Cultured Period-Dependent Change of Function and Expression of ATP-Binding Cassette Transporters in Caco-2 Cells

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ABSTRACT:

The objective of this study was to determine an appropriate culture period to assess whether a compound of interest is transported by efflux transporters such as human multidrug resistance 1 (hMDR1), human multidrug resistance-associated protein 2 (hMRP2), and human breast cancer resistance protein (hBCRP) in Caco-2 cells. Caco-2 cells were cultured on a Transwell for 1 to 6 weeks. The expression of these transporters in the mRNA and protein levels was examined using a real-time polymerase chain reaction and Western blotting, respectively. Transcellular transport activities using digoxin, ochratoxin A, olmesartan, and estrone-3-sulfate were also examined. Except for digoxin, the permeability coefficient (P_app) ratio of the three compounds at 2 weeks was the highest in the periods tested. The P_app ratio of digoxin at 2 weeks was higher than that at 3 weeks. The temporal expression profile of each transporter in the mRNA level was similar to that in the protein level, and the functions of hMRP2 and hBCRP were roughly correlated with the expression in the mRNA and protein levels, but that of hMDR1 was not. These data suggest that among all the culture periods evaluated a 2-week culture is the best culture period for transport studies to identify whether a compound is a substrate for hMDR1, hMRP2, and hBCRP.

Caco-2 cells, derived from human colorectal adenocarcinoma, form monolayers (as human intestinal epithelium) under conventional culture conditions and have been widely used as a potent in vitro model to predict drug absorption in humans to explore mechanisms of drug absorption and identify substrates or inhibitors of transporters (Artursson and Karlsson, 1991; Artursson et al., 1996; Yamashita et al., 1997). Various ATP-binding cassette transporters, including human multidrug resistance 1 (hMDR1), human multidrug resistance-associated protein 2 (hMRP2), and human breast cancer resistance protein (hBCRP), are well known efflux transporters among the transporters expressed in Caco-2 cells (Seithel et al., 2006; Hilgendorf et al., 2007).

These transporters play an important role in drug bioavailability as they contribute to drug efflux and drug-drug interactions. Active transport systems mediated by hMDR1 and hMRP2 have been well characterized in Caco-2 cells (Makhey et al., 1998; Döppenschmitt et al., 1999; Gutmann et al., 1999), and the transport function of hBCRP in Caco-2 cells has also been defined (Xia et al., 2005). Because hMDR1 has very broad substrate specificity (Fromm, 2002), numerous drug-drug interactions via hMDR1 such as between digoxin and quinidine have been reported (Pedersen et al., 1983). More recently, a regulatory viewpoint for the evaluation of hMDR1-mediated drug-drug interactions using Caco-2 cells was presented (Zhang et al., 2008), whereas at the moment the need to evaluate drug-drug interactions mediated by transporters other than hMDR1 is drug- or therapeutic class-specific. In this situation, the Caco-2 cell line is considered to be a useful model for the evaluation of the jejunal drug efflux processes of drugs.

The conventional culture period of Caco-2 cells for transport studies is 21 days, judging from the membrane integrity as assessed by the measurement of transepithelium electrical resistance (TEER) and the permeability of hydrophilic molecules such as mannitol (Artursson and Karlsson, 1991; Tavelin et al., 2002). The culture periods are considered to be important for the formation of a tight junction (Sambuy et al., 2005). Pinto et al. (1983) showed that the Caco-2 cell monolayers express high levels of several brush border hydrolases in human intestine and have well developed junctional complexes after 2 to 3 weeks in cell culture. However, up to now there have been few reports on appropriate culture periods from the viewpoint of not only the expression levels of hMDR1, hMRP2, and hBCRP but also the transport function using a typical substrate for each transporter (Hirohashi et al., 2000; Goto et al., 2003). There are only a few reports about the relationship between transport function and expression in Caco-2 cells (Hirohashi et al., 2000; Xia et al., 2005), despite being many reports about the expression levels of these transporters (Nakamura et al., 2003; Seithel et al., 2006; Hilgendorf et al., 2007).

