Functional Evaluation of 3D-culture of Human Hepatocytes on Cell-able under Newly Optimized Condition.

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A multi-well plate microfabricated with highly hydrophilic polymer, Cell-able facilitates 3-dimensional (3D) culture of primary hepatocytes and induces long-term well-functioning culture, comparing to conventional monolayer. We report here the improved outcome using novel culture medium specialized for human hepatocytes and mouse fibroblasts as feeder cells. [Methods] Primary human hepatocytes were isolated from surgically resected liver tissue (Etical permission No.385 and 396) or purchased as cryopreserved hepatocytes (Xenotech). Hepatocytes (2x10^4/well) were seeded on 96-well type Cell-able that had been precultured with mouse fibroblasts (JCRB9019 (same as ATCC CCL-92) or ATCC CCL-163). Cells were cultured with RM101 (Transparent) based on Williams medium E containing 1% fetal bovine serum. Hepatocyte functions were evaluated by testosterone metabolism and transportation of tritiated-taurocholic acid and carboxy-dichlorofluorescein diacetate (CDF-DA). [Results and Discussion] Hepatocytes cultured on Cell-able with RM101 showed 1.55 to 3.43 fold higher activity of testosterone 6-beta hydroxylation of other 5 media (3 commercially available and 2 previously reported media) examined on day 7 and the superiority increased thereafter until day 21. Activity of testosterone 6-beta hydroxylation was maintained well for 54 days. Noticeably, the cells on day 54 increased the activity from 0.15 to 0.80 fmol/cell/min in response to rifampicin. Time course changes in taurocholate uptake were 24.1 fmol/cell (day of isolation), 2.84 (day 3), and 6.83 (day 7), while those of monolayer hepatocytes were 0.49 (day 3) and not detectable on day 7. The increase of the activity from day 3 to 7 on Cell-able was thought to be due to maturation of spheroid structure. The evidence of bilially efflux was examined by cofocal microscope using CDF-DA. The clear fluorescent spots were detectable intercellular area of the spheroids. When calcium ion was withdrawn from the culture medium, the intensity and sharpness of fluorescent spots became weaker and vaguer, suggesting the formation of small bile duct-like structure between hepatocytes. The present results indicate that Cell-able culture system suits for the examination of a sequence of reactions of DMPK research.

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INTRODUCTION

Outline and Features of Cell-able
Appearance of Cell-Able

Interculture area coated by block co-polymer. Neither proteins nor cells can adhere.

Cell culture area
Standard Protocol of Primary Hepatocyte Culture with Feeder Cells

① Plate surface coated with Block co-polymer

② Feeder Cells

③ Feeder + Hepatocytes

Advantage of co-culture with feeder cells

➢ Long-lasting CYP activities
➢ Cryopreserved hepatocyte with low attaching capability can be cultured

Optimization of culture medium enabled long-lasting CYP activities in feeder-free culture (see below)
Microstructure of Hepatocyte-feeder cell heterospheroids formed on the cell array

Downward view

Horizontal view

Magnified horizontal view

Diesse space-like structure was observed between hepatocyte (Hp) and feeder (HH) cell with microvilli rooted from hepatocyte was observed. m; mitochondria.

Arrow heads indicate HH cells that migrate from the culture plate and enwrap spheroidal hepatocyte mass.
Materials

[Hepatocytes]
Fresh; isolated from surgically resected liver in National Center for Child Health and Development (IRB permission No.385, 396)
Cryopreserved; Xenotech (799, HC2-6, HC5-7), IVT (TSF, GHA)

[Feeder cells]
HH bovine aortic epithelial cells (JCRB0099), Mouse 3T3 fibroblasts (ATCC CCL-92, ATCC CCL-163), Rhesus monkey retinal epithelial cells (ATCC CRL-1780)

[Culture medium]
RM100; medium for rat hepatocytes (Transparent)
RM101; Medium for human hepatocytes (Transparent)
SE & YY; Williams E-base Matrigel-containing medium (reported by Enosawa and Yamada in JSSX2009, Kyoto)
IVT; InVitroGRO HI Medium
XENOTECH; Hepatocyte culture media
BD; BD Hepatocyte Culture Medium Kit
Methods

[Culture]
2x10^4 human hepatocytes /one well of 96-well type Cell-able
When feeder cells were used, 8x10^3 cells / one well of 96-well type Cell-able were seeded two days before hepatocytes inoculation. In addition, cryopreserved feeder cell-seeded plates were also used.

[CYP activity]
Hepatocytes were incubated with 100 micro-mol/L testosterone or phenacetin for 3 hrs. Formation of metabolites (6 beta hydroxytestosterone, testosterone glucronide, acetaminophen) were determined by UPLC (Waters). CYP induction was performed by 72-hr incubation with rifampicin (25 micro-mol/L) or omeprazole (5 micro-mol/L).

