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Carrier-mediated placental transport of cimetidine and valproic acid across differentiating JEG-3 cell layers

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Human choriocarcinoma has been used as a model to study trophoblast transcellular drug transport in the placenta. Previous models had limitations regarding low molecular weight drug transport through the intracellular gap junction. The purpose of this study was to evaluate placental carrier-mediated transport across a differentiating JEG-3 choriocarcinoma cell (DJEGs) layer model in which the intracellular gap junction was restricted. Cimetidine is the substrate of an efflux transporter, breast cancer resistance protein (BCRP). BCRP highly expressed in the placenta, and its function in the DJEGs model was investigated. In addition, the placental drug transport of another efflux transporter, multidrug resistance-associated proteins (MRPs), and an influx transporter, monocarboxylate transporter (MCT), were examined with various substrates. Cimetidine permeated from the fetal side to the maternal side at significantly high levels and saturated in a dose-dependent manner. The permeability coefficient of a MRP substrate, fluorescein, across the DJEGs model was significantly increased by inhibiting MRP function with probenecid. On the other hand, permeation in the influx direction to the fetal side with a substrate of MCT, valproic acid, had a gentle dose-dependent saturation. These findings suggest that the DJEGs model could be used to evaluate transcellular placental drug transport mediated by major placental transporters.

1. Introduction

Drug transport from the maternal to fetal blood across the placenta is mainly determined by the size and lipid solubility of the drug molecule and the transcellular transport pathway involved, which includes carrier-mediated transports. The main barrier in the placental transcellular pathway is the syncytiotrophoblast layer (Fig. 1). Previous studies investigated an *in vitro* placental drug transport model by using differentiating human choriocarcinoma JEG-3 cell layers (DJEGs) (Fig. 2). These studies demonstrated that the DJEGs model had several syncytiotrophoblast-like features: secretion of human chorionic gonadotropin (hCG); high levels of breast cancer resistance protein (BCRP), an efflux transporter expressed on the placenta; and cell-cell fusion function. The functionality of the barrier is also sufficient to evaluate transcellular drug transport across cell layers by using measures of transepithelial electrical resistance (TEER) (Ikeda et al. 2011, 2012). However, to evaluate drug transport with *in vitro* models, we have to confirm that the models have functioning transporters. In this study, we used the substrates of each transporter to investigate whether some transporters, which are known to be expressed on the syncytiotrophoblast, could also be functional in the DJEGs model.

In the DJEGs model, the confirmed high levels of BCRP expression demonstrated an active efflux function, suggesting that the model reflects the main efflux feature of exogenous materials across the placenta *in vivo*. It was reported that the rat placenta, as well as the human placenta, had high levels of BCRP expression, and the transport of cimetidine, a substrate of BCRP, was high levels in the pathway from the fetal to the maternal side. This predominant transport to the maternal side was restricted by BCRP inhibitors (Staud et al. 2006). Moreover, Pavek et al. (2005) reported that the intracellular incorporation of cimetidine increased under the coexistence of BCRP inhibitors in Madin-Darby canine kidney (MDCK) II cells transfected with human BCRP genes. This demonstrated definitely that cimetidine is also a substrate of human BCRP. Furthermore, cimetidine is not metabolized by the human placenta (Schenker et al. 1987), had low levels when transported passively, and was not a substrate of p-glycoprotein (p-gp, MDR1) (Staud et al. 2006). Therefore, cimetidine is a suitable substrate for evaluating the placental transport function of BCRP. The first purpose of this study was to validate cimetidine transport in the DJEGs model to evaluate whether it reflects efflux transporter function *in vivo*.

Our second purpose was to investigate another efflux transporter, multidrug resistance-associated proteins (MRPs). To validate its drug transport function in the DJEGs model, fluorescein was used. Fluorescein has been reported to be a substrate of MRPs, and probenecid an inhibitor (Utoguchi et al. 2000; Sun et al. 2001; Hamilton et al. 2007; Hawkins et al. 2007).

