Establishment and Characterization of a Novel Caco-2 Subclone with a Similar Low Expression Level of Human Carboxylesterase 1 to Human Small Intestine

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ABSTRACT

Caco-2 cells predominantly express human carboxylesterase 1 (hCE1), unlike the human intestine that predominantly expresses human carboxylesterase 2 (hCE2). Transport experiments using Caco-2 cell monolayers often lead to misestimation of the intestinal absorption of prodrugs because of this difference, as prodrugs designed to increase the bioavailability of parent drugs are made to be resistant to hCE2 in the intestine, so that they can be hydrolyzed by hCE1 in the liver. In the present study, we tried to establish a new Caco-2 subclone, with a similar pattern of carboxylesterase expression to human intestine, to enable a more accurate estimation of the intestinal absorption of prodrugs. Although no subclone could be identified with high expression levels of only hCE2, two subclones, #45 and #78, with extremely low expression levels of hCE1 were subcloned from parental Caco-2 cells by the limiting dilution technique. Unfortunately, subclone #45 did not form enterocyte-like cell monolayers due to low expression of claudins and β-actin. However, subclone #78 formed polarized cell monolayers over 4 weeks and showed similar paracellular and transcellular transport properties to parental Caco-2 cell monolayers. In addition, the intestinal transport of oseltamivir, a hCE1 substrate, could be evaluated in subclone #78 cell monolayers, including P-glycoprotein-mediated efflux under nonhydrolysis conditions, unlike parental Caco-2 cells. Consequently, it is proposed that subclone #78 may provide a more effective system in which to evaluate the intestinal absorption of prodrugs that are intended to be hydrolyzed by hCE1.

Introduction

Caco-2 cells derived from human epithelial colorectal adenocarcinoma form a polarized epithelial monolayer that is characterized by the presence of microvilli and tight junctions consisting of several transmembrane and intracellular proteins such as occludin, claudins, and zona occludens (ZO)-1 (Paris et al., 2008; Deli, 2009; Shen et al., 2011; Ulluwishewa et al., 2011). The fraction dose absorbed in human intestine correlates well with the permeability of compounds passively transported through Caco-2 cell monolayers (Cogburn et al., 1991; Rubas et al., 1993). It has been reported that Caco-2 cells also possess major ABC and SLC family drug transporters that are expressed in the human intestine (Nakamura et al., 2002; Hilgendorf et al., 2007). Caco-2 cells are therefore widely used as an in vitro model of human intestinal epithelium for screening the oral absorption of drug candidates at an early stage of drug development. However, expression levels of drug-metabolizing enzymes, such as cytochrome P450 and UDP-glucuronosyl transferase isomers, are extremely low in Caco-2 cells compared with human small intestine (Raiessi et al., 1999; Jeong et al., 2005; Bieche et al., 2007), indicating that the permeability of Caco-2 cells does not accurately reflect the intestinal absorption of compounds metabolized in the intestinal epithelium.

Carboxylesterase (CES) is a major metabolic enzyme responsible for the bioconversion of prodrugs. The expression level of CES is high in Caco-2 cells as well as in the human intestine; however, the pattern of CES isozymes expressed in each is rather different (Imai et al., 2005). The predominant human intestinal CES is hCE2 (UniProt ID: O00748), whereas hCE1 (UniProt ID: P23141), the major hepatic CES isozyme, is the predominant CES in Caco-2 cells. The two human CES isoforms, hCE1 and hCE2, show completely different substrate specificities, a fact that is used in the prodrug approach to drug delivery (Imai, 2006). To improve the oral bioavailability of parent drugs with poor intestinal absorption, some successful prodrugs, such as temocapril, oseltamivir, and mycophenolate mofetil, are designed to be resistant to intestinal hydrolysis by hCE2, but to be rapidly hydrolyzed by hCE1 in the liver, producing high plasma concentrations of the parent drugs (Takei et al., 1997; Shi et al., 2006; Fujiiyama et al., 2010). Unfortunately, the high levels of hCE1 expressed in Caco-2 cells hydrolyze these prodrugs, so that parent drug is transported into the luminal side as well as the basolateral side. Therefore, the human intestinal absorption of prodrugs cannot be accurately estimated in transport experiments using Caco-2 cells.

We have previously developed an evaluation system for the intestinal absorption of prodrugs using Caco-2 cells in which CES-mediated hydrolysis is inhibited by pretreatment with 200 μM bis-p-nitrophenyl phosphate (BNPP), a specific CES inhibitor (Ohura et al., 2010). This method is useful because of the only limited influence of the pretreatment procedure on paracellular and transcellular transport, including transporter-mediated transport. However, the necessity to add BNPP as an inhibitor is inconvenient for high-throughput screening.
systems due to its possible interaction with either cellular proteins or prodrug. Furthermore, because the characterization of Caco-2 cells is often changed by culture conditions, optimization of the protocol for pretreatment with BNPP may occasionally be required in each laboratory.

