Human Small Intestinal Epithelial Cells Differentiated from Adult Intestinal Stem Cells as a Novel System for Predicting Oral Drug Absorption in Humans

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ABSTRACT

Adult intestinal stem cells (ISCs) possess both a long-term proliferation ability and differentiation capability into enterocytes. As a novel in vitro system for the evaluation of drug absorption, we characterized a human small intestinal epithelial cell (HIEC) monolayer that differentiated from adult ISCs. Continuous proliferation/differentiation from ISCs consistently conferred the capability of maturation of enterocytes to HIECs over 25 passages. The morphologically matured HIEC monolayer consisted of polarized columnar epithelia with dense microvilli, tight junctions, and desmosomes 8 days after seeding onto culture inserts. Transepithelial electrical resistance across the monolayer was 9-fold lower in HIECs (98.9 Ω cm²) than in Caco-2 cells (900 Ω cm²), which indicated that the looseness of the tight junctions in the HIEC monolayer was similar to that in the human small intestine (approximately 40 Ω cm²). No significant differences were observed in the overall gene expression patterns of the major drug-metabolizing enzymes and transporters between the HIEC and Caco-2 cell monolayers. Furthermore, the functions of P-glycoprotein and breast cancer resistance protein in the HIEC monolayer were confirmed by the vectorial transport of marker substrates and their disappearance in the presence of specific inhibitors. The apparent drug permeability values of paracellularly transported compounds (fluorescein isothiocyanate-dextran 4000, atenolol, and terbutaline) and nucleoside transporter substrates (didanosine, ribavirin, and doxifluoridine) in the HIEC monolayer were markedly higher than those of Caco-2 cells, whereas transcellularly transported drugs (pindolol and midazolam) were equally well permeated. In conclusion, the HIEC monolayer can serve as a novel and superior alternative to the conventional Caco-2 cell monolayer for predicting oral absorption in humans.

Introduction

Oral absorption often needs to be predicted in humans to assess the feasibility of drug development so that development candidates can be selected and prioritized at the discovery stage. Many techniques have served as tools for the prediction of intestinal absorption and metabolism in humans. For example, Lennermäis et al. (1997) demonstrated that the human jejunal perfusion method could predict human oral absorption for both passively and carrier-mediated transported drugs. The Ussing-chamber model has also been used as a system that can not only accurately predict absorption, but can also transepithelial (Rozehl et al., 2012; Sjöberg et al., 2013). However, neither technique is frequently used as a routine assay at the discovery stage of new drug candidates because of the limited and irregular availability of human materials for these systems.

Cell-based assay methods such as Caco-2 cells (human colon adenocarcinoma cells), MDCK cells (Madin–Darby canine kidney cells), and 2/4/A1 cells (conditionally immortalized rat intestinal cells) have also been used to evaluate membrane permeability and the transport of drug candidates (Irvine et al., 1999; Tavelin et al., 2003b; Skolnik et al., 2010). The Caco-2 cell line is one of the most widely used cell systems. Although the Caco-2 cell line retains very similar morphologic properties to human enterocytes, its tight junctions are markedly tighter than those in the small intestine, which may reduce the permeability of drugs with significant paracellular absorption (Lennermäis et al., 1996; Saitoh et al., 2004), thereby resulting in the underestimation of intestinal absorption for those compounds (Tavelin et al., 2003b; Saitoh et al., 2004; Matsson et al., 2005). The utilization of human primary normal enterocytes was expected to be a promising solution for the aforementioned discrepancy between in vivo and in vitro models. Although several methods have been used to isolate primary enterocytes from the human small intestine (Perreault and Beauleiu, 1998; Aldhous et al., 2001; Grossmann et al., 1998), these systems have their limitations and irregular availability of human materials for these systems.