The third purpose was to evaluate the function of an influx transporter expressed on the placenta by using the DJEGs model. Teratogenicity was indicated during treatment with valproic acid in pregnancies, and it was known that the placental transport

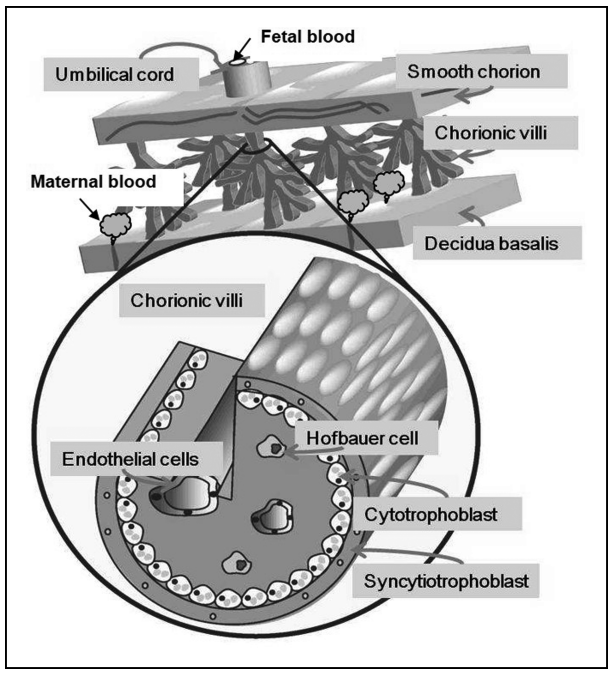

Fig. 1: Overview of the placental membrane.

rate of valproic acid was high (Forestier et al. 2003; Briggs et al. 2011). In addition to the physical properties such as small molecular size and high lipophilicity, it was hypothesized that the transport of valproic acid from the maternal to fetal side might be mediated by an influx transporter, monocarboxylate transporter (MCT) (Deguchi et al. 1997; Kumar et al. 2000; Utoguchi et al. 2000; Ushigome et al. 2001; Nakamura et al. 2002; Settle et al. 2004; Silva 2004). Moreover, in a report of valproic acid fetal transitivity with human materials, the fetal to maternal blood concentration (F/M ratio) of valproic acid was 1.38, indicating high levels in the fetus (Forestier et al. 2003). Based on these reports, valproic acid transport in the DJEGs model was investigated to further validate the model as a representative of placental drug transport.

Our study as a whole investigated whether the DJEGs model could reflect features of the placental carrier-mediated transport *in vivo*, by evaluating the characteristics of the DJEGs model with the substrates of several transporters expressed in the placenta.

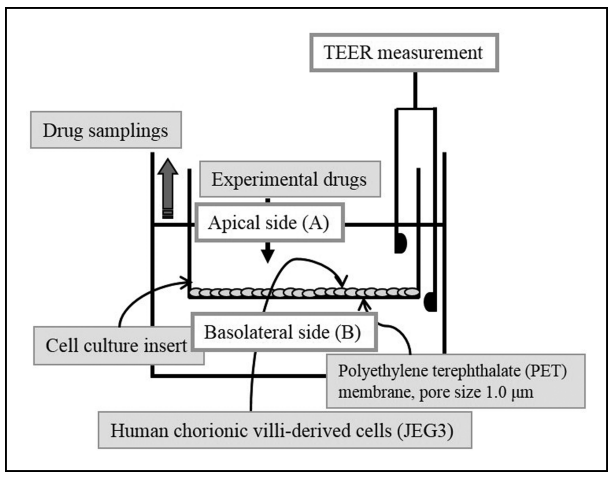

Fig. 2: Diagram of culturing human choriocarcinoma cell layers. TEER: transepithelial electric resistance.

