Directional Drug Transport through Membrane-Supported Monolayers of Human Liver-Derived Cell Lines

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Note

INTRODUCTION

In vitro assay systems are important for evaluating characteristics such as absorption, distribution, metabolism, excretion, and toxicity in the development of new drugs.1) Evaluation of hepatotoxicity, especially toxicity related to cholestasis, is especially important, because hepatotoxicity is one of the main reasons for the withdrawal of candidate drugs from clinical trials or the withdrawal of approved drugs from the market. Several evaluation systems for drug-induced cholestasis,2,3) drug-induced bile duct injury,4) and drug-induced liver injury5–7) have been reported. Moreover, it is important to know in the early stages of drug discovery whether a drug candidate is excreted in bile, as active biliary excretion of a drug may reduce liver burden and toxicity. However, although biliary canaliculus-like networks can be formed by culturing liver-derived cells,8–14) there is still a need for improved assays to screen candidate drugs for cholestatic liver disease.

Therefore, the aim of the present work is to develop a new assay system for biliary excretion drugs, using human liver-derived cell lines HepG2 and Huh-7 grown on insert membranes.

MATERIALS AND METHODS

Materials HepG2 and Huh-7 cells were obtained from JCRB Cell Bank (Osaka, Japan). The reagents and cell culture medium used in this study are described in the supplementary materials.

Cell Culture HepG2 and Huh-7 cells were cultured in a humidified atmosphere of 5% CO₂ in air at 37°C. They were seeded at a density of 2.2 × 10⁴ cells/well for HepG2 cells or 2.0 × 10⁵ cells/well for Huh-7 cells on 12-well membrane inserts, i.e., Transwell (Costar® 3494, Corning, NY, U.S.A.) or Vitrigel (ad-MED Vitrigel®2, Kanto Chemical, Tokyo, Japan) (Day 0). Where the Vitrigel is an insert using collagen Vitrigel® membrane, and the Transwell is an insert made of collagen-treated polytetrafluoroethylene membrane. At one and four days after seeding, the medium on the both apical and basal sides was replaced with fresh medium containing 0.1% dimethyl sulfoxide (DMSO). Incubation was continued for three days, and then experiments were performed (Day 7). In the case of the test of adding bile acid, when the medium was changed (Days 1 and 4), the medium on the apical side was replaced with fresh medium containing 0.1% DMSO with bile acid (100 µM), while the medium on the basal side was replaced with fresh medium containing 0.1% DMSO without bile acid.

Transport Assay The transepithelial electrical resistance (TEER) of the cell monolayers was measured using a Millicell®ERS-2 (Millipore, MA, U.S.A.). The inserts were washed with saline (9.0 g/L NaCl) and the cell monolayers were pre-incubated with the transport buffer i.e., HBSS-2-(4-(2-hydroxyethyl)piperazin-1-yl)ethanesulfonic acid (HEPES) (9.7 g/L Hanks’ Balanced Salts, 2.34 g/L HEPES, 0.34 g/L NaHCO₃, pH 7.4) for 10 min at 37°C. Transport experiments...
were initiated at 37°C by adding the transport buffer containing 24 μM sulfobromophthalein (BSP) to the donor side, while the receiver side was filled with transport buffer (the final DMSO concentration was 1% on both sides). During inhibition studies, 100 μM MK-571 was added to both sides as an inhibitor. Samples were collected at 15, 30, 45, and 60 min, and replaced with equal amounts of transport buffer. The method for quantifying the transported compound is described in the supplementary materials. The apical-to-basal direction was designated as A-to-B, and the opposite direction was designated as B-to-A. The apparent permeability coefficient ($P_{app}$, cm/s) was calculated according to the following formula (Eq. 1).

$$P_{app} = \frac{\frac{dQ}{dt} \times \frac{1}{A \times C_0}}{}$$

Where $\frac{dQ}{dt}$ is the rate of compound transfer (pmol/s) into the receiver side, $A$ is the surface area of the membrane insert (cm²), and $C_0$ is the initial concentration of the compound in the donor side. When the samples were taken at 15, 30, 45, and 60 min after the start of the transport assay, the amount of compound was plotted to obtain a regression line. The slope of the regression line was set as the initial rate of the compound transfer. The efflux ratio (ER) was calculated as the ratio of $P_{app,B-to-A}$ to $P_{app,A-to-B}$.