ABBREVIATIONS: BCRP, breast cancer resistance protein; BrdU, bromodeoxyuridine; CDX2, caudal type homeobox 2; CNT, concentrative nucleoside transporter; Ct, threshold cycle; DMEM, Dulbecco’s modified Eagle’s medium; ENT, equilibrative nucleoside transporter; ER, efflux ratio; Fa, fraction absorbed in humans; FBS, fetal bovine serum; FD-4, fluorescein isothiocyanate-dextran with an average molecular weight of 4000; HIEC, human small intestinal epithelial cell; IFABP, intestinal fatty acid-binding protein; IPS, induced pluripotent stem; ISC, intestinal stem cell; Ko143, 3-(6-isobutyl-9-methoxy-1,4-dioxo-1,2,3,4,6,7,12,12-octahydroprazinol[1’2’:1,6]pyrido[3,4-b]indol-3-yl)-propionic acid tert-butyl ester; LGR5, leucine-rich repeat-containing G protein–coupled receptor 5; MRP, multidrug resistance protein; P450, cytochrome P450; PAMPA, parallel artificial membrane permeability assay; Papp, apparent permeability coefficient; P-gp, P-glycoprotein; SI, sucrase-isomaltase; TEER, transepithelial electrical resistance; TM, transport medium; UGT, UDP-glucuronosyltransferase.
resistance (TEER) value of 98.9, junctions in the HIEC monolayer, which had a transepithelial electrical differentiation potency into enterocytes from ISCs. The looseness of tight the evaluation of oral drug absorption. The HIEC had the ability to form permeability of drug candidates to predict absorption in humans has not

The authors demonstrated that ISCs were maintained in culture with continuous proliferation and spontaneous differentiation into four distinct epithelial cell lineages including not only absorptive enterocytes, but also secretory lineages such as enteroendocrine cells, goblet cells, and Paneth cells. Suzuki et al. (2010) also found that these differentiated cells formed polarized monolayers with dome-like structures. However, to the best of our knowledge, the utility of this differentiated HIEC for evaluating the permeability of drug candidates to predict absorption in humans has not yet been assessed.

In this study, we characterized the HIEC monolayer as a novel tool for the evaluation of oral drug absorption. The HIEC had the ability to form a monolayer with a tight and matured in vivo-like morphology. HIECs also displayed the long-term proliferating ability of ISCs and continuous differentiation potency into enterocytes from ISCs. The looseness of tight junctions in the HIEC monolayer, which had a transepithelial electrical resistance (TEER) value of 98.9 $\Omega \times \text{cm}^2$, was similar to that of the human small intestine (TEER = approximately 40 $\Omega \times \text{cm}^2$) (Sjoberg et al., 2013), whereas that of Caco-2 cells was not (TEER = 900 $\Omega \times \text{cm}^2$). The HIEC monolayer also distinctly expressed concentrative nucleoside transporter (CNT) 3, which was absent in Caco-2 cells. As a result, the permeability values of paracellularly transported drugs and nucleoside transporter substrates were markedly higher in the HIEC monolayer than in Caco-2 cells, whereas transcellularly transported drugs were equally well permeated. This study demonstrated that the HIEC monolayer could serve as a novel and superior alternative to the conventional Caco-2 cell monolayer for predicting oral absorption in humans; its accuracy was more pronounced for paracellularly transported drugs and nucleoside transporter substrates.

