Secretion of Albumin and Induction of CYP1A2 and CYP3A4 in Novel Three-dimensional Culture System for Human Hepatocytes using Micro-space Plate

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Summary: We evaluated a novel primary three-dimensional culture system for human hepatocytes using micro-space plates. The functional activity of human hepatocytes in primary culture was determined by measuring albumin secretion from hepatocytes to medium and measuring expression levels of albumin, CYP1A2 and CYP3A4 mRNA. Albumin secretion was higher in micro-space plates compared with traditional plates after 72 h of culture; the levels of albumin secretion from hepatocytes to medium in culture using micro-space plates after 96 h of culture were 2.7-fold higher than those in culture using traditional plates, and secretion of albumin in micro-space plate culture subsequently remained constant. Expression levels of albumin, CYP1A2 and CYP3A4 mRNA in the culture of hepatocytes were significantly higher using micro-space plates than using traditional plates. The inducibility of CYP1A2 and CYP3A4 mRNA after exposure to inducers in hepatocyte culture on micro-space plates was comparable to that in culture on traditional plates, while expression of CYP1A2 and CYP3A4 mRNA after exposure to inducers was higher on micro-space plates than on traditional plates. The present study demonstrates that a novel primary three-dimensional culture system of cryopreserved human hepatocytes using micro-space plates could be used for evaluating the induction of drug-metabolizing enzymes in humans. This in vitro method may thus be useful for screening the induction potency of new drug candidates.

Keywords: three-dimensional culture system; micro-space plate; human hepatocyte; albumin secretion; CYP1A2 induction; CYP3A4 induction

Introduction

Drug-drug interactions are an important consideration in drug development. Human hepatocytes are used for the evaluation of induction of drug-metabolizing enzymes after exposure to new drug candidates.1–3) We have been studying the changes in mRNA levels for various drug-metabolizing enzymes in primary cultures of human hepatocytes following treatment with prototypical inducers such as rifampicin (Rif) and omeprazole (Ome).4) Rif induces CYP3A4 in primary cultures of human hepatocytes,5–7) while Ome is a potent inducer of CYP1A in primary cultures of human hepatocytes.8) We have evaluated induction potency of new drug candidates on CYP1A2 and CYP3A4 mRNA in primary monolayer cultures of human hepatocytes.9) However, the mRNA expression levels of drug-metabolizing enzymes, such as CYP1A2 and CYP3A4, are known to decrease in primary monolayer cultures of hepatocytes using traditional plates.1,10–12) Recently, a micro-space plate was developed by Yakemoto et al. as a tool for three-dimensional cell culture.13) The micro-space plate has regularly arranged compartments on its surface as shown in Figure 1. Therefore, we attempted to maintain the mRNA expres-
The micro-space plate has 200 μm × 200 μm × 50 μm square compartments on its culture surface.

Hepatocytes of Lot 100 were examined microscopically to determine cellular status after 3, 24, 72 and 120 h of culture. Arrows indicate spheroidal hepatocytes.

The present study investigated changes in the secretion of albumin; in expression levels of albumin, CYP1A2 and CYP3A4 mRNA; and in the induction potency of Rif and Ome for CYP3A4 and CYP1A2 mRNA in human primary hepatocyte cultures using traditional and micro-space plates.

**Materials and Methods**

**Materials:** Cryopreserved human hepatocytes (donor #1 [Lot 100], donor #2 [Lot LMP], and donor #3 [Lot VUA]) (Table 1) were purchased from In Vitro Technologies, Inc. (Baltimore, MD, USA). Rif and Ome were obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan); trypan blue was from Merck (Darmstadt, Germany); hepatocyte culture medium (CC–3198) was...
Table 1. Characteristics and preparation of human hepatocytes

<table>
<thead>
<tr>
<th>Donor</th>
<th>Age</th>
<th>Sex</th>
<th>Race/Strain</th>
<th>Viability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>#1 (Lot 100)</td>
<td>74</td>
<td>Female</td>
<td>Caucasian</td>
<td>92.3, 87.0</td>
</tr>
<tr>
<td>#2 (Lot LMP)</td>
<td>38</td>
<td>Female</td>
<td>Caucasian</td>
<td>82.6</td>
</tr>
<tr>
<td>#3 (Lot VUA)</td>
<td>68</td>
<td>Female</td>
<td>Caucasian</td>
<td>85.0</td>
</tr>
</tbody>
</table>

*Viability was determined by trypan blue dye exclusion.

from Lonza (Walkersville, MD, USA); RNeasy Mini Kit and QIAshredder were from Qiagen (Hilden, Germany); yeast tRNA was from Life Technologies, Inc. (Rockville, MD, USA); and TaqMan One-Step RT-PCR Master Mix Reagents were from Applied Biosystems (Foster City, CA, USA). All other chemicals used in this study were of reagent grade.