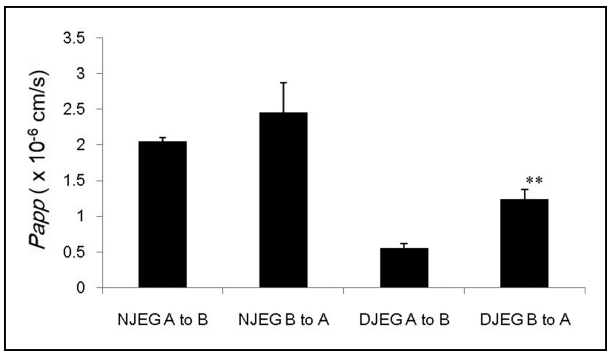

Fig. 3: Apparent permeability coefficient (*Papp*) of cimetidine (30 µM) across NJEG (normal JEG-3) and DJEG (differentiating JEG-3) layers both apical side to basolateral side (A to B) and basolateral to apical (B to A). Permeation studies were carried out for 60 min. $^{**}p < 0.01$ vs DJEG A to B, Student's *t*-test (n = 4).

2. Investigations and results

2.1. Evaluation of cimetidine transport in the DJEGs model

The DJEGs were cultured with CSC® culture media for differentiation. In this study, normally cultured JEG-3 cells (NJEGs), cultured with minimum essential media (MEM), were used as references. BCRP is an efflux transporter localized on the maternal side. In the DJEGs and NJEGs models, the maternal side and the fetal side are considered apical (A) and basolateral (B), respectively. We first compared the apparent transport coefficients (*Papp*) of cimetidine from A to B with those from B to A in these models (Figs. 2, 3). The cimetidine transport coefficient in the DJEGs model which had high TEER value (data not shown) was lower than in the NJEGs model in both directions. In the NJEGs model, the transport coefficients in both the directions did not have a significant difference. However, in the DJEGs model, the transport coefficient from B to A was significantly higher (Fig. 3). It was hypothesized that cimetidine transport from A to B in the DJEGs model was decreased due to the efflux function of BCRP.

Additionally, the cimetidine permeation rates in both the directions were examined with variable doses in the DJEGs model (Fig. 4). The rate from B to A, the efflux direction to the maternal side, demonstrated dose-dependent saturation with a sum of a Michaelis-Menten component (Km approximately 19.8 µM)

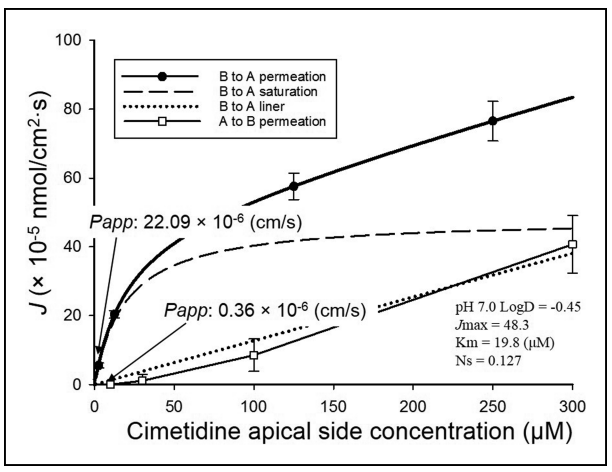

Fig. 4: Concentration dependent permeation rate (*J*) of cimetidine across DJEG (differentiating JEG-3) layers both apical side to basolateral side (A to B) and basolateral to apical (B to A). Apparent permeability coefficients (*Papp*s) indicate the values in DJEGs model around clinical cimetidine concentration. Km: Michaelis constant, Ns: Non-saturable constant, *J*max: maximum permeation rate.

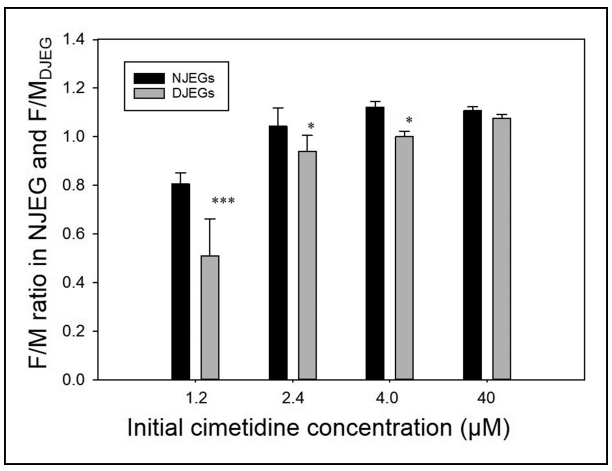

Fig. 5: Evaluation of an efflux function in the differentiating JEG-s cells (DJEGs) model. F/M (fetal/maternal) ratio in NJEG (normal JEG-3) layers and F/M$_{DJEG}$ (F/M ratio in the DJEGs model) were compared for evaluating. The transport studies were carried out for 90 min at 37 °C after cimetidine was administered both the apical and the basolateral sides at the concentration from 1.2 to 40 μM. $^{*}P < 0.05$, $^{***}P < 0.001$ versus NJEG, Student's *t*-test (n = 4).

and a linear component. This result suggests that the DJEGs model has a functional efflux transporter, BCRP.