**Materials and Methods**

HIECs (ACBR1519) and Caco-2 cells (HTB-37) were obtained from Cell Systems (Kirkland, WA) and the American Type Culture Collection (Rockville, VA), respectively. Dulbecco’s modified Eagle’s medium (DMEM), DMEM mixed 1:1 with Ham’s F-12 (DMEM/F12), 0.25% trypsin-EDTA, Hanks’ balanced solution, nonessential amino acids, penicillin-streptomycin, and GlutaMAX were obtained from Life Technologies (Carlsbad, CA). Bovine pituitary extract was purchased from Kohjin Bio (Saitama, Japan). Fetal bovine serum (FBS) was obtained from Life Technologies (Carlsbad, CA). Bovine pituitary extract was used to prepare cDNA using the PrimeScript RT reagent kit (Takara; Shiga, Japan). The quantitative real-time polymerase chain reaction amplification of cDNA corresponding to 25 ng of total RNA was performed in a reaction mixture (20 $\mu\text{l}$) containing 1× concentration of FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 0.2 $\mu\text{M}$ primer pairs (Table 1) for caudal type homeobox 2 (CDX2), intestinal fatty acid-binding protein (IFABP), apical sodium-dependent bile acid transporter, monocarboxylate transporter 1, and multidrug resistance protein (MRP) 3, or containing 1× concentration of the TaqMan fast universal PCR master mix (Applied Biosystems) and the following TaqMan Gene Expression Assay with individual assay IDs: LGR5 (Hs00173664_m1), sucrase-isomaltase (SI) (Hs00356112_m1), CNT1 (Hs00984403_m1), CNT2 (Hs00188407_m1), CNT3 (Hs00910439_m1), equilibrative nucleoside transporter (ENT) 1 (Hs01085704_g1), ENT2 (Hs00154262_m1), ENT3 (Hs00217911_m1), and glyceraldehyde-3-phosphate dehydrogenase (Hs00275891_g1) on a 7500 FAST Real-Time PCR system (Applied Biosystems). Thermal cycling conditions included 95°C for 20 seconds, 40 cycles of 95°C for 15 seconds, and 60°C for 1 min. Threshold cycle (Ct) values were determined with 7500 software (Applied Biosystems). Ct values for target genes

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</tr>
<tr>
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<td>TTGGCTCTTCACTCTTCATAG</td>
</tr>
<tr>
<td>ASBT Forward</td>
<td>TGGCCCCAAAAAGGAAA</td>
</tr>
<tr>
<td>ASBT Reverse</td>
<td>AACCCTGGCCACGTCTCAC</td>
</tr>
<tr>
<td>MCT1 Forward</td>
<td>CGCGCGATATAAGCATATTT</td>
</tr>
<tr>
<td>MCT1 Reverse</td>
<td>ATCCAACTTGACCTCCCAA</td>
</tr>
<tr>
<td>MRP3 Forward</td>
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</tr>
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ASBT, apical sodium-dependent bile acid transporter; MCT1, monocarboxylate transporter 1.

**Proliferation Analysis.** HIECs were seeded on type I collagen-coated 96-well plates at 5 × 10^4 cells/well. Cell proliferation analysis was performed 48 hours after seeding by using a colorimetric bromodeoxyuridine (BrdU) cell proliferation assay kit (Millipore) according to the manufacturer’s instructions. A cell viability assay was also performed 48 hours after seeding by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). BrdU incorporation into the DNA of proliferating cells as well as cell viability were measured with a microplate reader (SpectraMax Gemini; Molecular Devices, Sunnyvale, CA) at 450 nm with a reference wavelength of 650 nm. To compensate for variability of viable cell numbers, the absorbance in the BrdU incorporation assay was divided by the absorbance in the cell viability assay.

**mRNA Quantification.** Total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies) from HIECs and Caco-2 cells on 8 and 20 days, respectively, after cell seeding onto membrane inserts. Total RNA from subconfluent HIECs was also extracted in the same manner. These total RNAs and human small intestinal total RNA (5-donor pooled; BioChain Institute, Newark, CA) were used to prepare cDNA using the PrimeScript RT reagent kit (Takara; Shiga, Japan). The quantitative real-time polymerase chain reaction amplification of cDNA corresponding to 25 ng of total RNA was performed in a reaction mixture (20 $\mu\text{l}$) containing 1× concentration of FAST SYBR Green Master Mix (Applied Biosystems) and the following TaqMan Gene Expression Assay with individual assay IDs: LGR5 (Hs00173664_m1), sucrase-isomaltase (SI) (Hs00356112_m1), CNT1 (Hs00984403_m1), CNT2 (Hs00188407_m1), CNT3 (Hs00910439_m1), equilibrative nucleoside transporter (ENT) 1 (Hs01085704_g1), ENT2 (Hs00154262_m1), ENT3 (Hs00217911_m1), and glyceraldehyde-3-phosphate dehydrogenase (Hs00275891_g1) on a 7500 FAST Real-Time PCR system (Applied Biosystems). Thermal cycling conditions included 95°C for 20 seconds, 40 cycles of 95°C for 15 seconds, and 60°C for 30 seconds. Threshold cycle (Ct) values were determined with 7500 software (Applied Biosystems). Ct values for target genes

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ASBT, apical sodium-dependent bile acid transporter; MCT1, monocarboxylate transporter 1.
were normalized to the Ct value of glyceraldehyde-3-phosphate dehydrogenase (ΔCt). Relative mRNA expression was determined using the 2^ΔΔCt method. We selected Ct values > 35 as the cut-off for the absence of expression.

