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Development of a Human Mammary Epithelial Cell Culture Model for Evaluation of Drug Transfer into Milk

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In the present study, a human mammary epithelial cell (HMEC) culture model was developed to evaluate the potential involvement of carrier-mediated transport systems in drug transfer into milk. Trypsin-resistant HMECs were seeded on Matrigel®-coated filters to develop monolayers of functionally differentiated HMEC. Expression of the specific function of HMEC monolayers was dependent of the number of trypsin treatments. Among the monolayers with different numbers of treatment (treated 1 to 3 times), the monolayer treated 3 times (3-t-HMEC monolayer) showed the highest maximal transepithelial resistance and expression of β-casein mRNA as an index of differentiation. Transport of tetraethylammonium (TEA) across the 3-t-HMEC monolayer in the basolateral-to-apical direction was significantly higher than that in the apical-to-basolateral direction ($p < 0.05$), whereas such directionality was not observed for *p*-aminohippurate, suggesting the existence of organic cation transporters, but not organic anion transporters. In fact, expression of mRNAs of human organic cation transporter (OCT) 1 and 3 were detected in the 3-t-HMEC monolayer. These results indicate that the 3-t-HMEC monolayer is potentially useful for the evaluation of carrier-mediated secretion of drugs including organic cations into human milk.

Key words: Human mammary epithelial cell, Carrier-mediated drug transport, Differentiation, Trypsin-resistant cells

INTRODUCTION

Breastfeeding has great advantages for infants with respect to better nutrition and the development of immunity as well as helping prevent post-natal depression in the mothers (American Academy of Pediatrics, 1997; Anderson, 1991). However, 90% of mothers take medication during the first week after delivery and 17% during the first 4 months, and breastfeeding in such cases is generally avoided to prevent the chance of exposure of the infants to drugs (Bennet, 1996; Anderson, 1991). Evaluation of drug safety in the infants is a major problem to be solved in order to allow the mothers access to pharmacotherapy. Although some drugs, such as amoxicillin, cimetidine, and verapamil, have been shown to have no observable effect on infants after breastfeeding from mothers taking medication (American Academy of Pediatrics, 2001), current problems are due to the lack of available information regarding the potential exposure of infants to drugs because of ethical concerns associated with such clinical studies. Thus, development of a method to estimate the transfer of drugs and their metabolites into milk is an important issue as overviewed in the Food and Drug Administration (FDA) guidance (FDA, 2005).

Drug transfer into milk has been predicted based on physicochemical properties of drugs including the pH-partition theory, partition coefficient into the milk lipid phase and the degree of protein binding (Wilson, 1981; Fleishaker *et al.*, 1983; Atkinson and Begg, 1990; Fleishaker *et al.*, 2003; Ito and Lee, 2003). Atkinson and Begg (1990) showed that milk-to-plasma concentration ratio (M/P ratio) of drugs predicted from their physicochemical properties was very consistent with the observed

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ratio, indicating that the concentration of certain drugs in milk can be estimated based on their plasma concentration and M/P ratio. By contrast, the observed ratio for some drugs, such as cimetidine and nitrofurantoin, was markedly higher than the predicted ratio (Gerk *et al.*, 2001; Oo *et al.*, 1995), suggesting possible involvement of carrier-mediated transport systems. Thus, incorporation of the carrier-mediated processes into the prediction model in addition to the physicochemical parameters of drugs appears to be essential for precise prediction of the M/P ratio of drugs.

Cell culture models provide useful information for evaluation of carrier-mediated drug transport. Mammary cells in the lactating state are highly differentiated and their physiological functions are different from those in the non-lactating state (Neville *et al.*, 2001). Schmidhauser *et al.* (1990) reported that the cultured mammary cells not detached by trypsin treatment (trypsin-resistant cells, CID9 cells) had the ability to differentiate functionally as indicated by β-casein expression and this ability was increased on increasing the treatment period. Furthermore, Toddywalla *et al.* (1997) and Gerk *et al.* (2003) showed that CIT3 cells, a subline of CID9 cells, expressed carrier-mediated transport processes for nitrofurantoin. However, these cell culture models may not be appropriate for evaluation of the specific transport processes in humans because of species differences (McNamara *et al.*, 1992). Therefore, a human-specific culture model of mammary epithelial cells is essential to evaluate the possible carrier-mediated transport of drugs. Such a culture model has not been developed yet.