Therefore, we investigated the transport function and expression levels of mRNA and protein of hMDR1, hMRP2, and hBCRP simultaneously after various culture periods to determine the most appropriate culture period for the evaluation of the transcellular transport activities of these efflux transporters on the absorption process.
of drugs and drug candidates. In the present study, four test compounds were selected: 1) digoxin, a typical hMDR1 substrate (Pedersen et al., 1983); 2) ochratoxin A, a specific substrate for hMRP2 that showed transcellular transport activity across Caco-2 cells (Berger et al., 2003); 3) olmesartan, a highly hydrophilic substrate for hMRP2 (Nakagomi-Hagihara et al., 2006; Kamiyama et al., 2007); and 4) estrone-3-sulfate (ES), a typical substrate of hBCRP (Xia et al., 2005).

In addition, the correlation between transcellular transport activity and the expression level of each transporter was examined. The development of a tight junction was also confirmed by measuring TEER and the permeability of the small hydrophilic markers mannitol and Lucifer yellow (LY). Furthermore, we examined the mRNA and protein expression of zonula occludens (ZO-1) and occludin (a tight junction-associated protein), which are integral structural components of tight junctions.

Materials and Methods

Chemicals. Digoxin was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Ochratoxin A, ES, verapamil, and LY were purchased from Sigma-Aldrich (St. Louis, MO). Olmesartan [4-(1-hydroxy-1-methyl-ethyl)-2-propyl-1-[2-(1H-tetrazol-5-yl)phenyl]-4-ylmethyl]imidazole-5-carboxylic acid] was obtained from Sankyo Organic Chemical Co., Ltd. (Kanagawa, Japan). MK571 was purchased from Cayman Chemical (Ann Arbor, MI). Fumitremorgin C (FTC) was purchased from EMD Chemicals, Inc. (Gibbstown, NJ). \( ^{1}H \)Digoxin and \( ^{3}H \)ES were purchased from PerkinElmer Japan Co., Ltd. (Kanagawa, Japan). \( ^{3}H \)Ochratoxin A was purchased from American Radiolabeled Chemicals (St. Louis, MO). \( ^{3}C \)Mannitol was purchased from GE Healthcare Bio-Sciences Corporation (Tokyo, Japan). \( ^{3}H \)(Olmesartan (methyl-\(^1\)H)Holmesartan) was synthesized at Amersham Biosciences Inc. (Little Chalfont, Buckinghamshire, UK). Fetal bovine serum (lot 1233051) was purchased from Invitrogen (Carlsbad, CA). Anti-hMDR1 antibody (C219) was purchased from Signet Laboratories (Dedham, MA). Anti-hMRP2 antibody (M3,III-6) and anti-hBCRP antibody (BXP-21) were purchased from Kamiya Biochemical (Seattle, WA). All other reagents used were of regent grade.

Caco-2 Cells. Caco-2 cells (passage number 20; lot 2463681) were purchased from American Type Culture Collection (Rockville, MD). The Caco-2 cells (passage number 44–57) were cultured to be grown in Dulbecco’s modified Eagle’s medium (culture medium) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 0.1 mM minimal essential medium nonessential amino acids, 100 units/ml penicillin, and 100 \( \mu \)g/ml streptomycin and were cultured in an atmosphere of 5% CO\(_2\) at 37°C. The Caco-2 cells to determine the expression in mRNA and protein levels were seeded at a density of 10\(^4\) cells/0.5 ml/insert on a Transwell (Corning Japan K.K., Tokyo, Japan) insert and were cultured for 0, 1, 2, 3, 4, 5, and 6 weeks. On the other hand, the Caco-2 cells for the transcellular transport study were seeded with a density of 2.0 \( \times \) 10\(^4\) cells/0.2 ml/insert on an HTS Transwell and were cultured for 1, 2, 3, 4, 5, and 6 weeks.

Transcellular Transport Study. Transport buffer (TB) prepared by adding HEPEIS (Viso volume) to Hanks’ balanced salt solution was adjusted to pH 7.4. The culture medium added to the apical and basal sides of an HTS Transwell, where Caco-2 cells had been seeded, was removed by suction, and washed three times with TB maintained at 37°C. The culture medium was replaced by TB, and the plate was preincubated at 37°C for 20 min. After TEER in all the wells was measured using a Millicell (Nihon Millipore K.K., Tokyo, Japan), the TB of subculture volume on the receiver side, where the test solutions maintained at 37°C and the plate was incubated at 37°C (triplicate). After incubation for 1 h, TB of subduple volume on the receiver side, where TEER was also measured to check the influence of the test compounds on Caco-2 cells when the transcellular transport study was finished.