In the present study, we tried to establish a new evaluation system using Caco-2 cell subclones. Because Caco-2 cells are originally heterogenous, subcloning has been used in research on the morphology and function of proteins such as sucrose-isomaltase and P-glycoprotein (P-gp) in Caco-2 cells (Chantret et al., 1994; Horie et al., 2003). We isolated subclones from parental Caco-2 cells by the limiting dilution technique to obtain various subclones that differed in the expression of CES isozymes. The expression profile of CES isozymes in subclones was analyzed by staining for esterase activity after native PAGE and measurement of hydrolyase activities for hCE1 substrates. The trans-epithelial electrical resistance (TER) values, mannitol flux, and expression levels of tight junction proteins in subclones were then measured to evaluate the formation and integrity of their cell monolayers. The passive diffusion and carrier-mediated transport in the subclones were compared with parental Caco-2 cells. Finally, the permeability of oseltamivir was evaluated using subclone cell monolayers.

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Materials and Methods

Materials. p-Nitrophenyl (PNP) acetate and PNP were purchased from Nacalai Tesque (Kyoto, Japan). PNP butyrate was purchased from Sigma-Aldrich (St. Louis, MO). Tempocapril was provided by Daiichi Sankyo (Tokyo, Japan). Oseltamivir and its acid metabolite were gifts of T. Oghira (Takasaki University of Health and Welfare, Takasaki, Japan). Propranolol hydrochloride was obtained from Wako Pure Chemical Industries (Osaka, Japan). Pooled human small intestinal S9 fraction of BD Gentest (pool of three subjects, one male, PMSF-free) was obtained from Coming Life Sciences (Tewksbury, MA). Rabbit anti-hCE1 and anti-hCE2 antibodies were a gift of M. Hosokawa (Chiba Institute of Science, Chiba, Japan). Rabbit anti-glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) and mouse anti-β-actin antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-claudin-1 and anti–caludin-4 antibodies were purchased from Bioss (Beijing, China). [14C]D-Mannitol (1.96 GBq/mmol) and [3H]Ptaclutaxel (1.52 GBq/mmol) were purchased from Moravek Biochemicals (Brea, CA). [3H]Glyceraldehyde (2.22 GBq/mmol) was obtained from American Radiolabeled Chemicals (St. Louis, MO). [3H]Estrogene-3-sulfate ammonium salt (1.70 GBq/mmol) was obtained from PerkinElmer Life Sciences (Boston, MA). All other chemicals and reagents were of analytical grade.

Cell Culture. Caco-2 cells were purchased from American Type Culture Collection (Rockville, MD). Caco-2 cells (passage 25–40) were grown in 75-cm² culture flasks in a humidified incubator at 37°C under 5% CO₂ in air, in Dulbecco’s modified Eagle’s medium (Sigma-Aldrich) consisting of 10% heat-inactivated fetal bovine serum, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Medium was replaced every other day. Conditioned media were collected after 48 hours of incubation with subconfluent parental Caco-2 cells. The cells were trypsinized with 0.25% trypsin and 0.53 mM EDTA and plated onto flasks.

Cloning from Parental Caco-2 Cells. Caco-2 cells from passage 25–35 were cloned by the limiting dilution method. Trypsinized Caco-2 cells were seeded in 96-well plates (CellBIND surface; Coming Life Sciences, Tewksbury, MA) at a concentration of one cell/well. Single cells were cultured in a mixture of equal volumes of conditioned medium and standard culture medium with 20% fetal bovine serum. Medium was changed every week for 3–5 weeks until the cell formed a colony. The individual colonies were scaled up to 75-cm² flasks. The expression of CES isozymes was evaluated by staining for esterase activity after native PAGE and Western blot analysis, according to our previous method (Ohura et al., 2010).

Preparation of Subcellular Fraction from Parental Caco-2 Cells and Subclones. Three subcellular fractions, whole-cell lysate, 9000g supernatant (S9) fraction, and a fraction rich in membrane-bound proteins, were prepared from parental Caco-2 cells and each subclone. The cell monolayers grown on the 75-cm² flask or Transwell insert (3-μm pore size, 24-mm diameter; Coming Life Sciences) were washed with ice-cold phosphate-buffered saline (PBS) and then detached with a spatula. The S9 fraction was prepared by centrifugation of the harvested cells in SET buffer (292 mM sucrose, 1 mM EDTA, and 50 mM Tris), according to our previous method (Ohura et al., 2010). The protein content of the S9 fractions was determined by Bradford’s method (Bradford, 1976) with bovine serum albumin (BSA) as standard. The protein content of the membrane-bound protein fraction, the harvested cells were solubilized in ice-cold radioimmunoprecipitation assay buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) for 30 minutes and centrifuged at 16,000g for 15 minutes at 4°C. The supernatant was used as whole-cell lysate. For the preparation of the membrane-bound protein fraction, the harvested cells were lysed with ice-cold Cell Lysis Buffer M (Wako Pure Chemical Industries) for 15 minutes and centrifuged at 16,000g for 5 minutes at 4°C. The pellets were then solubilized in ice-cold radioimmunoprecipitation assay buffer, and the obtained supernatant was used as the membrane-bound protein fraction. The protein content of whole-cell lysate and the membrane-bound protein fraction was determined with BSA as standard by the bicinchoninic acid method (Smith et al., 1985). These preparations were stored at –80°C until use.