[Transporter activities]
Influx 1; tritiated ([3H(G)]) taurocholate (1 micro-mol/L) as a substrate and Rifamycin SV (100 micro-mol/L) as an inhibitor.
Influx 2; tritiated ([6,7-3H(N)])-estrone sulfate (1 micro-mol/L) as a substrate and taurocholate (100 micro-mol/L) as an inhibitor.
Efflux; carboxy-dichlorofluorescein diacetate (CDF-DA) (10 micro-mol/L)
**Optimum Culture Medium**

**ASSAY:** Testosterone 6 beta hydroxylation

**CULTURE CONDITION:**
- **Sph H+F;** spheroids formed with human cryopreserved hepatocytes (Xenotech 799) and feeder cells (HH) on Cell-able
- **Sph H;** feeder-free spheroids of human cryopreserved hepatocytes (Xenotech 799) on Cell-able
- **Monolayer;** monolayer culture of human cryopreserved hepatocytes on type I collagen-coated plate

<table>
<thead>
<tr>
<th>Condition</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 21</th>
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<tbody>
<tr>
<td>RM100</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>RM101</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SE&amp;YY(JSSX)</td>
<td></td>
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<tr>
<td>IVT</td>
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<tr>
<td>BD</td>
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</table>

**RECOMMENDED**

RM101 (Transparent) showed Excellent CYP activities even with feeder-free culture
Optimum feeder cell
Hepatocytes; TSF, HC2-6, HC5-7, GHA

Comparison of feeder cells (basal activity)
Testosterone→6 Hydroxytestosterone

Comparison of feeder cells (basal activity)
Testosterone→Testosterone glucronide

Cell lines examined

<table>
<thead>
<tr>
<th>Designation</th>
<th>Code No.</th>
<th>Origin</th>
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<tbody>
<tr>
<td>HH</td>
<td>JCRB0099</td>
<td>Bovine aortic epithelium</td>
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<tr>
<td>3T3-Swiss albino</td>
<td>ATCC CCL-92 (JCRB9019*)</td>
<td>Mouse fibroblast</td>
</tr>
<tr>
<td>BALB/3T3 clone A31</td>
<td>ATCC CCL-163</td>
<td>Mouse fibroblast</td>
</tr>
<tr>
<td>RF/6A</td>
<td>ATCC CRL-1780</td>
<td>Rhesus monkey retinal epithelium</td>
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</table>

*ATCC CCL-92 is also distributed by JCRB as JCRB9019 in Japan
Optimum feeder cell (Induction study)

Hepatocytes; Xenotech HC2-6

Induction study (Rifampicin)

Testosterone → 6 hydroxy testosterone

Induction study (Omeprazole)

Phenacetin → Acetaminophen

Optimum feeder cell (Induction study)

RECOMMENDED

Chromatography

1; 6-beta hydroxytestosterone
2; Testosterone glucronide
3; Testosterone
Long-lasting CYP Activity of Cryopreserved Human Hepatocytes Cultured on Cell-able Hepatocytes; Xenotech HC2-6 and HC5-7 Feeder cells; ATCC CCL-92

The initial activity of each lot was 549.6 and 214.8 pmol/2x10^4/h, respectively.
Influx Transporter Activity (1)

Hepatocytes; freshly isolated human hepatocytes
Feeder; ATCC CCL-163
Tracer; [3H(G)]-taurocholate
inhibitor; Rifamycin SV

Spheroid culture by Cell-able
Day 7

Monolayer culture by collagen-coated plate
Day 7

Hepatocyte spheroids showed good influx transporter activity.
Non-specific incorporation was determined under the existence of inhibitor (Rifamycin SV).
Hepatocyte spheroids showed good influx transporter activity.
Non-specific incorporation was determined under the existence of inhibitor (taurocholate).
Hepatocyte spheroid showing efflux transporter activities examined by CDF-DA exclusion into intercellular bile pools

hepatocytes; freshly isolated human hepatocytes
feeder cells; ATCC CCL-92

Culture day 2  day 4  day 7

Bile pool formation and CDF exclusion were becoming marked with the increase of culture days or maturation of spheroid. (Above)

Bile pools almost disappeared by removal of Ca^{2+} ions. (Left)
Conclusion

✧ The best medium for human hepatocyte spheroid culture on Cell-able is RM101 by Transparent.
✧ The RM101 medium can maintain CYP activities without feeder cells on Cell-able.
✧ Mouse 3T3 fibroblasts are more effective on hepatocytes culture as feeder cells than bovine endothelial of monkey epithelial cells on Cell-able.
✧ The human hepatocyte spheroids formed on Cell-able showed influx and efflux transporter activities.
Acknowledgment

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