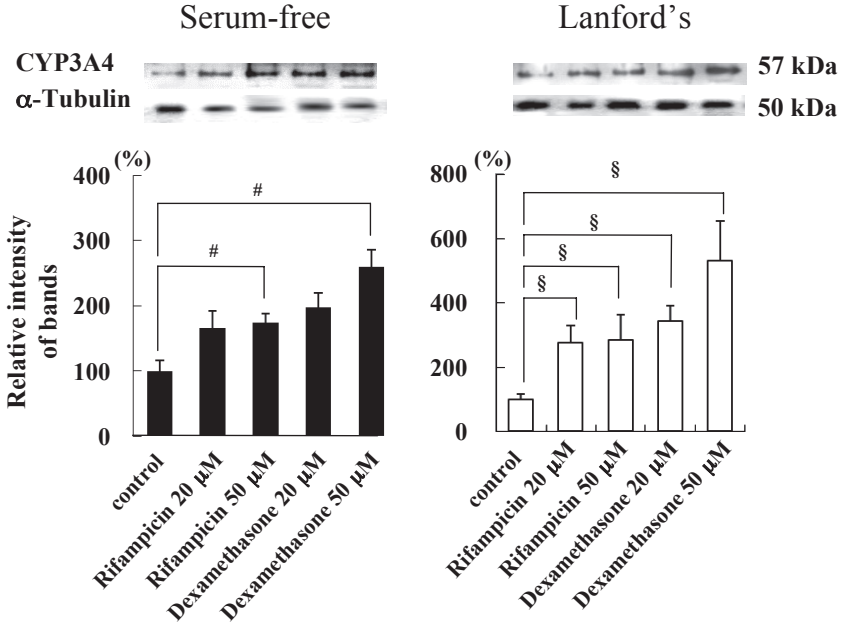

Fig. 5. Induction of CYP3A4 protein by CYP3A substrates in primary hepatocytes. Hepatocytes cultured with serum-free WEM and Lanford's were incubated with rifampicin and dexamethasone for 48 h. The final concentration of DMSO in culture medium was 0.1% (v/v), and 0.1% DMSO alone was added to the control culture. DMSO at 0.1% had no effect on CYP3A4 expression. CYP3A4 protein levels were analyzed by Western blotting. Quantitative results of detected proteins determined by densitometric analysis are shown. Protein levels are expressed as the percentage of the values in the control culture. Data are expressed as the mean ± S.E.M. and are representative of six independent experiments. $^{\#}P < 0.05$ vs. day 0 of serum-free WEM and $^{\S}P < 0.05$ vs. day 0 of Lanford's.

medium.

The primary culture of human hepatocytes is an important tool for evaluating drug-metabolizing ability of CYPs and transporters. In particular, as human CYP3A4 has very broad substrate specificity, many studies on therapeutic agents metabolized by CYP3A4 have been performed using primary cultured human hepatocytes in short-term culture of approximately 1 week because CYP3A activity is decreased more rapidly than those of other CYP family members in cultured human hepatocytes (24). CYP3A activity is reduced to 15% – 30% within the first 2 – 4 days in cultured human hepatocytes (7). Recently, several methods for maintaining hepatocytes function have been demonstrated: the use of defined culture medium (25), co-culture of hepatocytes with nonparenchymal cells (26), and the culture of hepatocytes as spheroids, etc. (27 – 29). However, published data on culture media that can maintain both CYP3A4 and transporter activities in a culture of freshly isolated human hepatocytes are limited. The choice of supplements including serum, growth factors, hormones, and other specific additives has been emphasized for cell survival and maintenance of liver function in primary cultured hepatocytes (30). Runge et al. established culture conditions allowing serum-free cultivation of human hepatocytes in the presence of hepatocyte growth factor (HGF) and epidermal growth factor (EGF) (31). With that medium, the cells maintained hepatocytes morphology for several weeks. Furthermore, the addition of serum or albumin to the medium was reported to support the growth of human hepatocytes (32 – 34).