Primary culture of human hepatocytes: Primary culture of cryopreserved human hepatocytes was carried out according to the method of Nishimura et al.6 Cell suspensions with viability rates of 82.6–92.3%, as assessed by trypan blue dye exclusion, were used in the experiments (Table 1). Cell suspensions were diluted to a final concentration of 2.5 × 10^6 viable cells/mL using hepatocyte culture medium, and inocula of 1.0 × 10^5 viable cells/0.4 mL/well were introduced into 24-well plates. Culture plates (24 wells) were type I collagen-coated traditional plates (AGC Techno Glass Co., Ltd., Chiba, Japan) and the special glass-coated micro-space plates (Fig. 1) (Kuraray Co., Ltd., Tokyo, Japan). The micro-space plates have 200 μm × 200 μm × 50 μm compartments on their surface. Culture medium was sampled for determination of albumin concentration. The hepatocytes were used for measurement of lactate dehydrogenase (LDH) activities or mRNA levels at 120 h after inoculation.

Treatment of primary cultures of human hepatocytes with inducers: Ninety-six hours after inoculation, hepatocytes were treated with inducers for 24 h. During inducer treatment, hepatocytes were cultured without hEGF, gentamicin or amphotericin B under 5% CO₂ and 95% air at 37°C. Hepatocytes were exposed to the inducers Rif and Ome (10 and 50 μM each) for 24 h. Both inducers were dissolved in dimethyl sulfoxide (DMSO) at a final vehicle concentration of 0.1% (v/v). Controls were exposed to DMSO at the same final concentration.

Measurement of intracellular LDH activity: The hepatocytes were washed twice with 1 mL of ice-cold phosphate-buffered saline (−) and dissolved by adding 0.4 mL of 0.1% Triton X-100. The samples (hepatocytes dissolved with Triton X-100) were analyzed for LDH using an autoanalyzer (Model 7170, Hitachi Co., Tokyo, Japan). LDH activities were measured by the Japan Society of Clinical Chemistry transferable method using t type WAKO LDH J (Wako Pure Chemicals).

Preparation of total RNA: Total RNA was extracted from hepatocytes using the QIAshredder and RNeasy Mini Kit.

Measurement of albumin: Albumin concentrations in culture media were measured using the Human Albumin EIA Kit (Takara Bio Inc., Shiga, Japan).

Oligonucleotides: Pairs of forward and reverse primers and TaqMan probes for hypoxanthine phosphoribosyltransferase 1 (HPRT1),14 albumin,15 CYP1A2 and CYP3A4 used in the RT-PCR sequences have been reported previously. The primers and TaqMan probes were synthesized by Sigma-Aldrich Japan K.K. Genosys Division (Ishikari, Japan). TaqMan probes contained FAM (6-carboxyfluorescein) at the 5’-end and TAMRA (6-carboxytetramethyl-rhodamine) at the 3’-end, and were designed to hybridize to sequences located between the PCR primers.

TaqMan RT-PCR conditions: Total RNA was diluted to about 4 μg/mL with 50 μg/mL yeast tRNA. Total RNA at about 12 ng per 20 μL of reaction mixture was used for measurement of the target mRNA in each sample. For RT-PCR, the TaqMan One-Step RT-PCR Master Mix Reagents Kit (Applied Biosystems) containing 300 nM forward primer, 900 nM reverse primer, and 200 nM TaqMan probe was used at 20 μL/tube. RT-PCR assay was performed using the Applied Biosystems 7500 Fast Real-Time PCR System with the following profile: 1 cycle at 48°C for 30 min, 1 cycle at 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min. The threshold cycle (Ct) was calculated by the instrument’s software (7500 Fast System ver. 1.3.1).

