ABSTRACT:

The function of breast cancer resistance protein (BCRP) and its role in drug absorption, distribution, and elimination has recently been evaluated. The objective of the present study was to examine the expression, localization, and functional characteristics of BCRP in Caco-2 cells, a widely used human intestinal epithelial cell model for investigating intestinal drug absorption. The expression of BCRP in Caco-2 cells was measured by Western blotting using the antibody BXP-21. Localization of BCRP was determined by an immunofluorescence technique using both antibodies BXP-21 and BXP-34. The drug efflux function of BCRP was evaluated via the epithelial transport of methotrexate (MTX) and estrone-3-sulfate (E3S) across Caco-2 cell monolayers in the presence or absence of the BCRP inhibitors Ko143 or GF120918. (N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide). Results from Western blot assay indicated that Caco-2 cells in the late passage (p56) expressed a higher level of BCRP as compared with the level in the early passages (p33). The total amount of BCRP protein did not change after the cells were confluent. Immunofluorescence studies revealed the positive staining of BCRP on the apical membrane of Caco-2 cells but not on the basolateral membrane after cell confluence. MTX and E3S showed a preferential basolateral-to-apical (B-to-A) transport across Caco-2 cell monolayers. Both BCRP inhibitors Ko143 and GF120918 increased the apical-to-basolateral (A-to-B) transport but decreased the B-to-A transport of MTX and E3S. Caco-2 cells may therefore be used as an in vitro model to study the transport characteristics of BCRP.

Expression, Localization, and Functional Characteristics of Breast Cancer Resistance Protein in Caco-2 Cells

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Breast cancer resistance protein (BCRP) was originally cloned and sequenced from genomic DNA, from highly mitoxantrone-resistant S1-M1-80 human colon carcinoma cells, and from MCF7 AdVp human breast cancer cells selected in doxorubicin (Doyle et al., 1998; Miyake et al., 1999). BCRP is a member of the ATP-binding cassette transporter G family and is also known as ABCG2 or ABCP or MXR (Ejendal and Hrycyna, 2002; Doyle and Ross, 2003). It is a 655-amino acid polypeptide (72 kDa), containing six putative transmembrane domains and four potential N-glycosylation sites. BCRP is similar to half the duplicated P-glycoprotein (P-gp) or multidrug resistance protein 1 (MRP1) molecule and functions as a homodimer bridged by disulfide bonds (Doyle et al., 1998; Kage et al., 2002).

BCRP is endogenously expressed at a high level in human placenta and to a lesser extent in liver, small intestine and colon, ovary, veins, capillaries, kidney, adrenal, and lung, with little to no expression in brain, heart, stomach, prostate, spleen, and cervix (Doyle et al., 1998; Litman et al., 2001; Maliepaard et al., 2001; Scheffer and Scheper, 2002). Importantly, BCRP is expressed in the human jejunum at levels considerably higher than those of many other ABC transporters (Taipalensuu et al., 2001). BCRP has been demonstrated to exist on the apical membrane of intestinal epithelium and has limited the oral absorption of topotecan in mice and humans (Jonker et al., 2000; Kruijtzer et al., 2002a). Given the liver and intestinal localization pattern, BCRP, similar to P-gp, may act as a barrier to uptake and absorption and limit the oral bioavailability of drugs as well as mediating hepatobiliary excretion of drugs (Jonker et al., 2000; Jorritsma et al., 2002; Kruijtzer et al., 2002b).

Caco-2 cells are derived from human colon adenocarcinoma cell line, and exhibit morphological and functional similarities to intestinal enterocytes. It has been widely used as a model of human intestinal epithelium for studies of intestinal drug absorption and metabolism. Many active transport systems such as P-gp (encoded by MDR-1 gene, also named ABCB1) and multidrug resistance-associated protein 2 (MRP2, or ABCC2) have been characterized in Caco-2 cells (Makhey et al., 1998; Doppenschmitt et al., 1999; Gutmann et al., 1999). BCRP mRNA has been detected in Caco-2 cells, although its level is 100-fold lower than that in human jejunum (Taipalensuu et al., 2001).