Primary cultured human hepatocytes are generally known to exhibit diminishing albumin secretion over time during culture. However under our culture conditions, the albumin levels were maintained for 7 days in hepatocytes cultured with four media, suggesting that liver function was maintained (Fig. 2). Hepatocytes longevity was nearly equal among the four media. The number of hepatocytes was maintained for 14 days in all media (data not shown). The hepatocytes cultured with FCS-WEM or HS-WEM were transformed into fibroblast-like cells from day 3 of culture. On the other hand, the hepatocytes cultured in serum-free WEM and Lanford's maintained their intrinsic polygonal shape for 7 days (Fig. 1). Hepatocytes that redifferentiate into fibroblast-like cells, which generally occur during the first 2 days of culture, are nonresponsive to p450 enzyme inducers (35). Spontaneous *CYP3A4* mRNA levels were maintained at up to 80% of the baseline level (day 0) on day 3 in the hepatocytes cultured with Lanford's (Fig. 3A). However, after day 3, the hepatocytes cultured with the other media only showed 50% or less of the spontaneous *CYP3A4* mRNA levels seen on day 0. (Fig. 3A) On the other hand, CYP3A4 protein levels did not show a marked decline compared with baseline but were maintained for 5 days in the hepatocytes cultured with Lanford's (Fig. 3B). *CYP3A4* mRNA expressions were rapidly decreased more rapidly than protein levels in the hepatocytes. Thus *CYP3A4* mRNA levels may not always reflect the levels of protein and catalytic activity in the cells (36, 37).

CYP3A4 in hepatocytes is modulated by several factors including the medium. The decrease of CYP3A4 gene expression may be accompanied by a decrease in

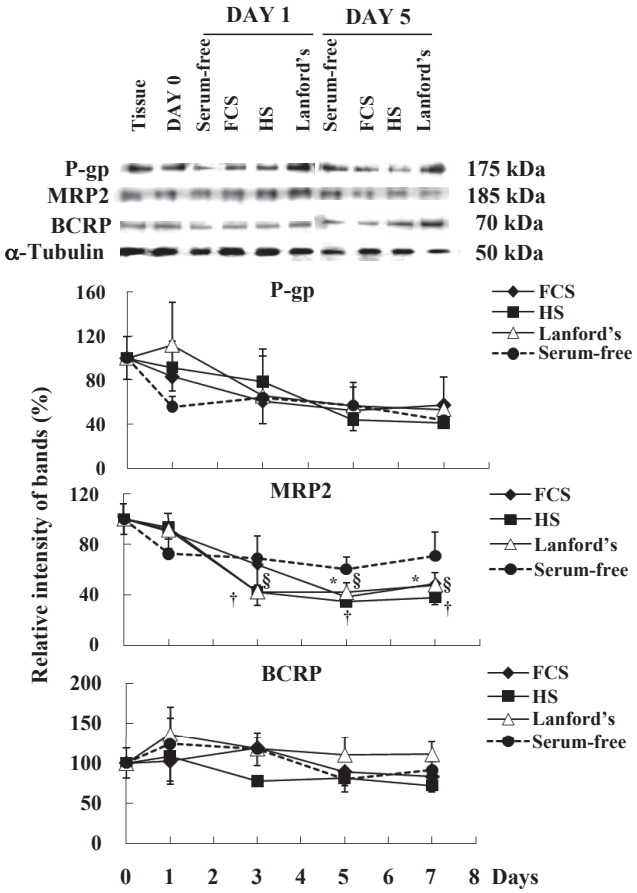

Fig. 6. Levels of P-gp, MRP-2, and BCRP analyzed by Western blotting. Quantitative results of detected proteins determined by densitometric analysis are shown. Protein levels are expressed as the percentage of the values in the day-0 group. Data are expressed as the mean ± S.E.M. and are representative of seven independent experiments. *$P < 0.05$ vs. day 0 of FCS-WEM, †$P < 0.05$ vs. day 0 of HS-WEM, §$P < 0.05$ vs. day 0 of Lanford's.

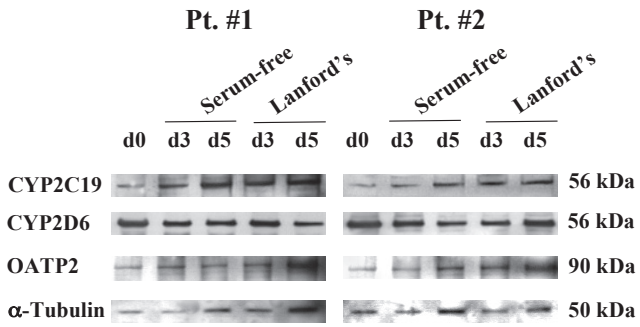

Fig. 7. Levels of CYP2C19, CYP2D6, and organic anion transporter polypeptide (OATP) analyzed by Western blotting. The protein levels were investigated in human hepatocytes cultured with serum-free WEM and Lanford's. Data are representative of four independent experiments and the results of two patients are shown in the figure.