Statistical analysis: Relative expression of each mRNA was calculated using the ΔΔCt method (where, ΔΔCt is the value obtained by subtracting the Ct value of HPRT1 mRNA from the Ct value of the target mRNA), as employed in previous studies.16,17 Specifically, the amount of target mRNA relative to HPRT1 mRNA is expressed as 2^−ΔΔCt. Data are expressed as the ratio of target mRNA to HPRT1 mRNA.

Experiments with hepatocyte cultures were performed in duplicate or triplicate, and the means or means ± SD were calculated. Statistical analysis was performed using Student’s t-test with Bonferroni correction. Significance was set at p < 0.05.

Results and Discussion

Cells in human primary hepatocyte cultures grown on micro-space plates have been reported to begin conglobating and showing some spheroids from day 3 (72 h) of culture, while spheroids were present in almost all micro-vessels on day 5 (120 h).13 In the present study, we evaluated a novel primary three-dimensional culture system using micro-space plates to determine the functional activity of human hepatocytes by measuring albumin...
secretion from hepatocytes to medium and measuring the expression levels of albumin, CYP1A2 and CYP3A4 mRNA.

During cultivation, the cells weakly adhered to the surface of the micro-space plate and were not suspended in the culture medium. Microscopic observation of the primary micro-space plate cultures of human hepatocytes is shown in Figure 2. Arrows after 72 and 120 h of culture indicate spheroidal hepatocytes. It was noted that the spheroidal hepatocytes at 120 h were larger than at 72 h.

The intracellular LDH activities, which are biomarkers for cell numbers, were comparable between traditional plates and micro-space plates at 120 h after inoculation (Fig. 3).

Albumin secretion was significantly smaller in micro-space plate culture than in traditional plate culture before 48 h. This may be due to a time-lag in achieving the secretion function, which may depend on the difference of cell adhesion in traditional plates and micro-space plates (Fig. 4). On the other hand, the liver-specific function of albumin secretion was higher in micro-space plate cultures after 72 h than in traditional plate cultures (Fig. 4). The level of albumin secretion of hepatocytes on micro-space plates after 96 h of culture was 2.7-fold higher than on traditional plates. The amount of albumin secreted in micro-space plate cultures subsequently remained constant.

In the present study, the changes in mRNA expression of albumin, CYP1A2 and CYP3A4 in primary cultures of the three Lots of cryopreserved human hepatocytes before inoculation and after 120 h of culture were investigated, and differences in mRNA expression levels between cultures using traditional plates and micro-space plates were compared (Figs. 5 and 6). As HPRT1 mRNA exhibited stable expression under various conditions and was confirmed to show the most stable expression of several housekeeping genes under the present experimental conditions (M. Nishimura, unpublished observation), it was selected as an endogenous control for the measurement of target mRNAs in the present study.

The ratios of albumin mRNA to HPRT1 mRNA in the hepatocyte cultures from Lots 100, LMP and VUA decreased to 1.6%, 1.9% and 5.8% of initial levels, respectively, using traditional plates after 120 h of culture, but decreased to 7.6%, 14% and 12%, respectively, using micro-space plates; the differences were statistically significant (Fig. 5).
Fig. 6. Changes in CYP1A2 and CYP3A4 mRNA expression in human primary hepatocyte cultures. The ratio of target mRNA to HPRT1 mRNA was determined using hepatocytes of all three Lots. Values are means±SD of triplicate human hepatocyte cultures. ***p<0.001 vs. traditional plates.

The ratios of CYP1A2 mRNA to HPRT1 mRNA in the hepatocyte cultures from Lots 100, LMP and VUA were 26%, 6.6% and 7.6% of initial levels, respectively, using traditional plates after 120 h of culture, but were 221%, 72% and 57%, respectively, using micro-space plates; the differences were significant (Fig. 6).

The ratios of CYP3A4 mRNA to HPRT1 mRNA in the culture of hepatocytes from Lots 100, LMP and VUA decreased to 1.3%, 0.22% and 0.098% of initial levels, respectively, using traditional plates after 120 h of culture, while the ratios decreased to 8.1%, 2.1% and 0.52%, respectively, using micro-space plates (Fig. 6). The differences were significant.