Real-Time PCR. Caco-2 cells cultured on the Transwell for the prescribed number of days were washed two times with phosphate-buffered saline (PBS) and suspended using an aliquot of RNA lysis buffer, and the suspension was collected. The total RNA was extracted from the collected cells using an SV total RNA isolation system (Promega K.K., Tokyo, Japan), and the cDNA was obtained by reverse transcription-PCR (37°C, 1 h). The cDNA diluted 10-fold was determined using each TaqMan probe by real-time PCR (7900HT Fast Real-Time PCR system; Applied Biosystems, Foster City, CA) in standard mode (cycle 50) and was analyzed by a sequence detection systems (7900HT, version 2.2.1; Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase mRNA was used as the housekeeping gene.

Western-Blotting. After Caco-2 cells cultured on the Transwell for the prescribed number of days were washed two times with PBS, the membranes of the Transwell were cut off. The cut off membranes were collected in aliquots of M-PER mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL). The collected Caco-2 cells were sonicated and centrifuged for 5 min at 1750 g. An aliquot of supernatant was collected to detect the tight junction-associated proteins. The other supernatant was centrifuged (100,000 g, 10 min), and the sediment was resuspended in PBS. The sample was diluted 50-fold with distilled water, and the protein concentration of the sample was determined using a DC protein assay kit (Bio-Rad Laboratories, CA). Ochratoxin A, ES, verapamil, and LY were purchased from Sigma-Aldrich (St. Louis, MO). 

Calculation of \( P_{\text{app}} \) and \( P_{\text{app}, A} \). The \( P_{\text{app}} \) and \( P_{\text{app}, A} \) ratio were calculated using Microsoft Office Excel 2003 (Microsoft, Redmond, WA). The transported amount of each substrate for hMDR1, hMRP2, or hBCRP across the monolayer was calculated by the value of the transported concentrations of the substrates multiplied by the volume, and \( P_{\text{app}} \) was calculated using eq. 1:

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

where \( dQ/dt \), \( A \), and \( C_0 \) represent the amounts of the test substrates transported within a given time period, the surface area of monolayer, and the initial concentrations of the substrates, respectively. The \( P_{\text{app}, A} \) ratio was calculated using eq. 2:

\[
P_{\text{app}, A} = \frac{P_{\text{app}, A}}{P_{\text{app}, B}}
\]

The 95% confidence intervals of the \( P_{\text{app}} \) ratios were estimated based on Fieller’s theorem using SAS software (version 8.2; SAS Institute, Cary, NC).

Results

Transcellular Transport of Digoxin, Ochratoxin A, Olmesartan, and ES. The \( P_{\text{app}, A} \) to B of digoxin, ochratoxin A, and ES, which are substrates for hMDR1, hMRP2, and hBCRP, respectively, at 2 weeks was the lowest among all the weeks and that of olmesartan, a substrate for hMRP2, at 2 weeks, was almost the same as that at 3 weeks (Fig. 1). The \( P_{\text{app}, B} \) to A of digoxin and ES at 1 week was the highest among all the weeks and was decreased until 6 weeks, and those of ochratoxin A and olmesartan at 2 weeks were almost the same as those at 1 week.

The \( P_{\text{app}} \) ratios of ochratoxin A, olmesartan, and ES at 2 weeks were the highest among all the weeks. Conversely, the \( P_{\text{app}} \) ratio of digoxin at 1 week was the highest among all the weeks but was almost the same as that at 2 weeks.

The value of TEER drastically increased until 3 weeks and decreased after that. The permeability of mannitol at 3 to 5 weeks was lower than that at 2 weeks and that of LY was kept constant and at a low level at 2 to 5 weeks.

Inhibitory Effect on Transcellular Transport of Each Substrate for hMDR1, hMRP2, and hBCRP at 2 and 3 Weeks. Verapamil, MK571, and FTC were used as typical inhibitors of hMDR1, hMRP2, and hBCRP, respectively (Allen et al., 2002; Balimane et al., 2004;
Xia et al., 2005). The $P_{\text{app}}$ ratio of each substrate at 2 weeks was shown to be higher than that at 3 weeks without inhibitors (Fig. 2).

The inhibitory effect of each inhibitor on the transcellular transport via hMDR1, hMRP2, and hBCRP at 2 and 3 weeks was similar (Fig. 2).