The mRNA levels of other transporters, including organic cation transporter 1, organic anion-transporting peptide 2B1, P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and MRp1 and MRp2, and the drug-metabolizing cytochrome P450 (CYP450) enzymes CYP2C9, CYP2C19, CYP3A4, and CYP3A5, and UDP-glucuronosyltransferases (UGTs) UGT1A1, UGT1A4, UGT1A6, and UGT2B7 were analyzed using the Quantigene Plex 2.0 Assay Kit (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions. HIECs and Caco-2 cells cultured on membrane inserts for 8 and 20 days, respectively, were lysed with the Quantigene Sample Processing Kit (Affymetrix). Samples were analyzed using a Bio-Plex 200 suspension array system (Bio-Rad, Hercules, CA). The expression level of hypoxanthine phosphoribosyltransferase was used to normalize mRNA expression data from the target genes.

**Transmission Electron Microscopy of HIECs.** HIECs seeded on culture inserts as described above were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and were then cooled to 4°C. Thereafter, they were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. After three washes with 0.1 M phosphate buffer, cells were postfixed with 2% osmium tetroxide in 0.1 M phosphate buffer at 4°C for 90 minutes, and dehydrated through a graded ethanol series (50–100%). The samples were embedded in resin, and ultrathin sections were stained with 2% uranyl acetate and lead stain solution. The specimens were examined using a JEM-1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

**Transport Assay.** Prior to the addition of substrates, growth medium was removed and monolayers were rinsed twice with transport medium (TM) (Hanks’ balanced salt solution with 4.2 mM NaHCO3; and 20 mM glucose), adjusted to pH 7.4 by 10 mM HEPES. Monolayers were preincubated in TM (pH 7.4) for 30 minutes at 37°C in 95% humidity. Transport assays were carried out with apical and basal buffers consisting of TM adjusted to pH 6.5 by 10 mM MES and TM (pH 7.4) with 4.5% (w/v) bovine serum albumin, respectively. The following apical and basal buffers consisting of TM adjusted to pH 6.5 by 10 mM MES were used for the elution of digoxin, mitoxantrone, vinblastine, topotecan, atenolol, terbutaline, pindolol, midazolam, 1’-hydroxymidazolam, and 4-hydroxymidazolam. The Capcellpak C18 AQ column (50 mm, 4.6 mm i.d.; Nacalai Tesque, Kyoto, Japan) was used for the chromatographic separation of analytes for digoxin, mitoxantrone, vinblastine, topotecan, atenolol, terbutaline, pindolol, midazolam, 1’-hydroxymidazolam, and 4-hydroxymidazolam. The Capcellpak C18 AQ column (50 mm, 4.6 mm i.d.; Analyticon, Tokyo, Japan) was used for the chromatographic separation of analytes for didanosine, ribavirin, and doxorubicin. The following gradient condition was used for the elution of digoxin, mitoxantrone, vinblastine, topotecan, didanosine, terbutaline, ribavirin, doxorubicin, pindolol, midazolam, 1’-hydroxymidazolam, and 4-hydroxymidazolam. The flow rate was 0.2 ml/min and the injection volume was 5 μl. Column temperatures were maintained at 40°C. All data processing was performed with Waters QuanLynx software (Waters, Woburn, MA).

**Calculations.** The apparent drug permeability (P_app) was calculated for cellular transport in the HIEC and Caco-2 cell monolayers according to the following equation:

\[
P_{\text{app}} = \frac{dQ}{dt} \times \frac{1}{A + C_0}
\]  

(1)

where \(dQ/dt\), A, and C_0 represent the total amount of the compound transported to the acceptor chamber per unit time, the surface area of the transport membrane (0.33 and 1 cm² for HIECs and Caco-2 cells, respectively), and the initial compound concentration in the donor chamber, respectively. The efflux ratio (ER) was determined from the ratio of P_app in secretory (basal-to-apical) to that in absorptive (apical-to-basal) directions.