The present study was aimed at characterization of the protein expression, localization, and efflux function of BCRP in Caco-2 cells. We have used BXP-21 monoclonal antibody (mAb) to determine the

ABBRVIATIONS: BCRP, breast cancer resistance protein; ABC, ATP-binding cassette transporter; A-to-B, apical-to-basolateral; B-to-A, basolateral-to-apical; E3S, estrone-3-sulfate; FBS, fetal bovine serum; MDR-1, human multidrug resistance gene 1; mAb, monoclonal antibody; MRP, multidrug resistance-associated protein; MTX, methotrexate; P_app, apparent permeability coefficient; P-gp, P-glycoprotein; TEER, trans-epithelial electrical resistance; GF120918, N-[4-[2-(1,2,3,4-tetrahydro-6,7-dimethoxy-2-isoquinolinyl)ethyl]-phenyl]-9,10-dihydro-5-methoxy-9-oxo-4-acridine carboxamide; LY335979, zosuquidar trihydrochloride; MK571, 3-[[3-[2-(7-chloroquinolin-2-y1)vinyl]phenyl]-2-dimethylcarbamoyl-ethylsulfanyl][methylsulfanyl] propionic acid; 2,4-DNP, 2,4-dinitrophenol; FITC, fluorescein isothiocyanate.
BCRP protein expression, and BXP-21 and BXP-34 mAbs to characterize the subcellular distribution of BCRP in Caco-2 cells. In addition, the efflux function of BCRP in Caco-2 cells was determined using estrone-3-sulfate (E3S) and methotrexate (MTX) as substrates. Knowledge of the properties of BCRP in Caco-2 cells is valuable to investigate the absorption mechanism of drug molecules using this in vitro model system.

Materials and Methods

Materials. [3H]Estrone-3-Sulfate (specific activity, 45 Ci/mmol) and [3H]methotrexate (specific activity, 33.5 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). GF120918 and LY335979 were synthesized at Millennium Pharmaceuticals, Inc. Ko143 was obtained from the Netherlands Cancer Institute (Amsterdam, Netherlands). MK571 was purchased from Alexis Biochemicals (San Diego, CA). Unlabeled E3S, MTX, prazocin, and 2,4-dinitrophenol (2,4-DNP) were purchased from Sigma-Aldrich (St. Louis, MO). Cell culture media and supplies were obtained from Invitrogen (Carlsbad, CA). BXP-21 and BXP-34 murine monoclonal antibodies were purchased from Signet Laboratories ( Dedham, MA). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was obtained from Abcam Inc. (Cambridge, MA). Horseradish peroxidase-conjugated goat antimouse IgG2a, mouse IgG2a, and mouse polyclonal antibody 119 for β-actin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). All supplies for Western blot studies were obtained from Invitrogen.

Caco-2 Cell Culture. The Caco-2 cells (passage 18) were obtained from American Type Culture Collection (Manassas, VA). The stock cells were cultured in 150-cm² tissue culture T-flasks for subsequent plating onto 24 Transwell plates (0.33 cm²/well, 0.4-μm pore size; Costar, Cambridge, MA). Briefly, 1 x 10⁶ cells were suspended in 0.2 ml of culture medium [Dulbecco’s modified Eagle’s medium with 0.1 mM nonessential amino acids, 2 mM L-glutamine, 4.5 g/l glucose, and 10% fetal bovine serum (FBS)] and added to the upper chamber of each filter membrane of a Transwell plate. One milliliter of cell-free culture medium was added to the lower chamber. The Transwell plates were then incubated at 37°C in an atmosphere of 5% CO₂ in air and 90% humidity. The culture media were changed every other day. Confluent cell monolayers were obtained within 5 to 7 days after plating. The transepithelial electrical resistance (TEER), as measured by an epithelial volt-ohm meter (World Precision Instruments, Inc., Sarasota, FL), gradually increased and reached a plateau after day 5, indicating the formation of tight junctions. Monolayers with TEER values greater than 250 ohm-cm² were used. Unless otherwise specified, cell passages between 20 and 40, other than specification otherwise specified, cell passages between 20 and 40, other than specification