transcription factors after hepatocytes isolation. CYP3A4 gene expression is controlled by targeting specific responsive elements in the regulatory region of the pregnane X receptor and constitutive androstane receptors (38). HNF-4*α* is enriched in the liver and associated with sustained CYP3A4 expression in hepatocytes (22, 23). Gómez-Lechón et al (37) reported that the mRNA levels of CYP3A4 decreased to 72%, while those of other CYPs were decreased to 97% in human hepatocytes during the first 20 h of culture under their conditions. In accordance with these findings, *HNF-4α* mRNA level was also decreased by approximately 80% at 21 h in the cultured hepatocytes (37). HNF-4*α* levels were similar to the *CYP3A4* protein level patterns (Fig. 4). These results suggest that maintaining CYP3A4 expression may be involved in HNF-4*α* function. The maintenance of spontaneous CYP3A4 and HNF-4*α* activity in our culture conditions could prolong the survival of hepatocytes compared with that in previous reports (37).

To investigate CYP3A4 function, analyses of the protein level and activity in hepatocytes were performed. However, CYP3A4 catalytic activity and protein measurements require a significant amount of sample material, which in the case of human in vitro studies is a serious drawback because the number of hepatocytes available is limited. In this study, we assessed the catalytic activity of CYP3A4 using the luciferase assay as a new technique (20, 21).

CYP3A4 activity was maintained up to 7 days in the hepatocytes cultured with three media, but not with HS-WEM (Fig. 3C). There was no decrease in CYP3A4 activity on day 7 in the hepatocytes cultured with any of the media compared with those of day 0, indicating that spontaneous CYP3A4 activity was maintained for 7 days (Fig. 3C). Overall, both serum-free WEM and Lanford's may be suitable for maintaining CYP3A4 activity.

To verify the induction of CYP3A4 expression, we investigated the CYP3A4 protein level after co-culture treated with the specific substrates, refampin and dexamethasone, in the hepatocytes cultured with serum-free WEM and Lanford's. The maintenance of spontaneous CYP3A4 protein expression and activity in the hepatocytes could be evaluated in the culture conditions used in the present study. Furthermore, the primary hepatocytes had the ability to induce p450 enzymes with the specific substrates. Rifampin and dexamethasone significantly increased CYP3A4 expression in the hepatocytes cultured with the serum-free WEM and Lanford's in dose-dependent manner (Fig. 5). The results were consistent with those reported in the literature (1, 2, 16). When cultured under appropriate conditions, human hepatocytes appear to respond to the p450 enzyme inducer

(35).

Hepatocellular transporters are also essential regulators of homeostasis and are involved in drug disposition and bile formation. Several ABC transporters have been observed to increase upon plating and cultivation in primary rat hepatocytes (39). Examining the process of the production of p-gp, MRP-2, and BCRP in the four media conditions, we demonstrated that the protein levels of these transporters were maintained for 7 days in the human hepatocytes cultured in all media (Fig. 6).

From our data, the human hepatocytes cultured by monolayer culture methods maintained both CYP3A4 and transporters. Considering, the maintenance of morphology, CYP3A4, and transporters expression, the culture methods using serum-free WEM or Lanford's may be useful for primary cultures of human hepatocytes. Finally, we also investigated the CYP2C19, CYP2D6 as typical other CYPs and OATP2 protein levels in the hepatocytes with serum-free WEM and Lanford's. The protein levels of CYP2C19, CYP2D6, and OATP2 were maintained at day 3 and 5, being similar to those of day 0 in the hepatocytes (Fig. 7). Further studies are needed to investigate whether the human hepatocytes can contribute to drug metabolism and toxicity in hepatocytes.

In conclusion, our culture methods may be useful for evaluating drug metabolism and transporters in primary cultures of human hepatocytes.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science, and Technology and a Grant for Research on Publicly Essential Drugs and Medical Devices from the Health and Labour Science Reseach Grants of Japan. The authors are grateful to Drs. Ken Matsumoto and Masahiro Mizuguchi of Nissui Pharmaceutical Co., Ltd., Diagnostic Research Department, for supplying Lanford's mediums.

References

1 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. 1995;23:415–421.

2 Silva JM, Morin PE, Day SH, Kennedy BP, Payette P, Rushmore T, et al. Refinement of an in vitro cell model for cytochrome P450 induction. Drug Metab Dispos. 1998;26:490–496.

3 Drobná Z, Walton FS, Harmon AW, Thomas DJ, Stýblo M. Interspecies differences in metabolism of arsenic by cultured primary hepatocytes. Toxicol Appl Pharmacol. 2010;245:47–56.

4 LeCluyse E, Madan A, Hamilton G, Carroll K, DeHaan R, Parkinson A. Expression and regulation of cytochrome P450 enzymes in primary cultures of human hepatocytes. J Biochem Mol Toxicol. 2000;14:77–88.

