Transcellular Transport of Organic Anions Across a Double-transfected Madin-Darby Canine Kidney II Cell Monolayer Expressing Both Human Organic Anion-transporting Polypeptide (OATP2/SLC21A6) and Multidrug Resistance-associated Protein 2 (MRP2/ABCC2)*

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Human organic anion transporting polypeptide 2 (OATP2/SLC21A6) and multidrug resistance-associated protein 2 (MRP2/ABCC2) play important roles in the vectorial transport of organic anions across hepatocytes. In the present study, we have established a double-transfected Madin-Darby canine kidney (MDCK II) cell monolayer, which expresses both OATP2 and MRP2 on basolateral and apical membranes, respectively. The basolateral-apical transport of 17β estradiol, 17β-estradiol-17β-D-glucuronide (E2/17βG), pravastatin, and leukotriene C4 (LTC4), which are substrates of OATP2 and MRP2, was significantly higher than that in the opposite direction in the double-transfected cells. Such vectorial transport was also observed for taurilithocholate sulfate, which is transported by rat oatp1 and Mrp2. The \( K_m \) values of E2/17βG and pravastatin for the basolateral-apical flux were 27.9 and 24.3 \( \mu \)M, respectively, which were comparable with those reported for OATP2. Moreover, the MRP2-mediated export of E2/17βG across the apical membrane was not saturated. In contrast, basolateral-apical transport of estrone-3-sulfate and dehydroepiandrosterone sulfate, which are significantly transported by OATP2, but not by MRP2, was not stimulated by MRP2 expression. The double-transfected MDCK II monolayer expressing both OATP2 and MRP2 may be used to analyze the hepatic vectorial transport of organic anions and to screen the transport profiles of new drug candidates.

One of the major functions of the liver is the removal of various endogenous and exogenous compounds from the blood circulation. This clearance process involves uptake across the basolateral membrane and excretion across the bile canalicular membrane (1–3). As far as the sinusoidal uptake of organic anions is concerned, a family of organic anion transporting polypeptides (OATPs) has been identified in rats and humans (4–11). OATPs mediate the transport of a wide variety of substrates in a sodium-independent manner (5–12). Among them, OATP2 (symbol SLC21A6), with its extensive expression in the liver, plays a key role in humans (5). Indeed, by using cRNA-injected Xenopus laevis oocytes and cDNA-transfected mammalian cells, it has been shown that OATP2 mediates the transport of bile acids, 17β estradiol-17β-D-glucuronide (E2/17βG), estrone-3-sulfate (E1S), dehydroepiandrosterone sulfate (DHEAS), glucuronide conjugates of bilirubin, and pravastatin, an hydroxymethylglutaryl-CoA reductase inhibitor (5–11).

These anionic compounds taken up into hepatocytes are then excreted into the bile via apically located ATP-dependent transporters, such as bile salt export pump (symbol ABCB11) (13) and multidrug resistance-associated protein 2 (MRP2, ABCB2) (4, 14, 15). Bile salt export pump is responsible for the biliary excretion of monovalent bile acids (13), whereas MRP2 mediates the excretion of a variety of anionic compounds, including conjugated metabolites (4, 14, 15). The substrate specificity of MRP2 has been determined by comparing the transport across the bile canalicular membrane between normal and MRP2-deficient mutant rats and by examining the ATP-dependent uptake into membrane vesicles expressing MRP2 (4, 14). Because of the similar substrate specificity between OATP2 and MRP2, it is considered that these transporters participate jointly in the transport of their substrates from blood into bile. However, no in vitro model to reflect the vectorial transport of organic anions has been reported. Evers et al. (16) and we ourselves (17) have established MDCK cell lines that stably express human and rat MRP2, respectively, and demonstrated the preferential apical excretion of intracellularly formed glutathione conjugates. These results are consistent with the apical localization of MRP2 in transfected MDCK cells (16). Although Evers et al. (16) have also shown that some hydrophobic cations (such as vinblastine), which may be cotransported with reduced glutathione by MRP2, are transported from the basal to apical compartments in an MRP2-expressing MDCK monolayer, the vectorial transport of organic polypeptide; MDCK, Madin-Darby Canine Kidney cells; MRP, multidrug resistance-associated protein; DMEM, Dulbecco's modified Eagle's medium; BSA, bovine serum albumin; PBS, phosphate-buffered saline; E2/17βG, 17β estradiol-17β-D-glucuronide; LTC4, leukotriene C4; E1S, estrone-3-sulfate; TCS, taurilithocholate sulfate; DHEAS, dehydroepiandrosterone sulfate.
Transport Across MDCK II Cells Co-expressing OATP2 and MRP2