Data Analysis. The cumulative amount of drug (Q) on the receiver side was plotted as a function of time. The steady-state flux J was then estimated from the slope (dQ/dt). The apparent permeability coefficient (Papp, A→B) for unidirectional flux for the test compound was estimated by normalizing the flux J (mol/s) against the nominal surface area A (0.33 cm²) and the initial drug concentration in the donor compartment C₀ (mol/ml), or Papp = J(A×C₀). The B/A ratio was equal to the Papp value for A-to-B transport (Papp A→B) divided by the Papp value for B-to-A transport (Papp B→A). The kinetic parameters for the E3S transport were estimated by fitting the flux against the donor E3S concentration using a nonlinear regression with a method of least squares fitting (GraphPad Software Inc., San Diego, CA).

Data are expressed as mean ± S.E.M. of three individual monolayers. Tests of significance of differences between mean values were made using a two-tailed unpaired Student’s t test. A probability of less than 0.05 (p < 0.05) was considered to be statistically significant.

Results

Expression and Localization of BCRP. BCRP protein expression was determined in Caco-2 cell lysate samples via Western blot analysis using BXP-21 as the primary antibody. As shown in Fig. 1A, both the half-transporter BCRP (~70 kDa) and its dimer (~140 kDa) were observed in lanes 1 and 4, in which the Caco-2 cell lysates were not treated with reducing agent (~7–13% of dithiothreitol). Lanes 2, 3, 5, and 6 are the lysates treated with reducing agent, which showed a strong band of BCRP monomer (~70 kDa). Lane 6 depicts a trace amount of BCRP dimer when a high concentrated unheated sample was loaded.

When Caco-2 cells were cultured in cell culture T-flasks, the expression of BCRP monomer and dimer in Caco-2 cells was more than 3-fold higher in the late passage [passages 33 (p33) or 36 (p36)] than in the early passage [passages 3 (p3) or 6 (p6)] after treatment with 19th days after the cell seeding on Transwell plates (Fig. 1B). The expression of BCRP monomer was about 3-fold higher and the dimer expression was more than 3-fold higher in the late passage [passages 56 (p56) or 59 (p59)].

When Caco-2 cells were cultured in cell culture T-flasks, the expression of BCRP monomer and dimer in Caco-2 cells was more than 3-fold higher in the late passage [passages 33 (p33) or 36 (p36)] than in the early passage [passages 3 (p3) or 6 (p6)] after treatment with 19th days after the cell seeding on Transwell plates (Fig. 1B). The expression of BCRP monomer was about 3-fold higher and the dimer expression was more than 3-fold higher in the late passage [passages 56 (p56) or 59 (p59)].

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membranes but also in the cytoplasm. On the 5-day-old culture, cells reached confluence and showed increased TEER values (indicative of the tight junction formation); BCRP staining was observed only on the apical membrane, and there was no detectable staining in the cytoplasm, indicating the localization of BCRP on the apical membrane.

The same phenomena were also noted during the cell differentiation period (i.e., the 12th and 19th days after cell seeding). When the late passage cells (p59) were grown on the Transwell plates for 12 days, multiple layers were observed as demonstrated by the multiple nuclei staining on the y-x and y-z views (Fig. 2B). BCRP staining, however, was still preferentially shown on the apical side of cell membranes (Fig. 2B). The absence of any green staining in the cell monolayers with mouse IgG2a and FITC-conjugated second antibody served as a negative control.