**Quantification of mRNA Expression Level** The mRNA expression levels of efflux transporters were measured by quantitative RT-PCR with reference to previous reports, as described in the supplementary materials. Plateable Human CryoHepatocytes (Lot No. 303, Corning) was used as a reference for mRNA expression levels of efflux transporters.

**Data Analysis** Quantitative data are presented as the mean ± standard deviation (S.D.). Statistical analysis was undertaken using Student’s t-test and Dunnett’s test as implemented in Pharmaco Basic software (Scientist Press Co., Ltd., Tokyo, Japan).

### RESULTS

The expression levels of efflux transporter mRNAs in the HepG2 and Huh-7 cell lines were compared with those in primary human hepatocytes (PHH). Multidrug resistance-associated protein 2 (MRP2) mRNA expression was 3.5-fold and 1.4-fold higher, respectively, in HepG2 and Huh-7 cells, compared with PHH, while the expression levels of P-glycoprotein (P-gp) and breast cancer resistance protein (BCRP) in these cell lines were markedly lower (Fig. 1).

When HepG2 and Huh-7 cells were seeded on Transwell and Vitrigel membranes (Supplementary Fig. 1), cell monolayers were formed. Transport of BSP through these monolayers was examined. The ER of BSP through the HepG2 cell monolayer on the Transwell was 1.39, while that through Huh-7 cells was 1.27. The MRP2 inhibitor MK-571 reduced both ERs to approximately 1 (Table 1), indicating the loss of directional transport.

The stimulating effect of bile acids on HepG2 cells was also examined. Several bile acids significantly increased ER or TEER, and Chenodeoxycholic acid significantly increased both (Table 2).

![Fig. 1. mRNA Expression of Efflux Transporters in HepG2 and Huh-7 Cells](image)

| Transwell | Vitrigel |
|-----------|----------|
| HepG2     | 0.95 ± 0.08 (1.10 ± 0.06) | 1.35 ± 0.09 (1.11 ± 0.08)* |
| Huh-7     | 1.27 ± 0.06 (1.00 ± 0.06)* | ND |

ER in the presence of the inhibitor MK-571 is shown in parentheses. Mean ± S.D. ($n = 3–4$).** $p < 0.05$, * $p < 0.01$ (Dunnett’s test, vs. Control).

| Stimulating Effect of Bile Acids on Directional Transport of BSP |
|---------------------------------------------------------------|
| *HepG2 on Transwell*                                         |

|                      | ER | TEER (Ω·cm²) |
|----------------------|----|------------|
| Control              | 1.38 ± 0.06 | 69 ± 9 |
| Cholic acid          | 1.61 ± 0.06* | 75 ± 21 |
| Deoxycholic acid     | 1.31 ± 0.07 | 91 ± 20 |
| Taurocholic acid     | 1.46 ± 0.09 | 215 ± 43** |
| Lithocholic acid     | 1.53 ± 0.07 | 80 ± 10 |
| Chenodeoxycholic acid| 1.62 ± 0.08** | 110 ± 16** |
| Glycochenodeoxycholic acid | 1.70 ± 0.03** | 52 ± 11 |
| Taurochenodeoxycholic acid | 1.48 ± 0.03 | 47 ± 8 |
| Taoursodeoxycholic acid | 1.23 ± 0.03 | 66 ± 18 |

The electrical resistance of a blank Transwell was approximately 108 Ω·cm². Mean ± S.D. ($n = 3–4$ for ER or 6–8 for TEER).** $p < 0.05$, * $p < 0.01$ (Dunnett’s test, vs. Control).