**Results**

**Morphology of HIECs.** HIECs survived and proliferated under our culture condition as previously demonstrated (Suzuki et al., 2010), and formed domes, which were similar to those in Caco-2 cells, after confluent on culture dishes (Fig. 1A). As monolayers were formed in HIECs and Caco-2 cells, TEER values increased and reached plateaus at
was examined by measuring TEER values (Fig. 2D). The TEER values
Moreover, the integrity of the HIEC monolayer during serial passage
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IFABP and SI on day 8 after seeding at any passage number indicated
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proliferating ability and formation capability of HIEC monolayers. To
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senescence (Fig. 2A), which implied that ISCs

In agreement with these results, transmission electron microscopy
confirmed the morphologic maturation of the HIEC monolayers after
8 days of growth on culture inserts (Fig. 1, C and D). HIECs grew as
monolayers of polarized columnar epithelia (Fig. 1C) with straight
dense microvilli, tight junctions, and desmosomes being observed
under a higher magnification (Fig. 1D). These results indicated that
HIECs differentiated into enterocytes with morphologically mature
characteristics under our culture condition.

Long-Term Proliferation Ability, Maintained Population
of ISCs, and Differentiation Potency into Enterocytes. Under our
culture conditions, HIECs constantly proliferated over 25 passages
without reaching senescence (Fig. 2A), which implied that ISCs
continuously existed and underwent cell division, leading to a long-term
proliferating ability and formation capability of HIEC monolayers. To
confirm this hypothesis, we examined the mRNA expression levels of
CDX2 and LGR5, a hindgut marker and an ISC marker (Barker and
Clevers, 2010; Iwao et al., 2014), respectively, at various passages. The
mRNA expression levels of CDX2 and LGR5 at subconfluence re-
mained almost constant up to 25 passages (Fig. 2B). In addition, there
was no consistent pattern of change in LGR5 levels between sub-
confluence and day 8 after seeding on culture inserts. These results
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long period of time, even after the formation of monolayers. Dif-
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the mRNA expression levels of IFABP and SI, markers for differ-
entiated enterocytes (Levy et al., 2009; Suzuki et al., 2010; Iwao et al.,
2014) (Fig. 2C). The increase observed in the expression levels of
IFABP and SI on day 8 after seeding at any passage number indicated
that ISCs had retained the potential to be differentiated into enterocytes.
Moreover, the integrity of the HIEC monolayer during serial passage
was examined by measuring TEER values (Fig. 2D). The TEER values
of HIEC monolayers on day 8 after seeding were almost constant over
the passage numbers tested, which suggested that HIECs formed mono-
layers with equal tightness over long-term continuous passages.

mRNA Analysis of Drug-Metabolizing Enzymes and Trans-
porters. The gene expression profiles of drug-metabolizing enzymes
and transporters in HIECs and Caco-2 cells are summarized in Fig. 3.
The mRNA expression levels of CYP3A5, UGT1A3, UGT1A6, or-
ganic cation transporter 1, organic anion-transporting peptide 2B1,
monocarboxylate transporter 1, CNT3, ENT1, ENT2, ENT3, P-gp,
BCRP, MRP1, MRP2, and MRP3 in HIECs were within 10-fold of
those in the human small intestine, whereas those of other P450/UGT
isoforms and transporters in HIECs were markedly lower (<10%) than
those in the human small intestine. UGT1A6 and CNT3 mRNA
expression levels were 6-fold and 17-fold higher in the HIEC mono-
layer than in Caco-2 cells, respectively, whereas the mRNA expression
levels of CYP2C9, CYP2C19, UGT2B7, and apical sodium-dependent
bile acid transporter in Caco-2 cells were >17-fold higher than those
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sion levels in both HIECs and Caco-2 cells were < 7.0% of those in
the human small intestine. Among the mRNA as for transporters
tested, CNT2 was not detected (Ct > 35) in either monolayers in this
study.

