Cytochrome P450-dependent drug oxidation activities in commercially available hepatocytes derived from human induced pluripotent stem cells cultured for 3 weeks

Norie Murayama and Hiroshi Yamazaki

Laboratory of Drug Metabolism and Pharmacokinetics, Showa Pharmaceutical University, Machida, Tokyo 194-8543, Japan

(Received December 21, 2017; Accepted January 20, 2018)

ABSTRACT — Hepatocyte-like cells differentiated from human induced pluripotent stem (iPS) cells are of great interest for applications in pharmacological research. For drug metabolism testing, commercially available hepatocytes derived from human iPS cells are generally recommended to be used 1 week after seeding on plates. In this study, however, after 3-4 weeks of culture according to the manufacturer’s instructions, human cytochrome P450 (P450) 2C9- and 2C19-dependent diclofenac 4′-hydroxylation and omeprazole 5-hydroxylation activities of the iPS-derived hepatocytes had significantly increased above the activities at 1 week and had reached levels similar to those in HepaRG cells, a human hepatocyte-like cell line. This increase in activities was associated with increasing P450 2C9 and 2C19 mRNA levels. Human P450 3A4-dependent midazolam 1′/4-hydroxylation activities in the iPS-derived hepatocytes were also enhanced after 3 weeks of culture, but the levels were low compared with those of HepaRG cells. These results indicate that the induction of mRNA of typical P450s in human iPS-derived hepatocyte-like cells occurred after 3 weeks of normal culture conditions. However, the induction levels varied considerably depending on the pregnane X receptor pathway and/or the P450 isoform. Our findings that the hepatic functions of human iPS-derived hepatocytes were enhanced by 3 weeks of simple culture could facilitate the use of these cells for drug metabolism and toxicity testing.

Key words: iPSC-derived hepatocytes, HepaRG cells, P450 3A4, P450 3A5, P450 2C9, P450 2C19

INTRODUCTION

Hepatocyte-like cells differentiated from human induced pluripotent stem (iPS) cells have the potential for application in regenerative medicine, pharmacological drug screening, toxicity testing, and the prediction of interindividual drug metabolism (Scott et al., 2013; Schwartz et al., 2014; Takayama et al., 2014; Nakamori et al., 2016). Investigations of drug metabolism mediated by human cytochrome P450 [P450, general term for cytochrome P450 (EC 1.14.14.1), or CYP] enzymes are important procedures carried out during drug discovery and development (Hewitt et al., 2007; Turpeinen et al., 2009). However, it remains uncertain whether human iPS cell-derived hepatocytes can reproduce normal human drug metabolism, especially that mediated by P450-dependent drug oxidation activities. iPS cell-derived hepatocytes reportedly do not have sufficient expression of a series of drug-metabolizing P450 isoforms (Takayama et al., 2014; Nakamori et al., 2016; Sasaki et al., 2013). Therefore, the aim of current study was to systematically investigate P450-dependent hepatic functions of human iPS-derived hepatocytes commercially available 1, 2, 3, and 4 weeks after simple culture for drug metabolism and toxicity testing. To follow up the observations, we used liquid chromatography/tandem mass spectrometry to analyze the drug metabolizing capacities of commercially available hepatocytes derived from human iPS cells. The results were compared with those from HepaRG cells, the first human cell line able to differentiate into mature hepatocyte-like cells (Turpeinen et al., 2009). Commercially available hepatocytes derived from human iPS cells are generally recommended to be used 1 week after seeding to plates. Consequently, after 3-4 weeks of culture according to the manufacturer’s recommended procedure, human P450 2C9- and 2C19-dependent diclofenac 4′-hydroxylation and omeprazole 5-hydroxylation activities in the iPS-derived hepatocytes were sig-
nificantly enhanced to levels similar to those of HepaRG cells. These activities were associated with increasing P450 2C9 and 2C19 mRNA levels in the iPS-derived hepatocytes. Human P450 3A4-dependent midazolam 1′/4-hydroxyl activity in the iPS-derived hepatocytes were also enhanced after 3 weeks of culture, but the levels were still below those of HepaRG cells, despite steady high levels of P450 3A5 mRNA during 4 weeks of culture in the iPS-derived hepatocytes.

**MATERIALS AND METHODS**

Human HepaRG cells (one lot, HPR116180-TA08) and hepatocytes derived from human induced pluripotent stem (iPS) cells (ReproHepato, two lots, PU03 and PL29) were obtained from Biopredic International (Rennes, France) and Reprocell (Kanagawa, Japan), respectively. HepaRG and iPS-derived cells (4.8 × 10^5 cells per well) were initially seeded in collagen-coated and matrigel-coated 24-well culture dishes, respectively, and cultured with ReproHP medium (Reprocell) under conditions recommended in the manufacturers’ instructions (instead of fetal bovine serum).