It is also known that the fetal/maternal concentration ratio *in vivo* (F/M ratio) of cimetidine was low. The F/M ratio in the DJEGs model (F/M$_{DJEG}$) of cimetidine was determined as the ratio of the concentration at B to the concentration at A. Though the low F/M ratio *in vitro* of cimetidine in the NJEGs was poorly detected, the F/M$_{DJEG}$ of cimetidine had a low value of 0.51 ± 0.15 (Fig. 5). The differences of the F/M ratios between the NJEGs and DJEGs had little effect on the high initial cimetidine dose. This result also suggests that in the DJEGs model, the saturation of BCRP occurs under the high doses of the substrate.

We also investigated the effect of inhibitors of p-gp, the other efflux transporter known to exist in the placenta, and BCRP on cimetidine transport from A to B in the DJEGs and NJEGs models. The cimetidine permeation was examined with or without a p-gp inhibitor, verapamil, a BCRP inhibitor, Ko143, and a p-gp and BCRP co-inhibitor, elacridar. In the NJEGs model, no effect was detected. However, the permeation of cimetidine from B to A in the DJEGs model was decreased when BCRP blockers, not the sole p-gp blocker, were applied (Fig. 6).

2.2. Evaluation of fluorescein transport in the DJEGs model

The *Papp* of fluorescein from A to B was investigated in the DJEGs model with or without a MRPs inhibitor, probenecid. The *Papp* of fluorescein across the DJEGs model was significantly increased by inhibiting MRPs function with probenecid (Fig. 7). This suggests that the transport to the fetal side of a substrate of the MRP, fluorescein, would be increased since the efflux function to the maternal side of the MRPs was inhibited by probenecid.

2.3. Evaluation of valproic acid transport in the DJEGs model

The permeation rates of a substrate of the influx transporter MCT, valproic acid, were examined with variable doses in both directions in the DJEGs model (Fig. 8). In contrast to cimetidine, a substrate of the efflux transporter BCRP, the permeation rate of valproic acid from A to B, the influx direction to the fetal side, indicated a dose-dependent saturation. The rate consisted