Hydrolysis Experiments. The S9 fractions of parental Caco-2 cells, Caco-2 subclones, and human small intestine were diluted with 50 mM HEPES buffer (pH 7.4) to appropriate protein concentrations. After precipitation of diluted S9 fraction for 5 minutes at 37°C, the reaction was started by adding substrates (tempocapril, oseltamivir, and PNP). The concentration of hydrolyses in the reaction solution was determined by high-performance liquid chromatography or the spectrophotometric method reported in our previous studies (Ohura et al., 2010, 2011; Bahar et al., 2012).

TEER Measurement of Cell Monolayers. Parental Caco-2 cells and subclones were seeded at a density of 8 × 10⁴ cells/cm² on a Transwell insert. The culture medium [1.5 mL in the apical (AP) compartment and 2.6 mL in the basolateral (BL) compartment] was replaced every other day for the first week and daily thereafter. TEER was measured every 2-3 days for 3–5 weeks using a Millicell-ERS ohmmeter (Millipore, Billerica, MA).

Transport Experiments. Transport experiments were performed using the cell monolayers whose TEER values reached a plateau. Each compound, dissolved in a transport buffer of Earle’s balanced salt solution (pH 6.0) or Hanks’ balanced salt solution (pH 7.4), was added to either the AP or BL side, and samples were taken sequentially from the donor and receiver compartments. All subsequent procedures were performed at 37°C. The concentration of 14C and 3H-labeled compounds and propranolol in the samples was determined by the methods reported in previous studies (Ohura et al., 2010, 2011). For the measurement of concentration of oseltamivir and its acid metabolite, each sample was mixed with phosphoric acid (final concentration 0.75 mM) and sodium pentanesulfonate (final concentration 60 mM) and analyzed by high-performance liquid chromatography.

The apparent permeability coefficient (Papp, cm/s) was calculated using the following equation: Papp = dQ/dt/Ao/Ci, where dQ/dt is the appearance rate of compound in the receiver compartment (steady-state flux), A is the surface area of the monolayer, and Ci is the initial concentration of compound in the donor compartment.

Fluorescence Staining of F-Actin. The parental Caco-2 cells and subclones were grown on a 35-mm glass-based dish (IWAKI, AGC Techno Glass, Chiba, Japan). Cells were fixed with 3.7% formaldehyde in PBS for 10 minutes at room temperature and treated with 0.1% Triton X-100 in PBS for 5 minutes. After preincubation with 1% BSA-PBS, cells were incubated with 0.1 μM Acti-stain 488 Phalloidin (Cytoskeleton, Denver, CO) in 1% BSA-PBS for 40 minutes at room temperature and counterstained with 10 μM Hoechst 33342 (Nacalai Tesque) in PBS for another 20 minutes. The specimens were observed under a fluorescence microscope (BIOREVO BZ-9000; Keyence, Osaka, Japan).

RNA Extraction and Quantification of mRNA Expression. Extraction of total RNA and synthesis of first-strand cDNA from cell monolayers were performed according to our previous study (Ohura et al., 2014). Real-time quantitative polymerase chain reaction (PCR) was performed using Bio-Rad iCycler iQ Real Time PCR detection system (Bio-Rad Laboratories, Hercules, CA) with SYBR Premix Ex Taq II (Takara Bio Inc., Shiga, Japan) and the specific primers listed in Table 1. Thermal cycling conditions were 95°C for 1 minute, followed by 40 cycles of 95°C for 10 seconds and 60°C for 45 seconds. Reactions
of hCE1 was high in parental Caco-2 cells, whereas its level was low in subclone #45 and #78. The expression levels of CES isozymes in subclones #45 and #78 were compared with parental Caco-2 cells by native PAGE and Western blot analysis of their cellular S9 fraction. The expression level of hCE1 was high in parental Caco-2 cells, whereas its level was negligible in the two subclones, as shown in Fig. 1A and B; hCE2 was present in both subclones at lower levels than in parental Caco-2 cells. To evaluate the mRNA expression of CES isozymes in the two subclones, the CES1 genotype of parental Caco-2 cells was first examined by reverse-transcription PCR analysis. In the human CES1 family, two functional genotypes, CES1A1 and CES1A2, have been identified (Fukami et al., 2008; Hosokawa et al., 2008). Because the sequences downstream of intron 1 in the CES1A1 gene are identical to those in the CES1A2 gene, the same mature protein, hCE1, is produced from both genes. As shown in Fig. 1C, parental Caco-2 cells mainly expressed CES1A1 mRNA. The CES1A1 and CES1A2 mRNA levels in parental Caco-2 cells and the two subclones were measured by real-time PCR. The results are shown in Fig. 1D. The mRNA expression profile of CES1A1 and CES1A2 was very similar to their protein expressions in each cell line.