**Efflux Function of BCRP.** The function of BCRP as an efflux transporter in Caco-2 cell monolayers was evaluated using a known substrate, E3S (Suzuki et al., 2002, 2003). As shown in Table 1, the $P_{app}$ value of [3H]E3S (0.02 μM) from B-to-A was $46.1 \times 10^{-6}$ cm/s and was 8-fold higher than the $P_{app}$ value (5.7 $\times 10^{-6}$ cm/s) for A-to-B in the 5-day culture (p33). In 12- and 19-day cultures (p33), the $P_{app}$ values for A-to-B decreased to 4.1 and 4.5 $\times 10^{-6}$ cm/s, and the $P_{app}$ value for B-to-A increased to 63.0 and 58.0 $\times 10^{-6}$ cm/s, respectively. In the presence of Ko143 (a BCRP inhibitor), the A-to-B and B-to-A transports of E3S were almost equal, with $P_{app}$ values around 9 to 11 $\times 10^{-6}$ cm/s. Figure 3 showed the comparison of BCRP-mediated [3H]E3S (0.02 μM) efflux in both early passage (p36) and late passage (p59) Caco-2 cell monolayers. In p36, the $P_{app}$ value for A-to-B transport of E3S was $2.3 \times 10^{-6}$ cm/s and was 32-fold less than the $P_{app}$ value for B-to-A transport. The $P_{app}$ value increased to $6.1 \times 10^{-6}$ cm/s in the presence of Ko143. However, $P_{app}$ values of E3S for A-to-B and B-to-A transport in p59 were 16.2 and 37.1 $\times 10^{-6}$ cm/s, respectively. The difference in the directional E3S transport was abolished by the BCRP inhibitor Ko143. The TEER value was 4-fold higher in p36 than in p59 in the present cell culture system (Fig. 3).

When [3H]E3S (0.03 μM) was coincubated with Ko143 (1 μM), GF120918 (2 μM) (an inhibitor for BCRP and P-gp), or prazocin (100 μM) (an inhibitor for BCRP and P-gp) in Caco-2 cell monolayers, the $P_{app}$ value of E3S for the A-to-B transport decreased and the $P_{app}$ value for B-to-A transport increased, resulting in the B/A ratio dropping from 27 to almost unity (Fig. 4). In contrast, the P-gp-specific inhibitor LY335979 (5 μM) did not significantly change the directional transport pattern of E3S ($p > 0.05$) (Fig. 4). The apical or basolateral presence of 2,4-DNP at 200 μM (known to inhibit mitochondrial ATP synthesis and deplete cellular ATP; Siekevitz and Potter, 1953) totally inhibited the efflux of E3S with an increase in the A-to-B transport and a decrease in the B-to-A transport (Fig. 4).

Figure 5 showed the unidirectional B-to-A flux ($J_{BA}$) of E3S across cultured Caco-2 cells at various concentrations in the absence or presence of a BCRP inhibitor, Ko143 (1 μM). The net efflux of E3S across Caco-2 cell monolayers in the B-to-A direction, the difference of B-to-A flux in the presence or absence of BCRP inhibitor ($J_{net} = J_{B-to-A} - J_{B\text{-to}\text{-}B}$), was saturable and followed Michaelis-Menten kinetics. The estimated apparent $K_m$ and $V_{max}$ were 13.1 μM and 10.8 pmol/s, respectively.

MTX was chosen to further investigate the BCRP efflux function in the Caco-2 cell monolayers. As shown in Fig. 6, the B-to-A transport of MTX was significantly decreased in the presence of MK571 (a MRP inhibitor; 50 μM), Ko143 (1 μM), and GF120918 (2 μM) ($p < 0.05$). However, neither the $P_{app}$ value for A-to-B nor the $P_{app}$ value for B-to-A of MTX was significantly affected after coincubation with LY335979 (5 μM).
The B-to-A efflux of rhodamine 123, which is a substrate for P-gp and mutant BCRP, was significantly decreased in the presence of LY335979 and GF120918 ($p < 0.05$), but was not changed by coincubating with Ko143 ($1 \mu M$) (Fig. 7).