Anions has not been demonstrated due to their poor penetration into cell monolayers across the basolateral membrane. We focused on the fact that OATP2 is expressed on the basolateral membrane of transfected MDCK cells (9) and, in the present article, we report the establishment of a double-transfected MDCK monolayer, which stably expresses OATP2 and MRP2 on basolateral and apical membranes, respectively, as an in vitro model to determine the transcellular transport of a series of organic anions. As model compounds, we used E₂₁⁷βG, pravastatin, and leukotriene C₄ (LTC₄), which are transported by OATP2 and MRP2 (5, 6), along with E₅ and DHEAS, which are transported by OATP2 but not by MRP2. We also examined the transcellular transport of taurocholate-sulfate (TLC-S), which is transported by rat oatp1 and Mrp2 (18).

Experimental Procedures

Materials—[³H]E₂₁⁷βG (1.6 TBq/mmol), [³H]E₅ (2.2 TBq/mmol), [³H]LTC₄ (4.81 TBq/mmol), and [³H]DHEAS (1.6 TBq/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). [³H]TLC-S was synthesized from taurocholate-3-sulfate (Sigma Chemical Co.) and [³H]DHEAS (PerkinElmer Life Sciences) as described previously (19). [³H]Pravastatin (1.6 TBq/mmol) was kindly donated by Sankyo Co. (Tokyo, Japan). pcDNA3.1/Zeo and Zeocin were purchase from Invitrogen (Carlsbad, CA). All other chemicals were commercially available and of reagent grade.

Construction of Plasmid Vector—Previously cloned human OATP2 cDNA (5), located between NcoI and SfiI sites of pBluescript SK II (−) vector, was subcloned into pcDNA3.1/Zeo vector according to the method of Lowry et al. (20). The previously described polyclonal antibody against OATP2 (diluted 50-fold in PBS) or the monoclonal antibody against MRP2 (Alexis Biochemicals, M211-2; diluted 40-fold in PBS) for 30 min at room temperature. Cells were then washed three times with PBS and incubated with Goat anti-rabbit IgG (Alexa 488) or Goat anti-mouse IgG (Alexa 546) (Molecular Probes, Eugene, OR, diluted 250-fold in PBS) for 30 min at room temperature. Nuclei were stained with SYTO61 dye (Molecular Probes, diluted 1000-fold in PBS). Membranes were cut from the inserts and mounted onto slides with 50% glycerol in PBS. Confocal laser-scanning immunofluorescence microscopy was performed using an LSM-410 apparatus from Carl Zeiss.

Immunofluorescence Microscopy of Transfected Cells—Transfected MDCK cells were cultured on Transwell membrane inserts (pore size of 3 μm; Falcon, Bedford, MA). Sodium butyrate was added to the culture medium 24 h before the experiment. After fixation with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 10 min and permeabilization with 0.1% Triton X-100 in PBS for 10 min, cells were incubated with the previously described polyclonal antibody against OATP2 (diluted 50-fold in PBS) or the monoclonal antibody against MRP2 (Alexis Biochemicals, M211-2; diluted 40-fold in PBS) for 30 min at room temperature. For the detection of MRP2, the membrane was allowed to bind to donkey anti-rabbit IgG conjugated with the horseradish peroxidase (Amersham Biosciences, Inc., Buckinghamshire, UK). The enzyme activity was assessed by using ECL Plus Western blotting Starter Kit (Amersham Biosciences, Inc.) with luminescent image analyzer (LAS-1000 plus, Fujifilm).