Our goal was to develop a cultured human mammary epithelial cell (HMEC) monolayer to evaluate carrier-mediated drug transport processes during lactation. In the present study, the transport of tetraethylammonium (TEA, a substrate for organic cation transporters (OCTs)), p-aminohippurate (a substrate for organic anion transporters), and D-mannitol (a paracellular transport marker) across the HMEC monolayer was measured in terms of directionality. In addition, the expression of OCT1 and OCT3 in mRNA was also determined. Our findings are the first report indicating that carrier-mediated directional transport occurs in functionally-differentiated HMEC monolayers.

MATERIALS AND METHODS

Chemicals

[^{14}C]-tetraethylammonium (55 mCi/mmol), [^{14}C]-*p*-aminohippurate (55 mCi/mmol), and [^{3}H]-mannitol (20 Ci/mmol) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, U.S.A.).

Cells and media

Normal HMEC (Cambrex Bio Science Walkersville Inc., Walkersville, MD, U.S.A.) were used in this study. The medium for cell proliferation was human mammary epithelial growth medium (MEGM®, Cambrex Bio Science Walkersville Inc.) supplemented with insulin, epidermal growth factor (EGF), hydrocortisone, amphotericin B, gentamicin, and bovine pituitary extract. To differentiate the cells, the MEGM® was modified by removal of EGF and addition of prolactin (Sigma Chemicals Co., St. Louis, MO, U.S.A.), which was used as the differentiation medium.

Cell culture

In order to obtain a trypsin-resistant cell population, the HMEC monolayer was cultured using the modified method by Schmidhauser *et al.* (1990). Normal HMECs were seeded at 2,500 cells/cm^2 on dishes coated with Matrigel® (Becton Dickinson Inc., Lincoln Park, NJ, U.S.A.) and grown in MEGM® in a humidified incubator (95% air/ 5% CO_2) at 37°C. When the cells were 80% confluent, they were treated with 0.025% trypsin/0.1% EDTA (Cambrex Bio Science Walkersville Inc.) and were removed until 20% confluent. Detached HMECs (trypsin-treated HMEC; t-HMEC) were collected by centrifugation, while the attached HMECs were allowed to continue growing on the same dish. When the attached HMECs were 80% confluent, trypsin/EDTA treatment was repeated. The processes of removing by trypsin/EDTA and continuing growth on the same dish were repeated 3 times. In each treatment with trypsin/EDTA the detached cells were referred to 1-, 2-, and 3-t-HMEC.

For transport studies, the 1-, 2-, and 3-t-HMEC (seeding density: 20,000 cells/cm^2) were grown on a FALCON cell culture insert (0.32 cm^2, pore size 1.0 μm, Becton Dickinson Inc.) coated with Matrigel® immersed in differentiation medium. Transepithelial resistance (TER) was sequentially monitored by a Millicell-ERS® (Millipore, Bedford, MA, U.S.A) for transport studies. The cells cultured on 10mm dishes (IWAKI, Tokyo, Japan) then underwent the reverse transcription-polymerase chain reaction.

Transport studies

Transport studies were performed when TER reached the maximal value.

Mammary epithelial basic medium (MEBM) containing TEA (1 μM) or PAH (1 μM) with co-presence of mannitol (20 nM), was added to the top (0.5 mL) or bottom (1.5 mL) chamber to initiate the transport experiment. The chamber with the model substrate is designated the donor chamber while the other one is the receiver chamber. MEBM was used as the receiver fluid. The plate setting the cell culture inserts was maintained at 37°C in the incubator throughout the transport study. Receiver fluid

(100 μL) was collected at 30, 60, 120, and 180 minutes, then an equal volume of MEBM was added to the chamber to replace the fluid removed. Radiolabel material transported was determined using a liquid scintillation counter (ALOKA, Tokyo, Japan). The apparent permeability coefficient (Papp) for model substances and mannitol were calculated from Eq. (1) (Toddywalla *et al.*, 1997):

$$P_{app} = \frac{[X]_{receiver}}{A \times t \times [C]_{donor}} \quad (1)$$

where $[X]_{receiver}$ is the amount of model substances or mannitol in the receiver chamber at time, t, A is the effective diffusion area of the cell culture insert, and $[C]_{donor}$ is the concentration of the model substrate or mannitol in the donor compartment. Then, mannitol transport served as an internal reference, and the permeability ratio was calculated by dividing the Papp of model substrates ($P_{app,\ model}$) by the Papp of mannitol ($P_{app,\ mannitol}$) in the same tissue (eq. 2, Marks *et al.*, 1991):