Expression in the mRNA Level. The expression levels of the various transporters and the tight junction-associated proteins in mRNA derived from Caco-2 cells at each culture period are shown in Fig. 3. The ratios of the expression levels (percentage of 3 weeks) of hMDR1, ZO-1, and occludin in Caco-2 cells showed the highest value at 4 weeks and that of hMRP2 increased at 2 weeks and was then almost constant until 4 weeks and decreased thereafter. The expression of hBCRP in the mRNA tended to increase until 4 weeks and decreased after that, although that at 2 weeks was almost the same as that at 3 weeks.

Expression in the Protein Level. The expression of hMRP2 in the protein level was the highest at 2 weeks (Fig. 4). The temporal expression profiles of hMDR1, hMRP2, and hBCRP in the protein level were similar to those in the mRNA level. The expression of ZO-1 and occludin in the protein levels at 4 weeks was the highest, the same as in the mRNA expression levels.

Relationship between the Transcellular Transport Activities and the Expression Levels of mRNA and Protein. The transcellular transport activity and expression of hMRP2 at 2 weeks were the highest or were similar to those at 3 weeks, and the activity of hMRP2 was roughly correlated with the expression in the protein levels (Fig. 5). The transcellular transport activity of hBCRP was also correlated with the expression in the mRNA and protein levels. The change in the transcellular transport activity of hMDR1 showed an inverse correlation with the expression in the mRNA and protein levels.

Discussion

The influence of the culture period on the transcellular transport activity and expression levels of hMDR1, hMRP2, and hBCRP was investigated. This is first report on the simultaneous determination of the expression and transport activities of three efflux transporters in Caco-2 cells cultured for periods ranging from 1 to 6 weeks. The $P_{\text{app}}$ ratio of each substrate for hMRP2 and hBCRP was the highest at 2 weeks among all the culture periods, whereas for hMDR1 was the highest at 1 week with a similar $P_{\text{app}}$ ratio at 2 weeks. Furthermore, the tight junction at 2 weeks was sufficiently formed to assess the transcellular transport activity, judging from the results of TEER and the permeability of LY. Therefore, our results demonstrated that a
2-week culture was the ideal condition to assess the transcellular transport activities of hMDR1, hMRP2, and hBCRP in Caco-2 cells compared with a conventional 3-week culture condition.

We performed an inhibition study using typical inhibitors for hMDR1, hMRP2, and hBCRP to compare the inhibitory effects between 2 and 3 weeks. In all the transports, the value of the percentage of no inhibitor at 2 weeks was almost the same as that at 3 weeks and the inhibitory effect at 2 weeks was not different from that at 3 weeks.
This result indicated little qualitative change in the transcellular transport activity of the efflux transporters in Caco-2 cells between 2 and 3 weeks, supporting the appropriateness of using Caco-2 cells cultured for 2 weeks.

The value of TEER at 3 weeks was the highest in all the culture periods, although the expression of tight junction-associated protein in the mRNA and the protein levels was the highest at 4 weeks (Fig. 1e). The formation of a tight junction is not always in proportion to the expression level of tight junction-associated proteins, and other tight junction-associated proteins such as claudins might contribute to the formation of a tight junction (Furuse et al., 1998; Morita et al., 1999). TEER values were reported to vary according to different clones or passage numbers of Caco-2 cells with a range from 62 to 1290 Ω × cm², and the TEER value at 30 to 40 passages, which was close to the passage number of Caco-2 cells in our studies, was approximately 350 Ω × cm² (Delie and Rubas, 1997; Tavelin et al., 2002). Therefore, the TEER value of 441 Ω × cm² at 2 weeks in this study was considered to be sufficiently high. The permeability of mannitol (mol. wt. 182) was lower at 3 to 5 weeks than that at 2 weeks, whereas that of LY (mol. wt. 521.58) was kept constant from 2 to 5 weeks (Fig. 1f). Even though the tight junction seemed not to be completely formed at 2 weeks, it might still be practically acceptable to assess the transcellular transport of various candidate drugs or drugs offered commercially because the TEER value was sufficiently high and the permeability of LY, the molecular weight of which is comparable to these drugs, was sufficiently low at 2 weeks. Considering the low TEER and the high permeability of mannitol and LY at 1 week, it is suggested that a culture for 2 weeks is preferable to assess the transcellular transport activity of digoxin.