5 Runge D, Köhler C, Kostrubsky VE, Jäger D, Lehmann T, Runge DM, et al. Induction of cytochrome P450 (CYP)1A1, CYP1A2, and CYP3A4 but not of CYP2C9, CYP2C19, multidrug resistance (MDR-1) and multidrug resistance associated protein (MRP-1) by prototypical inducers in human hepatocytes. Biochem Biophys Res Commun. 2000;273:333–341.

6 Lykkesfeldt J, Loft S, Poulsen HE. Simultaneous determination of urinary free cortisol and 6 beta-hydroxycortisol by high-performance liquid chromatography to measure human CYP3A activity. J Chromatogr B Biomed Appl. 1994;660:23–29.

7 Liddle C, Goodwin BJ, George J, Tapner M, Farrell GC. Separate and interactive regulation of cytochrome p450 3A4 by triiodothyronine, dexamethasone, and growth hormone in cultured hepatocytes. J Clin Endcrinol Metab. 1988;83:2411–2416.

8 Juliano RL, Ling V. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. Biochim Biophys Acta. 1976;455:152–162.

9 Doyle LA, Yang W, Abruzzo LV, Krogmann T, Gao Y, Rishi AK, et al. A multidrug resistance transpoter from human MCF-7 breast cancer cells. Proc Natl Acad Sci U S A. 1998;95:15665–15670.

10 Borst P, Evers R, Kool M, Wijnholds J. The multidrug resistance protein family. Biochem Biophys Acta. 1999;461:347–357.

11 Maliepaard M, van Gastelen MA, de Jong LA, Pluim D, van Waardenburg RC, Ruevekamp-Helmers MC, et al. Overexpression of the BCRP/MXR/ABCP gene in a topotecan-selected ovarian tumor cell line. Cancer Res. 1999;59:4559–4563.

12 Robey RW, Medina-Pérez WY, Nishiyama K, Lahusen T, Miyake K, Litman T, et al. Overexpression of the ATP-binding cassette half-transporter, ABCG2 (Mxr/BCrp/ABCP1), in flavopiridol-resistant human breast cancer cells. Clin Cancer Res. 2001;7:145–152.

13 Liang Y, O'Driscoll L, McDonnell S, Doolan P, Oglesby I, Duffy K, et al. Enhanced in vitro invasiveness and drug resistance with altered gene expression patterns in a human lung carcinoma cell line after pulse selection with anticancer drugs. Int J Cancer. 2004;111:484–493.

14 Sunouchi M, Sakemi K, Kubota K, Kita J, Kamikawa Y, Uchida K, et al. [Network for the collection and distribution of Japanese liver tissues resected surgically for drug development and research use]. Kokuritsu Iyakuhin Shokuhin Eisei Kenkyusho Hokoku. 2005;123:68–72. (text in Japanese with English abstract)

15 Lanford RE, Carey KD, Estlack LE, Smith GC, Hay RV. Analysis of plasma protein and lipoprotein synthesis in long-term primary cultures of baboon hepatocytes maintained in serum-free medium. In Vitro Cell Dev Biol. 1989;25:174–182.

16 Li AP, Rasmussen A, Xu L, Kaminski DL. Rifampicin induction of lidocaine metabolism in cultured human hepatocytes. J Pharmacol Exp Ther. 1995;274:673–677.

17 LeCluyse EL. Human hepatocyte culture systems for the in vitro evaluation of cytochrome P450 expression and regulation. Eur J Pharm Sci. 2001;13:343–368.

18 Taniguchi R, Kumai T, Matsumoto N, Watanabe M, Kamio K, Suzuki S, et al. Utilization of human liver microsomes to explain individual differences in paclitaxel metabolism by CYP2C8 and CYP3A4. J Pharmacol Sci. 2005;97:83–90.

19 Takeba Y, Kumai T, Matsumoto N, Nakaya S, Tsuzuki Y, Yanagida Y, et al. Irinotecan activates p53 with its active metabolite, resulting in human hepatocellular carcinoma apoptosis. J Pharmacol Sci. 2007;104:232–242.