$$P_{app}ratio = \frac{P_{app,model}}{P_{app,\mathrm{mannitol}}} \quad (2)$$

Detection of β-casein mRNA, hOCT1 mRNA, and hOCT3 mRNA by RT-PCR

For determination of the expression of β-casein mRNA, hOCT1 mRNA, and hOCT3 mRNA, total RNAs were extracted from 1-, 2-, and 3-t-HMEC monolayers using an RNeasy Mini Kit (QIAGEN, Valencia, CA, U.S.A.). RT-PCR was performed using a One-step RT-PCR Kit (QIAGEN). The mixture of a total volume of 50 μL with 0.5 μg total RNA was prepared according to the manufacturer's protocol. The specific primers for each gene are shown in Table I. RT-PCR was conducted using a Biometra® thermocycler (Biometra, Göttingen, Germany) according to the specific conditions shown in Table II. After RT-PCR, the samples were kept at 4°C until analysis. RT-PCR products were detected by electrophoresis on 2% agarose gel in tris-acetate-EDTA buffer. The gels were stained with ethidium bromide, visualized by UV light, and photographed with a Polaroid camera.

Statistical analysis

All data are presented as the mean ± S.D. Statistical comparison were analyzed by using a two-tailed paired *t*-test or by one-way ANOVA. When ANOVA showed significant differences between groups, Tukey's test was used to determine the specific pairs of groups between which statistically significant differences occurred. $P < 0.05$ was the accepted level of statistical significance.

RESULTS

Effect of trypsin treatment on maximal TER in HMECs

The maximal TERs were observed at day 11, day 14, and day 19 for 1-, 2-, and 3-t-HMECs, respectively, after seeding on cell culture inserts (data not shown). As shown in Fig. 1, the maximal TER significantly increased with the increasing number of trypsin treatments, and the highest value (227±11 $\Omega \cdot cm^2$) was obtained with 3-t-HMEC which seemed to be tight enough for transport

Table I. Primer sets used for RT-PCR

Transporter Gene	Alternative Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product Size
β-Casein	–	ATGAGGACCAGCAGCAAGGAGA	GGAGCAGAGGCAGAGGAAGATG	318
SLC22A1	hOCT1	GATTTCCTTTACTCCGCTCTGGTC	TTTCTTTGGGCTTTGCTTTTCTCC	487
SLC22A3	hOCT3	GACAAGAGAAGCCCCCAACCTGAT	CACTAAAGGAGAGCCAAAAATGTC	455
β-Actin	–	ATCTGGCACCACACCTTCTACAATGAG	CGCTCATACTCCTGCTTGCTGATCCACA	837

Table II. RT-PCR conditions for detection of β-casein mRNA, hOCT1 mRNA and hOCT3 mRNA in t-HMEC

	β-Actin		β-Casein		hOCT1		hOCT3	
	Time	Temperature	Time	Temperature	Time	Temperature	Time	Temperature
Reverse transcription	30 min	50°C	30 min	50°C	30 min	50°C	30 min	50°C
Initial PCR activation step	15 min	95°C	15 min	95°C	15 min	95°C	15 min	95°C
3-step cycling								
Denaturation	60 s	94°C	45 s	94°C	60 s	94°C	60 s	94°C
Annealing	60 s	53°C	45 s	60°C	60 s	61°C	60 s	60°C
Extension	60 s	72°C	30 s	72°C	60 s	72°C	60 s	72°C
Number of cycles	20		40		30		35	
Final extension	10 min	72°C	10 min	72°C	10 min	72°C	10 min	72°C