### Results

#### Expression of CES Isozymes in Caco-2 Subclones

The expression of hCE1 and hCE2 in the homogenates of 87 subclones isolated from parental Caco-2 cells was evaluated by native PAGE analysis. Each of the subclones showed a different phenotype for expression of hCE1 and hCE2 (Supplemental Fig. 1). Unfortunately, a subclone with predominant expression of hCE2 was not obtained. We therefore selected two subclones, #45 and #78, that showed low expression of both hCE1 and hCE2. The expression levels of CES isozymes in subclones #45 and #78 were compared with parental Caco-2 cells by native PAGE and Western blot analysis of their cellular S9 fraction. The expression level of hCE1 was high in parental Caco-2 cells, whereas its level was negligible in the two subclones, as shown in Fig. 1A and B; hCE2 was present in both subclones at lower levels than in parental Caco-2 cells.

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### Hydrolyase Activities in Caco-2 Subclones

The hydrolytic activities of subclones #45 and #78 were studied using temocapril and oseltamivir as hCE1 substrates. As shown in Fig. 2, A and B, the hydrolyses of these substrates in subclones #45 and #78 were less than 5% of the level in parental Caco-2 cells. The hydrolytic properties for hCE1 substrates in subclones #45 and #78 did not alter up to at least passage 25. These low hydrolytic activity levels were almost the same as those in the S9 fraction prepared from parental Caco-2 cell monolayers pretreated with BNPP, a specific inhibitor of CES (Supplemental Fig. 2A) (Ohura et al., 2011). In contrast, the hydrolysis of PNP derivatives, substrates for several esterases including CESs, in the two subclones showed 30–50% of the levels found in parental Caco-2 cells (Fig. 2C). The hydrolysis activity for PNP acetate was similar to the activity remaining (approximately 50%) in the S9 fraction of parental Caco-2 cell monolayers pretreated with BNPP (Supplemental Fig. 2B), suggesting that the expression of other esterases was retained in subclones #45 and #78.

#### Formation and Integrity of Caco-2 Subclone Cell Monolayers

The TEER values of cell monolayers of subclones #45 and #78 were evaluated after seeding on Transwells. As shown in Fig. 3A, the TEER value of parental Caco-2 cells increased over 3 weeks and reached a plateau at above 800 Ω·cm². The TEER values of subclones #45 and #78 increased only slightly in the first week due to their slow growth rate compared with parental Caco-2 cells. In subclone #45, TEER reached a
maximum value at 2 weeks, but this was still only a quarter of that of the parental Caco-2 cells. In contrast, the maximum TEER of subclone #78 was reached after 4 weeks and was above 1000 Ω × cm².

Mannitol flux, an indicator of adhesion among cells, was also measured in each cell monolayer when TEER had reached a plateau (Fig. 3B). The P_app value in subclone #45 was 37-fold higher than that in parental Caco-2 cells. In contrast, subclone #78 showed a larger mannitol flux than parental Caco-2 cells, although the difference did not reach statistical significance. These results indicate fragile and strong tight junctions in subclones #45 and #78, respectively.

Microscopic Observation and Expression of Housekeeping Genes in Caco-2 Subclones. To examine differences in intracellular adhesion between parental Caco-2 and subclone cell monolayers, cell morphologies were visualized by staining F-actin filaments under fluorescence phase-contrast microscope. As shown in Fig. 4A, parental Caco-2 cells closely adhered to adjacent cells, and their F-actin filaments were strongly stained as a continuous band encircling the cells at the cellular borders. The cell shape and distribution of F-actin filaments in subclone #78 were similar to those in parental Caco-2 cells. In contrast, the F-actin of subclone #45 was obscure, not only at the cell edge, but also over the whole cell, even at longer exposure times.