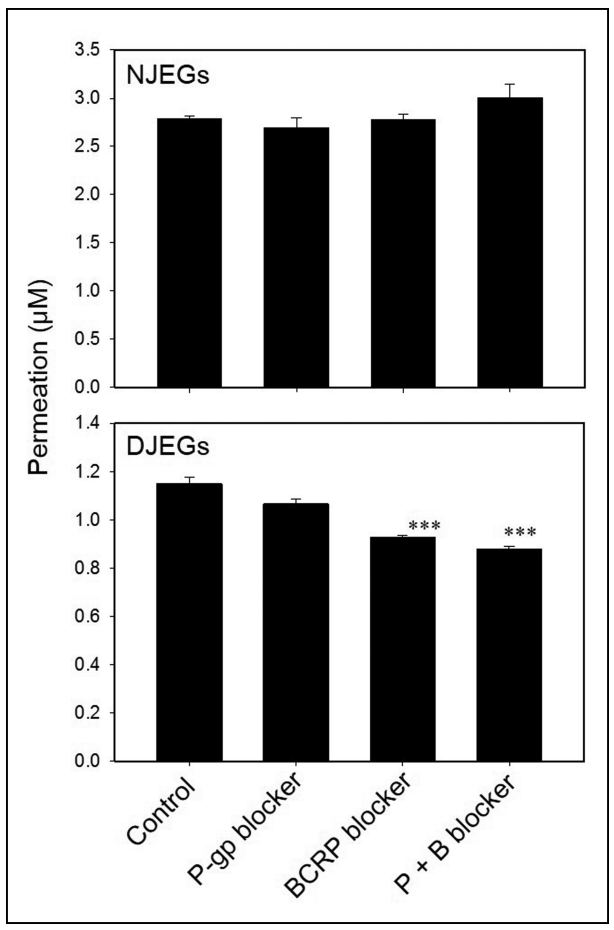

Fig. 6: . Inhibition of cimetidine transport from basolateral (B) side to apical side (A) in the differentiating JEG-s cells (DJEGs) model by breast cancer resistance protein (BCRP) inhibitors. Cimetidine (25 μM) was administered to the basolateral side. The permeation study of cimetidine from B to A was performed with or without a p-gp inhibitor, verapamil (25 μM), a BCRP inhibitor, Ko143 (1 μM), and a p-gp and BCRP co-inhibitor (P + B blocker), elacridar (2 μM), for 90 min after pre-treatment of the inhibitors for 30 min. $^{***}P < 0.001$ versus control, tukey multiple comparison test (n = 4).

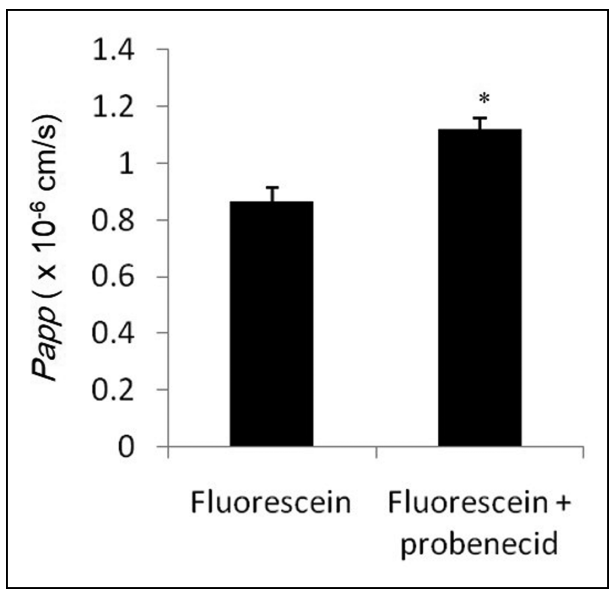

Fig. 7: Effect of a multidrug resistance-associated proteins (MRPs) inhibitor, probenecid (100 μM), on the fluorescein (15 μM) apparent permeability coefficient in the differentiating JEG-s cells (DJEGs) model from apical side (A) to basolateral (B) side. The transport study was carried out for 60 min with or without probenecid. $^{*}p < 0.05$, Student's *t*-test (n = 4).

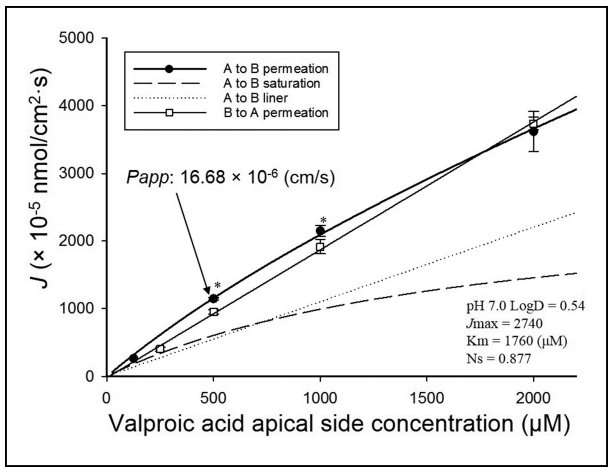

Fig. 8: Concentration dependent permeation rate (J) of valproic acid across the differentiating JEG-s cell (DJEG) layers from apical side to basolateral side (A to B) and from B to A for 60 min. Apparent permeability coefficient (*Papp*) indicates the value in clinical valproic acid concentration. Km: Michaelis constant, Ns: Non-saturable constant, Jmax: maximum permeation rate. $^*p < 0.05$ versus B to A, Student's t-test (n = 4).

of the sum of a Michaelis-Menten component (Km approximately 1.76 mM) and a linear component. Furthermore, the valproic acid transport from A to B was more significant than in the opposite direction around the clinical concentration, about 500 μM. This suggests that the DJEGs model reflects an influx transporter function by MCT.