Bidirectional Transport across HIEC and Caco-2 Cell Mono-
layers. Bidirectional (apical-to-basal and basal-to-apical) permeability
coefficients for digoxin, a P-gp marker substrate (Pauli-Magnus et al.,
2000), and mitoxantrone, a BCRP marker substrate (Matsson et al.,
2009), were determined in both HIEC and Caco-2 cell monolayers to
examine the functions of efflux transport mediated by these transporters.
The values of ER, calculated from the ratio of $P_{app}$ in secretory (basal-
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study.
Ko143, a specific BCRP inhibitor (Matsson et al., 2009). These results indicated that these efflux transporters were functionally active in the HIEC monolayer, similar to that in Caco-2 cells.

Permeability of 10 Test Compounds across HIEC and Caco-2 Cell Monolayers and PAMPA. The $P_{app}$ values for 10 test compounds across the HIEC and Caco-2 cell monolayers and in PAMPA are listed in Table 2. The compounds tested included those with diverse characteristics for intestinal absorption, such as the $F_a$, major route for absorption (paracellular versus transcellular), transporters involved in net absorption (P-gp, BCRP, and nucleoside transporters), and physicochemical properties (molecular weight, partition coefficient, and polar surface area). The $P_{app}$ values of 10 test compounds in HIECs, Caco-2 cells, and PAMPA ranged from $0.56 \times 10^{-6}$ to $30 \times 10^{-6}$ cm/s, $0.035 \times 10^{-6}$ to $170 \times 10^{-6}$ cm/s, and $0.0046 \times 10^{-6}$ to $40 \times 10^{-6}$ cm/s, respectively. Transcellularly absorbed drugs (pindolol and midazolam) showed similarly high $P_{app}$ values in all three models: $2.2 \times 10^{-6}$ to $7.9 \times 10^{-6}$ cm/s for pindolol and $30 \times 10^{-6}$ to $170 \times 10^{-6}$ cm/s for midazolam. The formation of the primary metabolites of midazolam (1$\beta$-hydroxymidazolam and 4-hydroxymidazolam) was less than the lower limit of quantification (3 nM) in the apical and basal buffers after a 2-hour incubation in both HIECs and Caco-2 cells, which was consistent with the low mRNA expression of CYP3A4 (Fig. 3). In agreement with the looseness of the tight junction in the HIEC monolayer being lower, as demonstrated by the lower TEER value than that of the Caco-2 monolayer (Fig. 1B), the $P_{app}$ values for paracellularly absorbed compounds (FD-4, atenolol, and terbutaline) in the HIEC monolayer were markedly higher than those in both the Caco-2 monolayer and PAMPA. Possibly due to the higher expression levels of nucleoside transporters (CNT3, ENT1, ENT2, and ENT3) in HIECs than in Caco-2 cells (Fig. 3), didanosine, ribavirin, and doxifluridine, all substrates of nucleoside transporters, permeated more than 5-fold, 45-fold, and 24-fold faster, respectively, across the HIEC monolayer than the Caco-2 monolayer and by more...
that in the human small intestine, whereas those of Caco-2 cells were not, consistent with the lack of both P-gp and BCRP in PAMPA. The looseness of the tight junctions in the HIEC monolayer was similar to those of columnar epithelia with dense microvilli, tight junctions, and desmosomes. From ISCs, displayed mature morphologic features consisting of polarized cells with tight junctions and desmosomes. The looseness of the tight junctions in the HIEC monolayer was similar to that in the human small intestine, whereas those of Caco-2 cells were not, and the HIEC monolayer also had a high permeability for paracellularly absorbed compounds. Furthermore, the presence of the functions of P-gp and BCRP, and abundant mRNA expression of CNT3 made HIECs a valuable tool for studies on the intestinal absorption of these substrates.