### DISCUSSION

We confirmed that HepG2 and Huh-7 cells highly express MRP2, and therefore we chose BSP, a substrate of MRP2, to evaluate directional transport in our system. The observed ER values indicate that $P_{app,B-to-A}$ is higher than $P_{app,A-to-B}$ (Supplementary Fig. 2). Moreover, this efflux transport was significantly suppressed by MK-571, an inhibitor of MRPs. Here, if the toxicity of MK-571 is such that it loosens tight junctions between adjacent cells, this could bring the ER closer to 1. Hence, we measured TEER after BSP transport study and found that MK-571 had no effect on TEER (Supplementary Fig. 2D). Therefore, we concluded that the effect of MK-571 on the ER value was due to the inhibition of MRP2, not toxicity. Further study with 5-(and-6)-carboxy-2'-7'-
dichlorofluorescein (DCF), another substrate of MRP2, indicated that this substrate is also directionally transported through Huh-7 monolayers on Vitrigel (Supplementary Fig. 3). In addition, there was a significant difference between \( P_{\text{app,A-to-B}} \) and \( P_{\text{app,B-to-A}} \) for BSP and DCF (Supplementary Figs. 2, 3). In contrast, there was not a significant difference between \( P_{\text{app,A-to-B}} \) and \( P_{\text{app,B-to-A}} \) for lucifer yellow and antipyrene, which are known as markers for the paracellular and transcellular routes, respectively (Supplementary Table 2). Hence, using this system we could evaluate the directional transport through monolayers of MRP2 substrate, although cell–cell adhesion may be weak.

Because HepG2 on Transwell inserts showed the highest ER of BSP among the combinations studied here, we next examined whether bile acid stimulation influenced the ER and TEER. It has been reported that bile acids induce the expression of efflux transporters.\(^{19}\) Therefore, we added bile acids to the substrate solutions expecting that the directional drug transport would be enhanced. Although several tested bile acids significantly increased the ER and/or TEER of HepG2 cells, we found no clear relationship between them. It has been reported that mouse cholestatic liver injury caused by bile duct ligation increases ezrin-radixin-moesin protein, which is a scaffold protein for tight junction.\(^{20}\) Hence, we inferred that the exposure of hepatocytes to a bile acid in our system may have increased TEER by increasing a scaffolding protein, but that additional studies would be needed to prove this.

Epithelial-derived cell lines such as Caco-2 and LLC-GAS-COL150 seeded on a membrane are reported to transport compounds faster in the B-to-A direction than in the A-to-B direction, and transport studies have been conducted using these cell lines.\(^{21–24}\) Our findings show that liver-derived cell lines behave similarly. Our observations are consistent with the fact that MRP2 is involved in the biliary excretion of many drugs in vivo.\(^{25,26}\) Therefore, our system may be suitable for assay of biliary excretion drugs. However, our assay system currently has several unresolved issues. It has been reported that hepatocytes are known to form apical membrane structures between adjacent cells,\(^{8,14}\) but we do not confirm how these structures are formed in our system. Thus, we will need to examine the localization of tight junction markers and of transporters by immunostaining. It is also necessary to investigate the effects of paracellular transport and influx transporters for test compounds. Further studies are in progress to validate this assay system.

In conclusion, our results show that monolayers of human liver-derived cell lines on a membrane exhibit directional drug transport mediated by MRP2. The advantage of our system is the potential to quantitatively evaluate biliary excretion of MRP2 substrates in vitro. Therefore, the assay system using these cell lines would be suitable for screening biliary excretion drugs and for investigating the hepatotoxicity of candidate drugs. Because directional drug transport was found to be enhanced by bile acids, we have not tested the system using bile acids as a substrate. The next step for the evaluation of this system is to verify that the bile acids themselves are exflxed to the bile-duct side. Drug induced cholestasis causes severe hepatotoxicity by inhibiting the bile acid transporters. Our system, using bile acids as substrates, might prove effective in screening of drugs that cause cholestasis.

Acknowledgments This research was funded by the Japan Agency for Medical Research and Development (AMED), Grant No. 16nk0101051h0201.

Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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