3. Discussion

Based on these results, some transport characteristics of the DJEGs model could reflect placental transport functions *in vivo*. Johnson et al. (1983) reported that the F/M ratio of cimetidine in pregnancies was approximately 0.5, and that cimetidine could be actively eliminated by efflux to the maternal side in the human placenta. Moreover, a report evaluating the BCRP-mediated placental transport characteristics of cimetidine in a two-layered perfusion model between the maternal and the fetal side across the rat placenta (Staud et. al. 2006) demonstrated that the cimetidine transport rate from fetal to maternal side saturated in a dose-dependent manner with a Km value of 16.7 μM. The F/M ratio of cimetidine equilibrated at about 0.5, but when an inhibitor of BCRP was applied, the F/M ratio increased to about 1.0, and no concentration difference between maternal and fetal cimetidine levels were detected. It was already well known that cimetidine was a substrate of the human BCRP (Pavek et al. 2005). These findings indicate that active cimetidine efflux to maternal side was regulated by BCRP. In this study, the transport of cimetidine from B (fetal) to A (maternal) in the DJEGs model was able to be saturated, and the F/M_{DJEG} ratio became significantly smaller than 1.0. The effect was also decreased by BCRP inhibitors. Furthermore, the Km value of the cimetidine transport rate from B to A was similar to the Km value reported by Staud et al. (2006) Therefore, it can be suggested that the characteristics of cimetidine transport in the DJEGs model reflects the efflux function of BCRP in the placenta.

The *in vitro* Caco-2 cell layers drug absorption model (Artursson et al. 1991) demonstrated that the TEER values of the cell layers had to be at least 300 ohms·cm^2 in order to achieve a satisfactory correlation between the drug absorption rate in the model layers and in small intestine epithelial cell layers. The TEER values on this DJEGs model were also about 300 ohms·cm^2 (Ikeda et al. 2011). Moreover, it was predicted that the fetal cimetidine amount after efflux to the maternal side for 60 min in the DJEGs model would be about one percent of the maternal in the effective blood concentration range. In a report using the *ex vivo* model with a term placenta (Hori et al. 2004), it was predicted that the amount transported after 60 min from the maternal to fetal side was about 4% of the maternal in the effective blood concentration range. Therefore, the DJEGs model is considered to have a barrier function at least as well as the *ex vivo* model, and thus be an adequate model for validating the correlation in *in vivo* placental drug transport.

Furthermore, the cimetidine transport rate across the DJEGs model was higher in the B to A direction than the A to B, and the B to A transport was saturated in a dose-dependent manner, indicating carrier-mediated transport. This suggests that BCRP in the DJEGs model would be normally functioning as an efflux transporter.

Next, other transporters that play a role in carrier-mediated drug transport in the placenta were investigated in the DJEGs model. The transport of fluorescein, a substrate of MRPs, from maternal to fetal blood was significantly increased by treatment with an MRP inhibitor, probenecid. Although the subtypes of MRPs were not defined in the DJEGs model, this result indicates that the DJEGs model had at least a partial MRP efflux transport function. Furthermore, the A to B transport of a substrate of an influx transporter MCT, valproic acid, was saturated in a dose-dependent manner in the DJEGs model. This demonstrates that the model would have MCT carrier-mediated influx. Moreover, in the effective blood concentration range, near 500 μM, the A to B transport rate was higher than B to A. This suggests that the DJEGs model can predict the high levels of valproic acid transport across a placenta as reported *in vivo*. Furthermore, the Km value of valproic acid transport by MCT described in previous reports ranged from 0.2 to 11.4 mM (Utoguchi et al. 2000; Ushigome et al. 2001; Nakamura et al. 2002). The Km value of the DJEGs model was 1.76 mM, close to reported values in human syncytiotrophoblast small vesicles (Nakamura et al. 2002). Therefore, it is suggested that the influx transport function mediated by the MCT expressed on the syncytiotrophoblast would be reflected in the DJEGs model, since saturation was demonstrated in the A to B transport of valproic acid across the DJEGs model. On the other hand, valproic acid is a small-molecule lipophilic drug, so the passive transport contributes to transport across cell layers. In the DJEGs model, the linear component in the A to B valproic acid transport contributed largely to total transport. This indicates that passive transport on the DJEGs model was not also negligible for the A to B valproic acid transport. These findings suggest that the DJEGs model can evaluate the placental transport caused both by passive transport and MCT carrier-mediated transport.