**Discussion**

Western blot analysis of Caco-2 cell lysates demonstrates the presence of both monomer (~70 kDa) and homodimer (~140 kDa) of BCRP in the Caco-2 cells (Fig. 1A). The 140-kDa BCRP complex dissociated to 70-kDa polypeptides in the presence of the reducing agent dithiothreitol, indicating that the BCRP dimer was linked by disulfide bonds. This finding is consistent with the recent discovery by Kage et al. (2002). The same group also demonstrates the necessity of homodimerization for the BCRP function when using a dominant-negative mutation of BCRP with a L554P alteration in the fifth transmembrane domain (Kage et al., 2002).

Immunohistochemical analysis of Caco-2 cells reveals the presence of BCRP on apical and basolateral plasma membranes as well as in the cytoplasm prior to cell confluence (Fig. 2). Upon cell confluence, BCRP was sorted to the “apical membrane” consistent with potential intracellular localization and redistribution of BCRP to the plasma membrane shown in BCRP-overexpressed MCF-7 AdVp3000 and S1-M1-80 drug-resistant cells (Litman et al., 2000). BCRP-transfected MDCK or LLC-PK epithelial cells also exhibited apical localization of BCRP (Jonker et al., 2000; Maliepaard et al., 2001), implying that BCRP may contain a certain endogenous sorting signal, which was recognized by epithelial cells (Jonker et al., 2000; Imai et al., 2003). The polarized apical BCRP distribution in Caco-2 cells continues during the cell differentiation period, which is consistent with the proposed role for BCRP as a secretory detoxifying transporter, contributing to the gastrointestinal epithelial barrier. Results from transport function assays using E3S as the BCRP substrate agreed with immunohistochemical study results. The directional transport of E3S across Caco-2 monolayers was observed immediately after cells reached confluence (5-day culture) and continued throughout the differentiation phase (day 12–19 cell culture) (Table 1). In view of the fact that total BCRP protein expression had not changed over day 5, day 12, and day 19 (Fig. 1B), the higher A-to-B transport and lower B-to-A transport of E3S across Caco-2 cells on day 5, compared with those on day 12 and day 19, indicated that BCRP continuously redistributed to the apical membrane during the differentiation phase.

Similar to P-gp, MRP, and lung-resistant protein (Yu and Sinko, 1997), the expression of BCRP is passage-dependent. The expression
of BCRP increased approximately 3-fold from the early passage to the late passage when Caco-2 cells were cultured in T-flasks, but decreased about 3- to 10-fold for monomer and dimer, respectively, from the early passage to the late passage when Caco-2 cells were grown on Transwell plates (Fig. 1, B and C). Although our findings are novel, they are supported by the previous reports that culture conditions and cell-growing matrices may affect efflux pump protein expression (Yu and Sinko, 1997). The polarized expression of BCRP in Caco-2 cells is consistent with the net secretory transport (B/A ratio >8) of E3S, a prototypical substrate of BCRP. Although there is a good agreement between the morphological and the functional measurements, the vectorial transport of E3S attributed to the BCRP expression in Caco-2 cells was further supported by the reduction of E3S efflux by the BCRP inhibitors Ko143, GF120918, and prazocin (Fig. 4). Ko143, a specific inhibitor of BCRP (Allen et al., 2002b), totally inhibited the E3S efflux and resulted in almost equal $P_{\text{app}}$ values for A-to-B and B-to-A of E3S across Caco-2 cell monolayers at the concentration of 1 $\mu$M. Under the same experimental conditions, Ko143 did not change the efflux properties of paclitaxel (a P-gp substrate) and vinblastine (a substrate for P-gp and MRP) in Caco-2 cells (data not shown), indicating that Ko143, at the concentration of 1 $\mu$M, did not inhibit P-gp and MRP. P-gp and BCRP inhibitors GF120918 and prazocin (Jonker et al., 2000; Cisternino et al., 2004) completely abolished the efflux of E3S. In contrast, the potent P-gp-specific inhibitor, LY335979 (Shepard et al., 2003), did not change A-to-B or B-to-A transport of E3S. Altogether, the modulation of GF120918 and prazocin on the E3S efflux across Caco-2 cells was due to the inhibition of BCRP. The uncoupling agent, 2,4-DNP, increased the A-to-B over B-to-A ratio of E3S from 26.7 to 0.6, suggesting that blocking the mitochondrial ATP synthesis and depleting the cellular energy abolished the BCRP efflux activity. (Data expressed as mean ± S.E.M., $n = 3$.)