The expression of housekeeping genes was subsequently analyzed by real-time PCR to evaluate basic cellular function and select the most suitable reference gene for the analysis of the mRNA expression profiles of tight junction components in parental Caco-2 cells and subclones. Generally, 18S rRNA and mRNAs of ribosomal protein L0 (RPL0), GAPDH, β-actin, and villin are regarded as housekeeping genes in parental Caco-2 cells. Figure 4B shows the expression levels of these mRNAs, normalized to the 18S rRNA level, in parental Caco-2 cells and two subclones. The relative mRNA levels of RPL0 and GAPDH were not significantly different among three cell lines. The mRNAs of β-actin and villin were also expressed at similar levels in parental Caco-2 cells and subclone #78, whereas their expression levels were remarkably low in subclone #45, indicating nonformation of microvilli in subclone #45. These results also show that 18S rRNA and RPL0 mRNA are suitable for use as reference genes. RPL0 mRNA was finally chosen because of the extremely high expression levels of 18S rRNA, in comparison with the mRNA levels of tight junction components.

Expression Levels of Tight Junction Proteins in Caco-2 Subclones Compared with Parental Caco-2 Cells. The mRNA expression profiles of tight junction components were evaluated in parental Caco-2 cells (Supplemental Fig. 3). With respect to peripheral

Fig. 1. The expression of CES isozymes in parental Caco-2 cells, subclone #45, and subclone #78. (A) Esterase activity staining after native PAGE using α-naphthylacetate. The S9 proteins (20 μg) were loaded in each lane. Arrows indicate bands corresponding to each CES isozyme. (B) Western blot analysis using anti-hCE1 and anti-hCE2 antibodies. The S9 proteins (5 μg and 30 μg) were subjected to SDS-PAGE for the detection of hCE1 and hCE2, respectively, and transferred electro-phytorically to polyvinylidene difluoride membrane for immunostaining. (C) The expression of CES1A1 and CES1A2 in parental Caco-2 cells was determined by reverse-transcription PCR analysis. (D) The mRNA expression levels of CES isozymes were determined by real-time PCR and normalized to the expression level of RPL0 mRNA. Values are means ± S.D. (n = 3). * indicates p < 0.05; ** indicates p < 0.01 in comparison with parental Caco-2 cells.

Fig. 2. Hydrolase activities for temocapril (A), oseltamivir (B), and PNP derivatives (C) in parental Caco-2 cells, subclone #45, and subclone #78. The S9 fractions were diluted with 50 mM HEPES buffer (pH 7.4) at 50–200 μg/ml. Substrates (temocapril, PNP derivatives: 500 μM; oseltamivir: 1 mM) were incubated with the diluted S9 fractions. Each column represents the mean ± S.D. (n = 3). # Indicates activities <2 pmol/min/mg protein. * and ** indicate p < 0.05 and p < 0.01, respectively, in comparison with parental Caco-2 cells. † indicates p < 0.05 in comparison with subclone #45.
membrane adaptor proteins, the mRNA level of ZO-1 was higher than that of ZO-2. With respect to the expression of claudin, an essential transmembrane protein of tight junctions, the highest expression level was observed for claudin-4 mRNA, followed by claudin-7 and claudin-1, and the mRNAs of claudin-2 and claudin-3 were barely expressed. The mRNA levels of other transmembrane proteins, occludin, junctional adhesion molecule (JAM)-1, and tricellulin, were comparable to those of claudin-1 and claudin-7.

The relative mRNA levels of tight junction components in subclones #45 and #78 were then compared with those in parental Caco-2 cells. The mRNA levels of claudin-2 and claudin-3 were negligible in both subclones, as well as in parental Caco-2 cells. As shown in Fig. 5A, tricellulin mRNA was expressed equally in parental Caco-2 cells and subclone #45. However, subclone #45 showed low mRNA expression levels of other tight junction components, especially claudin-1, claudin-4, and claudin-7, whose expression levels were close to the detection limits. In contrast, the mRNA levels of ZO-2, occludin, tricellulin, and claudin-1 in subclone #78 were almost the same as in parental Caco-2 cells. Interestingly, the mRNA levels of claudin-4 and claudin-7 in subclone #78 were approximately six- and four-fold higher, respectively, than in parental Caco-2 cells, although mRNA levels of ZO-1 and JAM-1 were low, as in subclone #45. Figure 5, B and C, shows the protein levels of claudin-1, claudin-4, β-actin, and GAPDH in the whole-cell lysate and the membrane-bound protein fraction, respectively, of parental Caco-2 cells and subclones. The expression levels of these proteins were correlated with their mRNA levels in both fractions. These results indicate that the quite low expression of claudins and β-actin causes incomplete assembly of tight junctions in subclone #45, whereas high expression levels of claudin-4 and claudin-7 in subclone #78 are sufficient to encourage intercellular adhesion.