20 Cali JJ, Ma D, Sobol M, Simpson DJ, Frackman S, Good TD,

et al. Luminogenic cytochrome P450 assays. Expert Opin Drug Metab Toxicol. 2006;2:629–645.
21 Stresser DM, Broudy MI, Ho T, Cargill CE, Blanchard AP, Sharma R, et al. Highly selective inhibition of human CYP3Aa in vitro by azamulin and evidence that inhibition is irreversible. Drug Metab Dispos. 2004;32:105–112.
22 Tirona RG, Lee W, Leake BF, Lan LB, Cline CB, Lamba V, et al. The orphan nuclear receptor HNF4alpha determines PXR- and CAR-mediated xenobiotic induction of CYP3A4. Nat Med. 2003;9:220–224.
23 Wortham M, Czerwinski M, He L, Parkinson A. Wan YJ. Expression of constitutive androstane receptor, hepatic nuclear factor 4 alpha, and P450 oxidoreductase genes determines interindividual variability in basal expression and activity of a broad scope of xenobiotic metabolism genes in the human liver. Drug Metab Dispos. 2007;35:1700–1710.
24 Suzuki S, Sato T, Yoshino H, Kobayashi E. Impact of warm ischemic time on microsomal P450 isoforms in a porcine model of therapeutic liver resection. Life Sci. 2004;76:39–46.
25 Kojima T, Mochizuki C, Tobioka H, Saitoh M, Takahashi S, Mitaka T, et al. Formation of actin filament networks in cultured rat hepatocytes treated with DMSO and glucagon. Cell Struct Funct. 1997;22:269–278.
26 Yamasaki C, Tateno C, Aratani A, Ohnishi C, Katayama S, Kohashi T, et al. Growth and differentiation of colony-forming human hepatocytes in vitro. J Hepatol. 2006;44:749–757.
27 Ryan CM, Carter EA, Jenkins RL, Sterling LM, Yarmush ML, Malt RA, et al. Isolation and long-term culture of human hepatocytes. Surgery. 1993;113:48–54.
28 Katsura N, Ikai I, Mitaka T, Shiotani T, Yamanokuchi S, Sugimoto S, et al. Long-term culture of primary human hepatocytes with preservation of proliferative capacity and differentiated functions. J Surg Res. 2002;106:15–23.
29 Riccalton-Banks L, Liew C, Bhandari R, Fry J, Shakesheff K. Long-term culture of functional liver tissue: three-dimensional coculture of primary hepatocytes and stellate cells. Tissue Eng. 2003;9:401–410.
30 Castell JV, Gomez-lechon MJ. Liver cell culture techniques. Methods Mol Biol. 2009;481:35–46.
31 Runge D, Runge DM, Jäger D, Lubecki KA, Beer Stolz D, Karathanasis S, et al. Serum-free, long-term cultures of human hepatocytes: maintenance of cell morphology, transcription factors, and liver-specific functions. Biochem Biophys Res Commun. 2000;269:46–53.
32 Ferrini JB, Pichard L, Domergue J, Maurel P. Long-term primary cultures of adult human hepatocytes. Chem Biol Interact. 1997; 107:31–45.
33 Hino H, Tateno C, Sato H, Yamasaki C, Katayama S, Kohashi T, et al. A long-term culture of human hepatocytes which show a high growth potential and express their differentiated phenotypes. Biochem Biophys Res Commun. 1999;256:184–191.
34 Baba T, Touchi A, Ito K, Yamaguchi Y, Yamazoe Y, Ohno Y, et al. Effects of serum albumin and liver cytosol on CYP2C9- and CYP3A4-mediated drug metabolism. Drug Metab Pharmacokinet. 2002;17:522–531.
35 Madan A, Graham RA, Carroll KM, Mudra DR, Burton LA, Krueger LA, et al. Effects of prototypical microsomal enzyme inducers on cytochrome P450 expression in cultured human hepatocytes. Drug Metab Dispos. 2003;31:421–431.
36 Sumida A, Kinoshita K, Fukuda T, Matsuda H, Yamamoto I, Inaba T, et al. Relationship between mRNA levels quantified by reverse transcription-competitive PCR and metabolic activity of CYP3A4 and CYP2E1 in human liver. Biochem Biophys Res Common. 1999;262:499–503.
37 Gómez-Lechón MJ, Donato MT, Castell JV, Jover R. Human hepatocytes in primary culture: The choice to investigate drug metabolism in man. Curr Drug Metab. 2004;5:443–462.
38 Drocourt L, Ourlin JC, Pascussi JM, Maurel P, Vilarem MJ2. Expression of CYP3A4, CYP2B6, and CYP2C9 is regulated by the vitamin D receptor pathway in primary human hepatocytes. J Biol Chem. 2002;77:25125–25132.
39 Luttringer O, Theil FP, Lavé T, Wernli-Kuratli K, Guentert TW, de Saizieu A. Influence of isolation procedure, extracellular matrix and dexamethasone on the regulation of membrane transporters gene expression in rat hepatocytes. Biochem Pharmacol. 2002;64:1637–1650.