However, some issues remain. First, although there are some reports that fluorescein is a substrate of MRPs, however affinities between fluorescein and the MRP subtypes was not shown clearly. Currently, fluorescein is described to at least be a substrate of MRP1 and MRP2 (Utoguchi et al. 2000; Sun et al. 2001; Hamilton et al. 2007; Hawkins et al. 2007). The fact that the fluorescein A to B transport coefficient was significantly increased by treatment with probenecid, a MRP inhibitor, but only by a little, supports the finding that some MRP subtypes would be functioning on the DJEGs model. Therefore, although the DJEGs model would reflect the efflux function by MRPs at least partially, in the future, it needs to be investigated further with other MRPs substrates and inhibitors to confirm that the DJEGs model reflects the MRPs transport features on the syncytiotrophoblast. Second, other influx transporter functions besides MCT should also be examined to further confirm placental features in the DJEGs model. Third, to add value to the DJEGs model when performing placental drug transport evaluations, correlations between the DJEGs model and clinical data must be made. For such an evaluation, correlational analysis between

F/M_{DJEG}s and F/M ratios of additional transporter substrates and inhibitors would be beneficial.

In conclusion, the DJEGs model was demonstrated to be able to evaluate transcellular placental drug transport mediated by major placental transporters. Additionally, by resolving the remaining problems, it is hoped that the DJEGs model could be an indicator of the fetal transitivity of medical drugs administrated to the maternal.

4. Experimental

4.1. Materials

Human choriocarcinoma JEG-3 cells, fetal bovine serum, and CSC® medium kits were obtained from Dainippon Sumitomo Pharma Co. Ltd. (Osaka, Japan). Eagle's minimum essential medium (MEM) was purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Cell culture plates and flasks were purchased from Becton Dickinson and Company (Franklin Lakes, NJ). Cellmatrix® (collagen type I-A) was obtained from Nitta Gelatin Inc. (Osaka, Japan). Millicell® 24-well hanging cell culture inserts (pore size, 1 μm; polyethylene terephthalate (PET) membrane, 0.33 cm^2) were purchased from Millipore Co. (Billerica, MA). Verapamil hydrochloride and Ko143 hydrate were purchased from Sigma-Aldrich, Inc. (MO, USA). Elacridar was purchased from Santa Cruz biotechnology, Inc. (TX, USA). All other reagents were purchased from Wako pure chemical Industries, Ltd. (Osaka, Japan).

4.2. Cell Culture

JEG-3 cells were cultured in MEM supplemented with 10% (v/v) fetal bovine serum and 1% (v/v) nonessential amino acids. These cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air. The PET membranes of Millicell® 24-well inserts were treated with 50 μL of collagen type-I:60% ethanol (1:3), and dried overnight at room temperature. And then the JEG-3 cells were seeded in 100 μL media of the apical side and 600 μL of the basolateral side at approximately 40,000 cells/cm^2 on the PET membranes of the 24-well inserts (Ikeda et al. 1999, 2011). For the transport studies, the seeded JEG-3 cells were cultured with MEM for 3 d, and then further cultured under each condition, MEM (NJEGs) and CSC® (DJEGs) for 4 d. These two types JEG-3 cell layers, NJEGs and DJEGs, were used to the drug transport studies.