Transcellular Transport in Subclone #78. We finally selected subclone #78, with its comparatively low expression levels of hCE1 and similar paracellular flux to parental Caco-2 cells. To evaluate the suitability of subclone #78 for transport assays, the mRNA expression levels of drug transporters and transcellular transport were compared between parental Caco-2 cells and subclone #78. As shown in Table 2, the mRNAs of efflux and influx transporters in subclone #78 were expressed at almost the same levels as parental Caco-2 cells. Figure 6 shows the P_app values for the transport of several compounds across parental Caco-2 and subclone #78 cell monolayers. The P_app values for bidirectional transport of propranolol, a marker of passive diffusion in the transcellular route, in subclone #78 were similar to parental Caco-2 cells. Furthermore, pH-dependent uptake of [3H]Gly-Sar by human...
peptide transporter 1 (PEPT1) and the efflux transport of \([3H]\)paclitaxel via P-gp was not significantly different between parental Caco-2 cells and subclone #78. In the transport of \([3H]\)estrone-3-sulfate, a substrate for organic anion-transporting polypeptide 2B1 and breast cancer–resistant protein on the AP membrane, and for organic solute transporter \(\alpha/\beta\) on the BL membrane, no statistical difference was observed between parental Caco-2 cells and subclone #78. Furthermore, the transport characteristics of subclone #78 did not change at high passage numbers.

Transport of Oseltamivir across Cell Monolayers of Subclone #78. The transport of prodrug across cell monolayers of subclone #78 was studied using oseltamivir as a model prodrug. The kinetic parameters for enzymatic hydrolysis of oseltamivir were compared in the S9 fractions of human small intestine, parental Caco-2 cells, and subclone #78. As shown in Fig. 7, nonsaturable hydrolase activities were observed over the concentration range of 50–1000 \(\mu\)M in all three S9 fractions. The intrinsic clearance obtained as the slope of the linear regression of hydrolase activity versus the concentration of oseltamivir was 10-fold higher in parental Caco-2 cells (0.570 ± 0.00824 \(\mu\)L/min/mg protein) than in human small intestine (0.0618 ± 0.0167 \(\mu\)L/min/mg protein), whereas subclone #78 also showed low intrinsic clearance (0.0477 ± 0.0115 \(\mu\)L/min/mg protein), indicating that subclone #78 may more accurately predict human intestinal absorption of oseltamivir than parental Caco-2 cells.

Figure 8 shows the results of the AP-to-BL transport experiment of oseltamivir. Oseltamivir was hydrolyzed in the cells during transport across parental Caco-2 cell monolayers, and its acid metabolite was transported into both AP and BL sides (Fig. 8A). In contrast, as expected, oseltamivir was transported into the BL side in subclone #78 cell monolayers, and the acid metabolite was not detected on either side (Fig. 8B), suggesting that oseltamivir is absorbed as an ester in human small intestine. The \(P_{app}\) value in the AP-to-BL transport was increased only 1.4-fold in subclone #78 compared with parental Caco-2 cells (Table 3). In contrast, the BL-to-AP transport of oseltamivir was significantly affected by hydrolysis, and its \(P_{app}\) value was 4.7-fold higher in subclone #78 than parental Caco-2 cells. The efflux ratio of permeability (BL-to-AP/AP-to-BL) in subclone #78 was 3.4-fold higher than in parental Caco-2 cells, indicating that oseltamivir has a high affinity for intestinal efflux transporters.

Table 2: Relative mRNA expression levels of transporters in parental Caco-2 cells and subclone #78

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fold Induction</th>
<th>% of Parental Caco-2</th>
</tr>
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<tbody>
<tr>
<td>MRP2</td>
<td>0.0737 ± 0.0121</td>
<td>108</td>
</tr>
<tr>
<td>MRP3</td>
<td>0.0197 ± 0.0004</td>
<td>98</td>
</tr>
<tr>
<td>MDR1</td>
<td>0.0121 ± 0.0006</td>
<td>92</td>
</tr>
<tr>
<td>BCRP</td>
<td>0.0046 ± 0.0005</td>
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<td>OATP2B1</td>
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<td>117</td>
</tr>
<tr>
<td>PEPT1</td>
<td>0.0026 ± 0.0007</td>
<td>132</td>
</tr>
<tr>
<td>OATP1A2</td>
<td>nd</td>
<td>nd</td>
</tr>
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Discussion

We have previously established a system for estimating human intestinal absorption using Caco-2 cells whose CES activity has been inhibited by pretreatment with BNPP (Ohura et al., 2010). However, it
Transport via transcellular routes across parental Caco-2 and subclone 
#78 cell monolayers. (A) The transport of propranolol (50 μM) in Hanks’ balanced salt solution (pH 7.4) to the AP and/or BL sides. (B) The AP-to-BL transport of [3H]Gly-Sar (1 nM) in Hanks’ balanced salt solution (pH 7.4) or in Earle’s balanced salt solution (pH 6.0) in the AP side and Hanks’ balanced salt solution (pH 7.4) in the BL side. (C) The transport of [3H]paclitaxel (2 nM) in Hanks’ balanced salt solution (pH 7.4) in both sides. (D) The transport of [3H]estrone-3-sulfate (4 nM) in Earle’s balanced salt solution (pH 6.0) in the AP side and Hanks’ balanced salt solution (pH 7.4) in the BL side. Each columns represent the mean ± S.D. (n = 3).