The transcellular transport activities of hMRP2 and hBCRP were roughly related to their expression levels, although functional change was disproportionately large compared with the change in the expression levels. On the other hand, the vectorial transport activity of hMDR1 showed an inverse relation to the expression level. At the present time, we do not have any convincing explanation to account for these relationships. The involvement of other transporters at the
influx and/or efflux of digoxin, octratoxin A, and ES such as organic solute transporter, which transports digoxin and ES (Seward et al., 2003; Ballatori et al., 2005), is a possible reason. The involvement of transporters other than hBCRP in the transport of ES was suggested by the fact that the value of the P_app ratio in the presence of FTC was less than 1 (Fig. 3c), as was also observed in a previous report (Wang et al., 2008). Another previous report suggested that basolateral transporters and apical influx transporters were involved in digoxin transport in MDCK-hMDR1 cells (Acharya et al., 2008). Taken together, we cannot rule out the possibility that minimal change in the expression levels of influx transporters on the apical side, or influx or efflux transporters on the basal side, would significantly affect the P_app and P_app ratio of these substrates. However, the predominant contribution of hMDR1, hMRP2, or hBCRP to digoxin, octratoxin A, or ES transport was suggested, considering the inhibitory effect of respective typical inhibitors on its transport activity. Therefore, a 2-week culture, under which the transcellular transport activities of these substrates were highest or were higher than those in a 3-week culture was suitable to assess the transcellular transport activities of these efflux transporters in Caco-2 cells.

The expression levels of these efflux transporters in human intestine were different in various reports (Englund et al., 2006; Seithel et al., 2006; Hilgendorf et al., 2007), indicating difficulty in performing a correlation analysis of the expression level in Caco-2 cells and human intestine. These differences could be caused by the sampling condition of the biopsy sample, ethnic differences in the patients or various conditions of the patients. However, the kinds of transporters expressed in Caco-2 cells cultured for 2 or 3 weeks were still similar to those in human intestine (Seithel et al., 2006). Therefore, we considered that Caco-2 cells cultured for 2 weeks are not different from those cultured for 3 weeks from the viewpoint of the similarity of the expression profiles of the transporters.

Culture period-dependant change of expression and/or transport activity of hMRP2 and hMDR1 was examined in Caco-2 cells in a plastic flask or dish (Hirohashi et al., 2000; Goto et al., 2003; Nakamura et al., 2003). This culture condition was completely different from ours using a Transwell, the condition under which conventional experiments for the evaluation of transcellular transport are conducted. Thus, it was impossible to simply compare the results. The age-dependent protein expression and function of hMDR1 and hBCRP were determined under experimental conditions similar to ours (Hosoya et al., 1996; Xia et al., 2005). However, in each case the authors focused only on hMDR1 or hBCRP and assessed a shorter culture period compared with that in our study. In addition, they did not determine the expression of each transporter in terms of the mRNA level (Hosoya et al., 1996; Xia et al., 2005). Therefore, we considered that the function and expression of the three efflux transporters in our study were assessed simultaneously and systematically, focusing on the culture period, compared with the studies mentioned above.

The transcellular transport activity of each substrate for these three efflux transporters in Caco-2 cells cultured for 1 week was also observed. It is possible to assess the transport activity via these efflux transporters using Caco-2 cells cultured for 1 week as a first tier screening assay. However, it is difficult to correctly assess the transcellular transport activity of high hydrophilic compounds in Caco-2 cells cultured for 1 week, because the transcellular transport activity of olmesartan, a good substrate for hMRP2 (Nakagomi-Hagihara et al., 2006) and also a hydrophilic compound (Kamiyama et al., 2007), was observed only in the 2-week culture. Therefore, we suggest that it is better to perform a transport study as a first tier screening assay using Caco-2 cells cultured for 2 weeks rather than 1 week.

In conclusion, the transcellular transport activities of hMDR1, hMRP2, and hBCRP expressed in Caco-2 cells cultured for 2 weeks are sufficiently high compared with those of other culture periods, including the conventional culture period of 3 weeks, although each transporter had individual expression patterns in the mRNA and protein levels. Consequently, it was demonstrated that 2 weeks is the best culture period to perform transport studies to identify whether a compound is a substrate for hMDR1, hMRP2, and hBCRP.

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