4.3. Transport experiment with cimetidine

The culture media of JEG-3 cell layers were replaced with hank's balanced salt solution (HBSS) supplemented with 4.5 g/L D-glucose for the drug transport studies. The cimetidine liquid solutions were added to the apical or the basolateral side of the cell layers at the final concentration from 10 to 300 μM. The transport studies were carried out for 60 min at 37 °C and cimetidine sample liquids transported across the NJEGs or the DJEGs layers were measured with high performance liquid chromatography (HPLC). The permeation rate (J) was calculated by the following equations with Sigma Stat®.

$$J = dQ/(dT \cdot A),\ J = j\max \times C_0/(Km + C_0) + Ns \cdot C_0$$

where J is the permeation rate (nmol/cm^2·s), Ns is the non-saturable constant, Km is the michaelis constant, Jmax is the maximum permeation rate, A is membrane area (cm^2) and C_0 is the cimetidine initial administrated concentration (μM).

The apparent permeability coefficient was calculated by the following equation.

$$Papp = dQ/dT/(A \cdot C_0)$$

where $Papp$ is the apparent permeability coefficient (cm/s), dQ/dT is the permeation rate (nmol/s), A is membrane area (cm^2) and C_0 is the donor side initial concentration (μM).

F/M_{DJEG} is the ratio of a basolateral side to a apical side in the DJEGs model. F/M ratio in NJEG layers and F/M_{DJEG} were compared for evaluating an efflux function in the DJEGs model. The transport studies were carried out for 90 min at 37 °C across the DJEGs model after cimetidine was administrated both the apical and the basolateral sides at the concentration from 1.2 to 40 μM.

The permeation of cimetidine (25 μM) from B to A in the DJEGs model was investigated with or without a p-gp inhibitor, verapamil (25 μM), a BCRP inhibitor, Ko143 (1 μM), and a p-gp and BCRP co-inhibitor, elacridar (2 μM), for 90 min. The DJEG layers were pre-treated with the inhibitors for 30 min and cimetidine of the apical side was measured with HPLC after the transport examination.

4.4. Quantification of cimetidine with HPLC

The concentrations of cimetidine were determined by the HPLC method reported previously (Macnamara et al. 1992; Gerk et al. 2001). Briefly, 40-μL aliquots of cimetidine transport study samples and cimetidine standard samples (2.5, 1.25, 0.61, and 0.31 μM) dissolved in HBSS were injected into the HPLC system. The HPLC system consisted of two solvent delivery pumps (Model LC-20AD, Shimadzu Co., Japan), a UV-Vis detector (SPD-M20A), a system controller (CBM-20A), a degasser (DGU-20A) and an autoinjector (SIL-20AC). The UV detector was set at 228 nm. The mobile phase consisted of acetonitrile/distilled water (containing 2 mM triethylamine and 25 mM acetic acid) (20:80). Separations were carried out on a Cosmosil C18 column (5 μm, 2.1 × 150 mm, Nacalai Tesque, Inc.) with a guard column. The retention time of cimetidine at a flow rate of 1.0 ml/min was 6.1 min. The lower limit of quantification for cimetidine in HBSS was 0.1 μM.

4.5. Transport experiment of fluorescein

Fluorescein of 15 μM final concentration was added to the apical side with or without a MRPs inhibitor, probenecid, of 100 μM in the DJEGs model prepared for the drug transport studies. The transport studies were carried out for 60 min at 37 °C and 10-μL aliquot of fluorescein sample transported to the basolateral side was diluted by 190-μL HBSS. The diluted fluorescein samples were measured with a spectrofluorometer (Powerscan HT, Dainippon Pharmaceutical, Osaka, Japan). The fluorescence detection was performed using excitation and emission wavelengths of 470 and 580 nm.

4.6. Transport experiment with valproic acid

Valproic acid from 125 to 2,000 μM final concentration was added to the apical or the basolateral side in the DJEGs model prepared for the drug transport studies. The transport studies were carried out for 60 min at 37 °C and 10-μL aliquot of the transported valproic acid solution was diluted by 190-μL HBSS. The diluted valproic acid concentrations were determined by an enzymatic competitive immune assay apparatus (VIVA-E, Siemens, Inc., PA, USA).

4.7. Statistical analysis

Student's *t*-test and Tukey multiple comparison test were performed to calculate the statistical significance of the difference between the means of the control group and the other sample group. The data indicated in the figures denote mean ± SD values.

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