Paracellular absorption refers to permeation across cell monolayers through pores in the tight junction, and the oral absorption of hydrophilic molecules generally relies on the paracellular pathway. In this study, the permeability values of paracellularly absorbed compounds (FD-4, atenolol, and terbutaline) were higher in HIECs than in Caco-2 cells (Table 2). These results indicated that the HIEC monolayer had a leakier paracellular route than Caco-2 cells. Furthermore, the permeability for these compounds of PAMPA, which completely lacks a paracellular pathway, was even lower. This result was consistent with the lower TEER value in the HIEC monolayer (98.9 Ω cm²) than in Caco-2 cells (900 Ω cm²) (Fig. 1). TEER values in the human duodenum, jejunum, and ileum have been reported to be 45, 34, and 37 Ω cm², respectively (Sjöberg et al., 2013), and indicate that the TEER value of HIECs was closer to the human small intestine than that of Caco-2 cells. Previous studies reported that the oral absorption of compounds that permeate via the paracellular route was poorly predicted in the Caco-2 monolayer, and this was attributed to its excessively tight junctions (Tavelin et al., 2003b; Saitoh et al., 2004; Mattson et al., 2005). The monolayer of 2/4/A1 cells, conditionally immortalized rat intestinal cells, has been reported to possess a radius of tight junction pores and TEER value that are similar to those of the human small intestine (Tavelin et al., 1999, 2003a). These HIEC-like characteristics led to the similarly high P_app values of paracellularly absorbed drugs between the 2/4/A1 cell monolayer and human jejunum (Tavelin et al., 1999, 2003b). However, in contrast with HIECs, the activities of efflux transporters, such as P-gp, BCRP, and MRP, are completely absent in 2/4/A1 cells (Tavelin et al., 2003a). Therefore, the utility of the HIEC monolayer as a superior alternative to the 2/4/A1 cell monolayer remains to be confirmed by side-by-side comparisons with diverse compounds including paracellularly absorbed compounds and the substrates of these efflux transporters.

The permeability of the HIEC monolayer to doxorubicin, ribavirin, and doxifluoridine, substrates of multiple nucleoside transporters (CNT, MRP, and NRTN, are completely absent in 2/4/A1 cells (Tavelin et al., 2003a)). Therefore, the utility of the HIEC monolayer as a superior alternative to the 2/4/A1 cell monolayer remains to be confirmed by side-by-side comparisons with diverse compounds including paracellularly absorbed compounds and the substrates of these efflux transporters.

TABLE 2

| Compound | 
|---|---|
| FD-4 | Vinblastine | Topotecan | Didanosine | Atenolol | Terbutaline | Ribavirin | Doxifluoridine | Pindolol | Midazolam | 
| % | 0.56 ± 0.01 | 2.6 ± 0.1 | 0.78 ± 0.14 | 0.62 ± 0.12 | 0.68 ± 0.09 | 0.70 ± 0.05 | 4.1 ± 0.3 | 4.7 ± 0.6 | 7.9 ± 0.7 | 30 ± 3 |
| $P_{app}$ | 0.035 ± 0.009 | 0.57 ± 0.02 | 0.35 ± 0.06 | 0.11 ± 0.02 | 0.34 ± 0.05 | 0.069 ± 0.006 | 0.092 ± 0.003 | 0.20 ± 0.03 | 0.36 ± 0.3 | 170 ± 7 |
| $F_a$ | 4.1 ± 0.8 | Transcellular/P-gp | Transcellular/BCRP | Nucleoside transporters | Paracellular | Nucleoside transporters | Nucleoside transporters | Transcellular | Transcellular |
| Molecular Weight | 4000 | 811.0 | 421.4 | 236.2 | 266.3 | 225.3 | 244.2 | 248.3 | 325.8 |
| LogP | 3.7 | 3.7 | 0.8 | 1.9 | 1.4 | 0.9 | 3.1 | 1.8 | 1.0 |
| PSA | 154.1 | 103.2 | 88.7 | 143.7 | 84.6 | 72.7 | 99.1 | 57.3 | 30.2 |

LogP, partition coefficient; PSA, polar surface area.

Discussion

In this study, we showed that the HIEC monolayer, which differentiated from ISCs, displayed mature morphologic features consisting of polarized columnar epithelia with dense microvilli, tight junctions, and desmosomes. The looseness of the tight junctions in the HIEC monolayer was similar to that in the human small intestine, whereas those of Caco-2 cells were not, and the HIEC monolayer also had a high permeability for paracellularly absorbed compounds. Furthermore, the presence of the functions of P-gp and BCRP, and abundant mRNA expression of CNT3 made HIECs a valuable tool for studies on the intestinal absorption of these substrates.