Fig. 7. S-V plot for hydrolysis of oseltamivir in the S9 fraction of human small intestine, parental Caco-2 cells, and subclone #78. Oseltamivir (50–1000 μM) was incubated for 120 minutes with S9 fractions of human small intestine, parental Caco-2 cells, and subclone #78 (200 μg/mL). Each symbol represents the mean ± S.D. (n = 3).

has been reported that BNPP inhibits not only CESs, but also other esterases, such as carboxymethylethylenebutenolidase (Ishizuka et al., 2010). Furthermore, because the cellular properties of Caco-2 cells are different under different culture conditions, laboratories may occasionally need to optimize pretreatment conditions using BNPP. It would therefore be advantageous to develop a method that would not require pretreatment with BNPP. In the present study, we aimed to establish a novel Caco-2 subclone with the same expression pattern of CES isozymes as human small intestine to allow us to evaluate the intestinal absorption of prodrugs without the use of BNPP.

A successful prodrug is resistant to hydrolysis by intestinal hCE2 and bioconverted by hepatic hCE1. An ideal subclone would therefore have a high level of hCE2 and a low level of hCE1. We obtained several subclones with different phenotypes from heterogenous Caco-2 cells using a dilution technique. Although we were unable to obtain a subclone with high levels of hCE2, we could obtain two subclones, #45 and #78, which expressed hCE1 at very low levels in comparison with parental Caco-2 cells (Fig. 1). A Caco-2 subclone with low levels of hCE1 would still offer a useful advantage over parental Caco-2 cells in the evaluation of the intestinal absorption of prodrugs. However, subclone #45 proved not to be suitable for the evaluation of intestinal permeability due to its weak membrane barrier (Fig. 3). Meanwhile, subclone #78 formed a polarized epithelium-like monolayer and showed

similar paracellular and transcellular permeabilities to parental Caco-2 cells. The major differences in the barrier properties of cell monolayers between parental Caco-2 cells and subclone #45 were found in the expression of Claudins and actins (Figs. 4 and 5). In several studies, it has been clarified that claudin families are the main structural components of tight junctions (Furuse et al., 1998; Suzuki and Hara, 2009; Doi et al., 2011; Lameris et al., 2013). Furthermore, depletion of β-actin impairs the formation of normal cell monolayers (Baranwal et al., 2012). The weak membrane barrier of subclone #45 may therefore be due to the low expression levels of Claudins and actins.

Interestingly, subclone #78 also showed a different expression profile of tight junction components to parental Caco-2 cells (Fig. 5). The expression levels of ZO-1 and JAM-1 were low in subclone #78 as well as in subclone #45. Although the reason for this was unclear, low expression of ZO-1 mRNA was common in other subclones with slower growth rates than parental Caco-2 cells (Supplemental Fig. 4). Chen et al. (2008) have reported that transcriptional repression of ZO-1 occurs by an interaction of JunD, a transcription factor, with the ZO-1 promoter region. It has also been reported that JunD inhibits the proliferation of Caco-2 cells (Li et al., 2002). The suppression of ZO-1 therefore might be related to JunD in these subclones.

The expression levels of Claudin-4 and -7 in subclone #78 were markedly higher than those in parental Caco-2 cells, despite equal expression levels of Claudin-1. However, in spite of their higher levels, subclone #78 showed only a slight increase of TEER and almost the same mannitol flux as parental Caco-2 cells. It has been reported that the expression of Claudin-4 is frequently upregulated by various compounds derived from fruit and vegetables in Caco-2 cells (Suzuki and Hara, 2009; Vreeburg et al., 2012; Noda et al., 2013). Furthermore, Langlois et al. (2010) have reported that the expression of Claudin-4 increases after reaching fully confluent Caco-2 cell monolayers, in spite of the relatively stable expression of Claudin-1. It has also been reported that TEER in Caco-2 cell monolayers is not changed by overexpression of Claudin-7 (Fujita et al., 2008). Taken together, our results imply that Claudin-1 may be an essential component of tight junctions in Caco-2 cells, and that the expression of Claudin-4 and -7 may reinforce the tight junction barrier.