**FIG. 4.** The effects of efflux pump inhibitors on the transport of E3S. LY335979 (5 $\mu$M), a P-gp-specific inhibitor, did not affect the E3S permeability. However, GF120918 (2 $\mu$M) (an inhibitor for P-gp and BCRP), prazocin (100 $\mu$M) (an inhibitor for P-gp and BCRP), and Ko143 (1 $\mu$M) (a BCRP inhibitor) increased A-to-B transport and decreased B-to-A transport of E3S, indicating that BCRP and P-gp pumps E3S out of Caco-2 cells. The uncoupling agent, 2,4-DNP, changed the ratio of B-to-A over A-to-B of E3S from 26.7 to 0.6, suggesting that blocking the mitochondrial ATP synthesis and depleting the cellular energy abolished the BCRP efflux activity. (Data expressed as mean ± S.E.M., $n = 3$.)

**TABLE 1**

<table>
<thead>
<tr>
<th>Cell passage No.</th>
<th>P36</th>
<th>P59</th>
</tr>
</thead>
<tbody>
<tr>
<td>TEER (ohm cm$^{-2}$) (n=6)</td>
<td>461±97</td>
<td>79±12</td>
</tr>
<tr>
<td>$P_{\text{app}} \times 10^6$ (cm/s)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A-to-B</td>
<td>B-to-A</td>
<td>Ratio (B/A)</td>
</tr>
<tr>
<td>5 days after cell seeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[^{3}H]$E3S</td>
<td>5.7±0.3</td>
<td>46.1±1.0</td>
</tr>
<tr>
<td>$[^{3}H]$E3S + Ko143 (1 $\mu$M)</td>
<td>9.2±1.4</td>
<td>8.0±0.3</td>
</tr>
<tr>
<td>12 days after cell seeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[^{3}H]$E3S</td>
<td>4.1±0.3</td>
<td>63.0±1.4</td>
</tr>
<tr>
<td>$[^{3}H]$E3S + Ko143 (1 $\mu$M)</td>
<td>11.2±0.7</td>
<td>11.0±0.9</td>
</tr>
<tr>
<td>19 days after cell seeding</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$[^{3}H]$E3S</td>
<td>4.5±0.1</td>
<td>58.0±0.2</td>
</tr>
<tr>
<td>$[^{3}H]$E3S + Ko143 (1 $\mu$M)</td>
<td>8.7±0.9</td>
<td>6.0±0.4</td>
</tr>
</tbody>
</table>

*Data are expressed as mean ± S.E.M. ($n = 3$).*
Ko143 (1 μM) (a BCRP inhibitor) did not inhibit MTX- or E3S-gated using MTX, a substrate for MRP and BCRP (Volk and Schneider, 2003) (Fig. 6). Ko143 (a BCRP inhibitor) and GF120918 (1 μM) (an inhibitor for P-gp and BCRP) but not Ko143 (1 μM) (a BCRP inhibitor) decreased the B-to-A transport of rhodamine 123, indicating that BCRP and MRP mediated the rhodamine efflux across Caco-2 cells. (Data expressed as mean ± S.E.M., n = 3.)

In conclusion, both BCRP monomer and dimer are expressed in the Caco-2 cells. BCRP is polarized at the apical side of Caco-2 cells and can efficiently transport its substrate, such as E3S and MTX, out of cells. Therefore, besides BCRP-transfected cell lines, Caco-2 cells can also be used as an in vitro model to study the transport function of BCRP.

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References


CHARACTERIZATION OF BCRP IN CACO-2 CELLS


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