The medium was replaced every 24 hr. P450-dependent drug oxidation activities of the hepatocytes were determined using 50 μM phenacetin (CAS registry number 62-44-2, > 99.0%, Tokyo Chemical Industry, Tokyo, Japan), 50 μM diclofenac (15307-86-5, > 98.0%, Tokyo Chemical Industry), 20 μM omeprazole (73590-58-6, > 98.0%, Tokyo Chemical Industry), 20 μM metoprolol (56392-17-7, > 98.0%, Tokyo Chemical Industry), and 5.0 μM midazolam (59467-70-8, > 98.0%, Sigma-Aldrich, St. Louis, MO, USA) in combination at days 8, 15, 22, and 29 of culture. After incubation for 1 hr at 37°C (5% CO2, v/v), the media were collected. The rates of metabolite formation were determined by liquid chromatography/tandem mass spectrometry as described previously (Murayama et al., 2014, 2015; Yajima et al., 2014). mRNA levels of human P450 1A2 (Hs01070374_m1), 2C9 (Hs04260376_m1), 2C19 (Hs00426380_m1), 3A4 (Hs01546612_m1), 3A5 (Hs00241417_m1), 3A7 (Hs00426361_m1), and pregnane X receptor (PXR, Hs1114267_m1) in three independent amplifications. Results are expressed as mean values (± S.D.) obtained from four to six wells of cultured iPS-derived cells.

**RESULTS AND DISCUSSION**

Because P450 1A2, 2C9, 2C19, 2D6, and 3A4/5 are typical human drug-metabolizing enzymes, P450-dependent probe drug oxidation activities were determined in HepaRG cells and in two lots of hepatocytes derived from human iPS cells (Table 1). After basic culture of 1-4 weeks from plating, HepaRG and iPS-derived cells were exposed to phenacetin, diclofenac, omeprazole, metoprolol, and midazolam (the probe drugs for P450 1A2, 2C9, 2C19, 2D6, and 3A4/5 recommended by US FDA drug interaction guidance) in combination for 1 hr (Table 1). All drug oxidation activities tested could be quantified in the HepaRG and iPS-derived cells. With respect to diclofenac, omeprazole, and metoprolol, there were statistically significant increases in oxidation activities of iPS-derived cells after 4 weeks of culture compared with those after 1 week of culture. These findings suggested good catalytic function of iPS-derived cells after 4 weeks of culture. Phenacetin O-deethylase activities at 1 and 4 weeks were comparable to those in HepaRG cells after 1 week of culture. However, P450 3A4/5-dependent midazolam hydroxylase activities in the iPS cell-derived cultures were much lower than those in HepaRG cells under the present conditions (Table 1). There were no differences in drug oxidation activities of the iPS-derived cells in the current assay conditions between simultaneous addition of the substrates and the separate individual addition of substrates to the culture media (data not shown). These results indicate that P450 1A2, 2C9, 2C19, and 2D6-dependent catalytic functions in the iPS-derived cells were present for up to 4 weeks in culture, despite the manufacturer’s recommended maximum 1 week of culture.

The induction of P450 1A2, 2C9, 2C19, and 3A4/5/7 mRNA and catalytic activities in human iPS-derived cells after 1, 2, 3, and 4 weeks of culture were assessed (Fig. 1). Microscopic examinations revealed the presence of binuclear iPS-derived hepatocytes after 2 weeks of culture and cell-cell interactions after 3 weeks (data not shown). There were statistically significant differences between the oxidation activities of diclofenac 4′-hydroxylase, omeprazole 5-hydroxylase, and midazolam 1′/4-hydroxylase between 1 week of culture and 3 or 4 weeks of culture. In terms of metoprolol O-demethylation activities, two sets of data at day 8 as control values were slightly different (Table 1 and Fig. 1D), this may result in the significance between 1 and 4 weeks (Table
### Table 1. Drug oxidation activities of HepaRG and iPS-derived cells at days 8 and 29.

<table>
<thead>
<tr>
<th>Hepatocyte</th>
<th>Day</th>
<th>Cell numbers per well</th>
<th>Phenacetin O-deethylation</th>
<th>Diclofenac 4'-hydroxylation</th>
<th>Omeprazole 5-hydroxylation</th>
<th>Metoprolol O-demethylation</th>
<th>Midazolam 4-hydroxylation</th>
<th>Midazolam 1'-hydroxylation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HepaRG</td>
<td>8</td>
<td>$1.8 \times 10^5$</td>
<td>0.93 ± 0.03</td>
<td>0.040 ± 0.004</td>
<td>4.1 ± 1.0</td>
<td>11 ± 2</td>
<td>1200 ± 280</td>
<td>20 ± 2</td>
</tr>
<tr>
<td>iPS-derived cells, lot PL29</td>
<td>8</td>
<td>$1.5 \times 10^5$</td>
<td>0.93 ± 0.24 (1.0)</td>
<td>0.004 ± 0.001 (1.0)</td>
<td>1.2 ± 0.2 (1.0)</td>
<td>7.0 ± 2.0 (1.0)</td>
<td>17 ± 4 (1.0)</td>
<td>94 ± 20 (1.0)</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>$1.1 \times 10^5$</td>
<td>1.2 ± 0.1 (1.3)</td>
<td>0.053 ± 0.006 (13*)</td>
<td>7.9 ± 8 (6.6*)</td>
<td>24 ± 4 (3.4*)</td>
<td>9 ± 1 (0.5)</td>
<td>26 ± 2 (0.3)</td>
</tr>
<tr>
<td>iPS-derived cells, lot PU03</td>
<td>8</td>
<td>$1.5 \times 10^5$</td>
<td>0.78 ± 0.19 (1.0)</td>
<td>0.002 ± 0.0004 (1.0)</td>
<td>1.0 ± 0.1 (1.0)</td>
<td>4.4 ± 1.1 (1.0)</td>
<td>7 ± 3 (1.0)</td>
<td>77 ± 6 (1.0)</td>
</tr>
<tr>
<td></td>
<td>29</td>
<td>$1.3 \times 10^5$</td>
<td>1.2 ± 0.1 (1.5)</td>
<td>0.027 ± 0.005 (14*)</td>
<td>6.5 ± 0.7 (6.5*)</td>
<td>16 ± 1 (3.6*)</td>
<td>4 ± 1 (0.6)</td>
<td>24 ± 3 (0.3*)</td>
</tr>
</tbody>
</table>