We also examined the reason for hCE1 downregulation in subclone #78. As shown in Supplemental Table 1, neither 5-azacytidine, a
DNA-demethylating agent, nor sodium butyrate, a histone-acetylating agent, induced the expression of CES1A1 mRNA, indicating no involvement of epigenetic regulation in the low expression of hCE1 in subclone #78. Because the promoter region of the CES1A1 gene contains binding sites for Sp1 and C/EBP, changes in their expression levels may lead to the decrease of hCE1 expression in subclone #78. However, the mRNA expression levels of other proteins regulated by Sp1 and/or C/EBP, such as P-gp, MRP2, PEPT1, and GAPDH (Claeyssens et al., 2003; Scotto, 2003; Hisaeda et al., 2004; Shimakura et al., 2005), were similar in subclone #78 and parental Caco-2 cells (Table 2), suggesting the unlikelihood of transcriptional regulation by Sp1 and C/EBP. The regulatory mechanism of hCE1 expression in subclone #78 could unfortunately not be determined.

Finally, we evaluated the transport of oseltamivir across subclone #78 cell monolayers. Although oseltamivir was slightly hydrolyzed in the S9 fraction of subclone #78 by an esterase other than hCE1 (Fig. 2), the acid metabolite of oseltamivir was not detected in either the AP or BL side during transport across subclone #78 cell monolayers (Fig. 8). Because the extremely slow hydrolysis rate of oseltamivir in the S9 fraction of subclone #78 was the same as that in human intestinal S9 fraction (Fig. 7), the hydrolysis of oseltamivir is presumed to be almost absent during absorption in human small intestinal mucosa. Oseltamivir was highly effluxed into the AP side in subclone #78 compared with parental Caco-2 cells (Table 3). Although it has been reported that oseltamivir is not only a substrate for P-gp, but also for PEPT1 (Morimoto et al., 2008; Hoffmann et al., 2009; Oghihara et al., 2009), the contribution of P-gp was mainly observed in subclone #78 under our experimental conditions. It is interesting that such conditions in subclone #78, that is, without CES-mediated hydrolysis, enabled us to evaluate clearly the transporter-mediated efflux of oseltamivir.

The \( P_{\text{app}} \) value for the AP-to-BL transport of oseltamivir in subclone #78 was 14.8 ± 3.56 \( \times 10^{-7} \) cm/s. This \( P_{\text{app}} \) value indicates that 50–60% of oseltamivir is absorbed in human intestine, judging from the correlation between the fraction dose absorbed in human intestine and Caco-2 cell permeability (Cogburn et al., 1991; Rubas et al., 1993). However, it has been reported that the absolute bioavailability of oseltamivir is 80% (He et al., 1999). This mismatch is presumably due to P-gp–mediated efflux of oseltamivir in subclone #78. Substrates of P-gp often show high intestinal absorption, because of their absorption at proximal regions of the small intestine, where the expression of P-gp is relatively low, and saturation of P-gp–mediated efflux by high concentrations of P-gp substrates at the site of absorption (Lin and Yamazaki, 2003; Varma et al., 2005; Ohashi et al., 2006). The in vivo intestinal absorption of oseltamivir may be little affected by P-gp. Therefore, although subclone #78 may slightly underestimate the intestinal absorption of oseltamivir due to P-gp–mediated efflux, subclone #78 is still expected to be suitable for use as an in vitro model of human intestinal epithelial cells in the development process of prodrugs.

### Table 3

<table>
<thead>
<tr>
<th>Subclone</th>
<th>( P_{\text{app}} ) (( \times 10^{-7} ) cm/s)</th>
<th>Ratio (BL-to-AP/AP-to-BL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parental Caco-2 cells</td>
<td>10.7 ± 0.770</td>
<td>52.5 ± 6.88</td>
</tr>
<tr>
<td>Subclone #78</td>
<td>14.8 ± 3.56</td>
<td>249 ± 69.4**</td>
</tr>
</tbody>
</table>

** indicates \( p < 0.01 \) in comparison with parental Caco-2 cells in each direction of transport.
In conclusion, the use of subclone #78 makes it possible to analyze the intestinal transport of prodrugs without prior addition of BNPP, as the transport characteristics and hydrolysis activities of subclone #78 are very similar to those of our previous system which required pretreatment with BNPP (Ohura et al., 2010). This transport assay system will not only be useful for the prediction of the human intestinal absorption of prodrugs designed to be hydrolyzed by hCE1, but also for analyzing the intestinal absorption behavior of ester-type drugs that are hydrolyzed by hCE1, such as methylphenidate and clopidogrel. We also demonstrated the usefulness of subclone #78 for evaluating the transport mechanism of these drugs and prodrugs. Thus, although we were unable to obtain an ideal subclone that expressed hCE1 and hCE2 at low and high levels, respectively, subclone #78 still offers useful advantages over parental Caco-2 cells. In the future, it is planned to develop subclone #78 using transfection technology to enable it to also stably express hCE2.

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Participated in research design: Ohura, Imai. Conducted experiments: Ohura, Nishiyama, Saco, Kurokawa. Contributed new reagents or analytic tools: Ohura, Imai. Performed data analysis: Ohura. Wrote or contributed to the writing of the manuscript: Ohura, Imai.

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