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![Fig. 4. Bidirectional permeability for digoxin and mitoxantrone across HIEC and Caco-2 cell monolayers in the presence or absence of inhibitors. (A) Efflux transport of digoxin (a P-gp substrate) in the presence or absence of verapamil. (B) Efflux transport of mitoxantrone (a BCRP substrate) in the presence or absence of Ko143. Data represent the mean ± S.D. (n = 3).](image-url)
CNT3, ENT1, and ENT2 for didanosine; CNT2, CNT3, ENT1, and ENT2 for ribavirin; CNT1, CNT3, ENT1, and ENT2 for dfoxifuridine (Mangravite et al., 2003; Yamamoto et al., 2007; Zhang et al., 2007; Young et al., 2013) was considerably higher than that of Caco-2 cells and PAMPA (Table 2). Na\(^+\)-dependent CNTs and Na\(^+\)-independent ENTs are expressed in the human small intestine (Meier et al., 2007). Nucleosides and their analog drugs generally have hydrophilic properties and diffuse slowly across the cell membrane; therefore, nucleoside transporters expressed in the small intestine largely contribute to their oral absorption (Endres et al., 2009; Okayama et al., 2012; Ishida et al., 2013). Previous studies reported that Caco-2 cells did not express CNT1, CNT2, or CNT3 (Ward and Tse, 1999; Bourgine et al., 2012), which was consistent with the very low expression of CNTs observed in our study (Fig. 3B). In contrast with the Caco-2 monolayer, the significantly high expression of CNT3 mRNA (16% of human small intestine) in HIECs may have conferred an absorption capability for didanosine, ribavirin, and dfoxifuridine to the HIEC monolayer (Table 2). Ribavirin and dfoxifuridine, which are good substrates for CNT3 (Hu et al., 2006), had higher \(P_{app}\) values than didanosine, a relatively poor substrate for CNT3 (Hu et al., 2006), in the HIEC monolayer, and the rank order of their \(P_{app}\) values was in agreement with that of \(Fa\) values.

Directional transport by P-gp and BCRP was confirmed in the HIEC monolayer by the efflux transport of the corresponding selective substrates (digoxin and mitoxantrone, respectively) and decreases in vectorial transport in the presence of specific inhibitors (verapamil and Ko143, respectively) (Fig. 4). Therefore, the HIEC monolayer can serve as a useful in vitro tool for the evaluation of P-gp– and BCRP–mediated transport during the absorption process. The ratio of \(P_{app}\) for the basol-to-apical direction to that for the apical-to-basal direction (ER) of mitoxantrone was approximately 10-fold higher in HIECs (38.2) than in Caco-2 cells (4.0) despite the lower mRNA expression of BCRP in HIECs than in Caco-2 cells (Fig. 3B). Ohtsuki et al. (2012) reported that the protein levels of several transporters including BCRP in the plasma membrane did not correlate with the respective mRNA levels in the human liver, and suggested that a post-transcriptional process and/or intracellular trafficking may play a key role in regulating the functional protein levels of transporters.
our knowledge, this is the first study to describe the application of a HIEC monolayer to the permeability assay for diverse compounds that are known to be absorbed paracellularly or transcellularly, and/or transported by P-gp, BCRP, and nucleoside transporters. The HIEC monolayer had similarly loose tight junctions to the human small intestine and the distinct mRNA expression of CNT3, which was absent in Caco-2 cells. This study demonstrated that the HIEC monolayer can serve as a novel and superior alternative to the conventional Caco-2 monolayer for predicting oral absorption in humans.

Authorship Contributions

Participants in research described: Takenaka, Harada, Kuze, Chiba, Iwao, Matsunaga.

Conducted experiments: Takenaka, Harada.

Performed data analysis: Takenaka, Harada.

Wrote or contributed to the writing of: Takenaka, Harada, Kuze, Chiba, Iwao, Matsunaga.

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