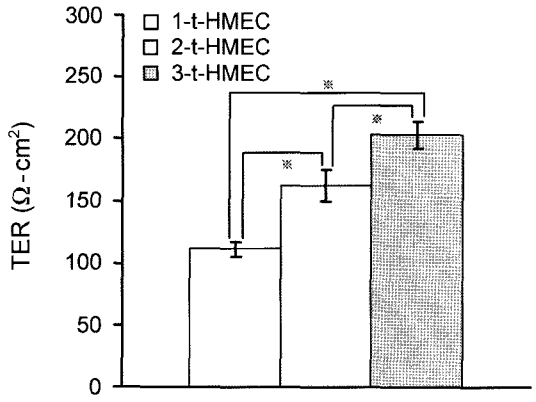

Fig. 1. Effect of trypsin treatment on transepithelial resistance in HMEC monolayers. Columns and vertical bars represent the mean±S.D. (n=6-10). $^{※}p < 0.05$, compared with the value in 1-t-HMEC.

studies. A further increase in trypsin treatment, *i.e.* 4-t-HMEC (resistant cells obtained by 4 treatments with trypsin) did not form a monolayer on the cell culture insert (data not shown).

Expression of β-casein mRNA in t-HMEC monolayers

The expression of β-casein mRNA (a differentiation marker of mammary cells) was detected in 2-, and 3-t-HMEC monolayers, but not in 1-t-HMEC (Fig. 2), suggesting that trypsin-resistant HMEC is differentiated and probably reflects the lactating state.

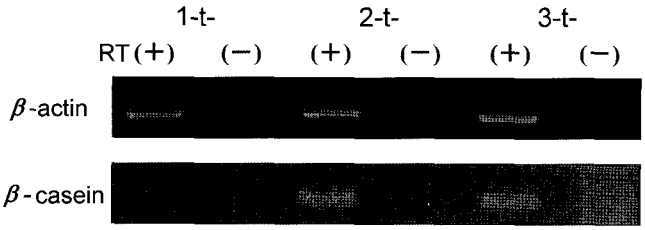

Fig. 2. Effect of trypsin treatment on β-casein mRNA expression in t-HMEC monolayers. Expression of β-casein mRNA was determined in 1-t-HMEC (1-t-), 2-t-HMEC (2-t-) and 3-t-HMEC (3-t-). RT-PCR analysis was performed with the specific primer sets shown Table I. Reactions were performed against total RNA with (+) or without (-) reverse transcription (RT).

Transport of model substrates across t-HMEC monolayers

Transport of TEA and PAH in both apical-to-basolateral and basolateral-to-apical directions were observed in t-HMECs (Table III). The corrected TEA transport in the basolateral-to-apical direction in 3-t-HMEC was significantly higher than that in the apical-to-basolateral direction ($p < 0.05$), suggesting the potential involvement of carrier-mediated processes in TEA transport. Similar directionality was also observed in 1- and 2-t-HMEC monolayers, but this was not significantly different between both directions. On the other hand, directionality was not observed in any of the t-HMEC monolayers examined.

Expression of hOCT1 and hOCT3 mRNA in t-HMEC monolayers

Since TEA is known to be a substrate of organic cation transporters, the expression of hOCT1 and hOCT3 mRNAs was determined (Fig. 3). The hOCT1 mRNA expression had a tendency to increase on increasing the

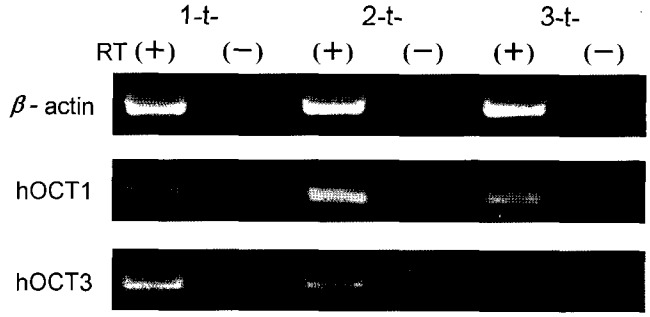

Fig. 3. Expression of trypsin treatment on the transporters related to vectorial transport. RT-PCR analysis was performed with the specific primer sets shown Table I for the detection of hOCT1 mRNA and hOCT3 mRNA in 1-t-HMEC (1-t-), 2-t-HMEC and 3-t-HMEC (3-t-). Reactions were performed against total RNA with (+) or without (-) reverse transcription (RT).