In the present study, 0.1% DMSO was used to dissolve the inducers, and hepatocytes were exposed to the inducers for 24 h; we previously found that 0.1% DMSO had no effects on induction. Moreover, we confirmed that the induction of drug-metabolizing enzyme and transporter mRNAs could be evaluated after 24 h of exposure to these inducers. The changes in CYP1A2 mRNA levels induced by exposure to Ome in primary cultures of cryopreserved human hepatocytes using traditional and micro-space plates are shown in Figure 7. On the traditional plate cultures, the level of CYP1A2 mRNA in hepatocytes from Lots 100, LMP and VUA increased by 74-, 25- and 101-fold, respectively, after exposure to 50 μM Ome compared with that in 0.1% DMSO-treated controls, while on micro-space plate cultures, the level increased by 34-, 32- and 41-fold, respectively. Therefore, the inducibility of CYP1A2 mRNA by exposure to

Fig. 7. Effects of exposure to Ome on CYP1A2 mRNA expression in human primary hepatocyte cultures. Hepatocytes were treated with Ome for 24 h at 10 or 50 μM. The ratio of CYP1A2 mRNA to HPRT1 mRNA was determined using all three Lots. Inset graphs use a logarithmic scale. Values are means of duplicate human hepatocyte cultures.
Novel Three-dimensional Culture for Human Hepatocytes

Ome using micro-space plates is similar to that using collagen-coated plates, and the amount of CYP1A2 mRNA expressed at 24 h of exposure to Ome using micro-space plates was higher than that using traditional plates.

The changes in CYP3A4 mRNA level induced by exposure to Rif in primary cultures of cryopreserved human hepatocytes using traditional and micro-space plates are shown in Figure 8. On the traditional plate cultures, the levels of CYP3A4 mRNA in hepatocytes from Lots 100, LMP and VUA increased by 9.4-, 12- and 24-fold, respectively, after exposure to 50 μM Rif, compared with that in 0.1% DMSO-treated controls, while on micro-space plate cultures, the levels increased by 12-, 48- and 25-fold, respectively. Therefore, the inducibility of CYP3A4 mRNA after exposure to Rif using micro-space plates was comparable to that using traditional plates, and the amount of CYP3A4 mRNA expressed after 24 h of exposure to Rif using micro-space plates was higher than that using traditional plates.

The novel primary three-dimensional culture system for human hepatocytes using micro-space plates maintains cell suspensions in the plates that are stationary under a CO₂ atmosphere, which allows for spheroid (three-dimensional) culture. This system therefore presents a simple method for three-dimensional culture of human hepatocytes, and is useful for maintaining mRNA expression levels of drug-metabolizing enzymes such as CYP1A2 and CYP3A4. Furthermore, we adopted a one-step real-time RT-PCR method in order to measure the mRNA expression levels of drug-metabolizing enzymes and transporters in human hepatocytes and tissues. This method has the advantages of high sensitivity, simplicity and linearity of quantification over a wide range of mRNA concentrations, making it particularly suitable for evaluating large numbers of samples. Therefore, the combination of this novel primary three-dimensional hepatocyte culture system and real-time one-step RT-PCR is a promising tool for evaluating the induction potency of new drug candidates for numerous drug-metabolizing enzymes and transporters.

Several studies have attempted rat hepatocyte spheroid formation in culture dishes and have reported possible beneficial effects for intercellular interactions on the viability of hepatocytes. In other experiments, the activities of drug-metabolizing enzymes such as CYP1A2 and CYP3A4 of human hepatocytes in culture were found to be significantly higher in micro-space plates than in traditional plates. In the present study, we also validated albumin secretion from hepatocytes to medium and the maintenance of mRNA expression levels of drug-metabolizing enzymes such as CYP1A2 and CYP3A4 in a novel primary three-dimensional culture system, suggesting that spheroid formation (three-dimensional formation of hepatocytes) mimics the cellular architecture found in native liver tissue.

In conclusion, the results of the present study demonstrated that the novel primary three-dimensional culture system for cryopreserved human hepatocytes using micro-space plates would be useful in preclinical drug development to evaluate candidates for the induction of drug-metabolizing enzymes in humans. Furthermore, this system can be used for the preliminary evaluation of the toxicity of drugs and chemicals.
References