Numbers in parentheses indicate fold-changes in drug oxidation activities of iPS-derived cells at day 29 compared with those at day 8.  
*Significantly different ($p < 0.05$) from the activities at day 8 by unpaired t-test (n = 4).
Good catalytic function of the iPS-derived cells after 3 weeks of culture was indicated. Comparisons of mRNA levels during 1-4 weeks of culture revealed significant induction (> 2-fold) of P450 1A2, 2C9, 2C19, and 3A4 between 1 and 3 weeks of culture. In contrast, P450 3A5 and 3A7 levels after 4 weeks of culture were roughly similar to those after 1 week. These results indicated the induction of typical P450 mRNAs in cultured human iPS-derived cells. Human adult and fetal forms of P450 3A4 and 3A7 are regulated by levels of nuclear receptors such as PXR (Betts et al., 2015) which has been also reported to influence the regulation of P450 2C9 and 2C19 (Kojima et al., 2007). However, the induction levels under the recommended normal culture conditions in the present study substantially varied depending on the PXR pathway and/or the P450 isof orm. Ontogenesis of P450 2C enzymes in the human liver has been reported (Treluyer et al., 1997). Apparent abundance of P450 2C9 or 2C19 and P450 2C-dependent activities in the iPS cell-derived cultures after 3 weeks of culture in this study may roughly resemble this developmental expression of P450 2C in human livers (Treluyer et al., 1997) and could be one of good factors for P450 induction study, which were almost unable to investigate P450 2C enzyme induction using human hepatocytes commercially available (Yajima et al., 2014).

Although several studies have already reported efficient methods for differentiating human iPSC cells into hepatocyte-like cells (Kondo et al., 2014; Fukuda et al., 2017), the differentiation process is generally time consuming, cost intensive, complicated, and unstable. Human iPS-derived hepatocyte-like cells have reportedly expressed low levels of human P450 3A4, 3A5, and 3A7 mRNAs when cultured in a three-step culture protocol that resulted in limited numbers of differentiated cells (Kondo et al., 2014). These findings were partly inconsistent with the present results in which P450 3A4 mRNA was more abundant than P450 3A7 mRNA (Figs. 1I, 1L), and levels of PXR mRNA were at high 2-4 weeks of incubation (Fig. 1M). It was recently reported that induction of P450 3A4/5 mRNA and corresponding drug oxidation activities were detected even in human placental BeWo cells cultured with a modified medium containing 5% charcoal.

![Fig. 1](image_url)

**Fig. 1.** Drug oxidation activities (A-F) and mRNA expression levels (G-M) of cultured hepatocytes derived from iPS cells at days 8, 15, 22, and 29, along with the cell numbers per well (N). Results are expressed as mean values (± S.D.) obtained from four to six wells of cultured iPS-derived cells (lot PL29) (*p < 0.05 and **p < 0.01, one-way analysis of variance with Dunnett’s post tests).
Cytochrome P450 activities of iPSC-hepatocytes

Stripped fetal bovine serum to reduce any masking effects of endogenous cortisol-like hormones present in the recommended media (Murayama et al., 2017). Although the mechanism by which iPS-derived hepatocytes achieve good P450-dependent catalytic function after 3 weeks of culture is currently not known, endogenous hormones present in these media might be one of causal factors after 3 weeks of culture. Some changes in P450 activity due to culture time were consistent with reported findings (Yu et al., 2012). Human P450 1A2, 2C9, 2C19, and 2D6-dependent drug oxidation activities in iPS-derived hepatocytes after 3 weeks of culture were at levels similar to those of HepaRG cells. These findings indicate that multiple drug oxidation activities of human iPS-derived cells were enhanced by 3 weeks of simple culture that appeared to promote hepatic maturation. These cells, after prolonged culture, could be of use in drug metabolism and toxicity studies.

ACKNOWLEDGMENTS

The authors thank Drs. Makiko Shimizu, Yusuke Kamiya, and Yuichi Okuda for their advice on English language usage. This work was supported in part by the Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research 17K08426 (N.M.) and 17K08425 (H.Y.).

Conflict of interest— The authors declare that there is no conflict of interest.

REFERENCES