Table III. Permeability of model substances and mannitol across t-HMEC

		Papp Ratio		$Papp_{Mannitol}$ ($\times 10^{-6}$ cm/s)	
		Apical to Basal	Basal to Apical	Apical to Basal	Basal to Apical
TEA	1-t-HMEC	1.139 ± 0.083	1.227 ± 0.042	5.734 ± 1.382	5.980 ± 1.111
	2-t-HMEC	1.112 ± 0.156	1.230 ± 0.073	2.299 ± 0.270	2.193 ± 0.080
	3-t-HMEC	0.940 ± 0.287	1.356 ± 0.076※¶	1.395 ± 0.256	1.622 ± 0.230
PAH	1-t-HMEC	0.860 ± 0.106	0.860 ± 0.028	7.387 ± 0.560	7.304 ± 0.500
	2-t-HMEC	0.864 ± 0.100	0.738 ± 0.045	3.693 ± 0.280	3.652 ± 0.250
	3-t-HMEC	0.865 ± 0.106	0.860 ± 0.028	2.629 ± 0.419	2.442 ± 0.119

Each value represent means±S.D. (n=4-5).
$^{※}p < 0.05$, compared with the value in the apical- to- basal direction.

number of trypsin treatments, whereas hOCT3 expression was reduced on increasing the number of trypsin treatments.

DISCUSSION

In the present study, we developed a human mammary epithelial cell monolayer for evaluation of transepithelial drug transport involving carrier-mediated transport processes. Our tight monolayer system is likely to be useful for evaluation of both passive diffusion and carrier-mediated transport across epithelial cells in the lactating state.

Mammary function is known to be differentiated in the lactating state to provide nutrition, immunity, and other essential materials to infants (McManaman *et al.*, 2003). Thus, it is important to evaluate drug transfer into milk using cell culture models reflecting the lactating state. Production of β-casein in mammary cells may be a good index of mammary differentiation because β-casein is specifically produced only in the lactating state (Shipman *et al.*, 1987). Schmidhauser *et al.* (1990) studied mouse mammary cells and reported that β-casein-producing cells were contained in a population of trypsin-resistant cells which could not be detached by immersion in trypsin solution, and the proportion of the producing cells was increased depending upon the treatment period. Our data showed a trend that the expression of mRNA of β-casein increased in the 2- and 3-t-HMEC (Fig. 2). This might be attributed to the increase in the β-casein-producing cell population after treatment with trypsin solution. In addition, mammary epithelial cells are reported to become tight in the lactating state in the goat (Lnzell and Peaker, 1971; 1974), and mouse (Berga, 1984). The higher TER value of the 3-t-HMEC monolayer compared with that of 1- and 2-t-HMECs (Fig. 1) could be consistent with progression of mammary differentiation.

A tight monolayer similar to *in vivo* conditions is generally required to evaluate the transcellular transport of drugs (Adson *et al.*, 1994). In addition to the TER value (Fig. 1), the mannitol Papp of 2.6×10^{-6} (cm/s) across the 3-t-HMEC monolayer (Table III) was comparable with that obtained in the mouse mammary monolayer (3.9×10^{-6} cm/s) (Toddywalla *et al.*, 1990), indicating that the 3-t-HMEC monolayer is tight enough to evaluate transcellular drug transport. The observed directionality of TEA transport in the 3-t-HMEC monolayer (Table III) indicates the possible contribution of carrier-mediated processes in the case of organic cationic compounds. This may be associated with the potential function of hOCT1 and hOCT3 since their mRNAs were detected in this monolayer. Alcorn et al. (2002) reported that the expression of mRNA of hOCT1 in human mammary cells isolated from human milk (lactating cells) was 7.8-fold higher than that in non-lactating cells, while it was 2.9-fold lower in lactating cells compared with non-lactating cells for hOCT3. Their observations appear to be in agreement with our findings in Fig. 3, although a quantitative analysis is required. On the other hand, the potential involvement of organic anion transporter was not clear based on the present study using PAH as a substrate. Because mRNA of multidrug resistance-associated protein and organic anion-transporting polypeptide families which potentially mediate transport of organic anions has been observed in lactating cells in humans (Alcorn *et al.*, 2002), further studies using other types of substrates having high specificity for their transporters are required to examine drug transport processes in HMEC during the lactating state.

CONCLUSION

Trypsin-resistant HMEC monolayers appear to be useful for evaluation of carrier-mediated drug transport during the lactating stage. Integration of the carrier-mediated processes with the prediction models in addition to the passive diffusion process based on the physicochemical properties of drugs enables us to accurately predict drug transfer into human milk. Development of such integrated prediction models is now required.

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