Transcellular Transport Study—Transfected MDCK II cells were seeded in 24-well plates at a density of 1.4 × 10⁶ cells per well and cultured with 10 μM sodium butyrate for 24 h (8). For uptake studies, cells were washed three times and preincubated with Krebs-Henseleit buffer or Na⁺-free buffer, prepared by substituting Na⁺ by choline, at 37°C for 5 min. Krebs-Henseleit buffer consisted of 142 mM NaCl, 23.8 mM Na₂CO₃, 4.83 mM KCl, 0.96 mM KH₂PO₄, 1.20 mM MgSO₄, 12.5 mM HEPES, 5 mM glucose, and 1.53 mM CaCl₂ adjusted to pH 7.3. The experiments were initiated by replacing the medium at the either apical or basolateral side of the cell layer with complete medium containing [³H]E₂₁⁷βG (1 μM), [³H]pravastatin (1 μM), [³H]LTC₄ (5 μM), [³H]TLC-S (1 μM), [³H]E₅ (1 μM), or [³H]DHEAS (1 μM). The cells were incubated at 37°C, and aliquots of medium were taken from each compartment at several time points. Radioactivity in 100 μL of medium was measured in a liquid scintillation counter (7000 CSC, Inc., Fullerton, CA) after addition of 8 mL of scintillation fluid (Hionic flow, Packard Instrument Co., Downers Grove, IL). At the end of the experiments, the cells were washed three times with 1.5 mL of ice-cold Krebs-Henseleit buffer and solubilized in 450 μL of 1 N NaOH. After addition of 500 μL of distilled water, 800-μL aliquots were transferred to scintillation vials. 50-μL aliquots of cell lysate were used to determine protein concentrations by the method of Lowry et al. (21) with BSA as a standard.

Data Analysis—For the kinetic analysis, the transcellular transport of ligands determined over 2 h was used. The kinetic parameters for transcellular transport of [³H]E₂₁⁷βG and [³H]pravastatin were estimated from the following Michaelis-Menten equation:

\[ v_p = \frac{V_{max} \times S}{K_m + S} + P_{non} \times S, \]

where \( v_p \) is the initial uptake rate of substrates (picomoles/min/mg of protein), \( S \) is the substrate concentration in medium (μM), \( K_m \) is the Michaelis constant (μM), \( V_{max} \) is the maximum uptake rate (pmol/min/mg of protein), and \( P_{non} \) is the non-transcellular accumulation (picomoles/min/mg of protein). The uptake data were fitted to this equation by a nonlinear least-squares method with a MULTFIT program (22) to obtain estimates of the kinetic parameters. The input data were weighted as the reciprocals of the squares of the observed values.

The permeability-surface area product across the apical membrane \( P_{as} \) was calculated by dividing the rate for the transcellular transport of [³H]E₂₁⁷βG determined over 2 h by the cellular concentration of [³H]E₂₁⁷βG determined at the end of the experiments (2 h).

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RESULTS

Expression of OATP2 and MRP2 in MDCK II Cells—The expression of OATP2 and MRP2 in MDCK II cells was determined by confocal immunofluorescence laser scanning microscopy. As shown in Fig. 1, OATP2 and MRP2 were localized on the lateral and apical membranes of transfected MDCK II cells, respectively, and their pattern of expression was not superimposable in the double transfecant. The expression level of these transporters was also confirmed by Western blot analysis (Fig. 2).

Transcellular Transport of E217G Across MDCK II Monolayers—Transcellular transport of E217G across MDCK II monolayers expressing OATP2 and MRP2, along with that across the control monolayer, was compared with that across the control monolayer. As shown in Fig. 3, a symmetrical flux of E217G was observed across the control and MRP2-expressing MDCK II monolayer. The basal-to-apical flux of E217G across the OATP2-expressing monolayer was approximately two times higher than that in the opposite direction (Fig. 3), whereas the basal-to-apical flux of E217G was ~9 times higher than that in the opposite direction in the double-transfected cells (Fig. 3). The cellular accumulation of E217G, determined at 2 min, in the double-transfected cells was significantly lower than that in OATP2 expressing cells (17.8 ± 0.8 pmol/mg of protein versus 34.0 ± 4.9 pmol/mg of protein, n = 3, p < .05).

The basal-to-apical flux of E217G across the MDCK II monolayer expressing OATP2 and the double transfecant was saturable (Fig. 4). Kinetic analysis revealed that the saturation can be best described by assuming the presence of one saturable and one non-saturable components. The analysis gave $K_{m}$, $V_{max}$ and $P_{diff}$ values of 23.8 ± 6.1 μM, 249 ± 60 (pmol/min/mg of protein) and 0.81 ± 0.41 (μl/min/mg of protein), respectively, for the OATP2 expressing MDCK II monolayer. This $P_{diff}$ value was comparable to that determined in the parental MDCK II monolayer (1.10 ± 0.03 μl/min/mg of protein). On the other hand, $K_{m}$, $V_{max}$ and $P_{diff}$ values of 27.9 ± 4.1 μM, 560 ± 89 (pmol/min/mg of protein), and 6.01 ± 0.61 (μl/min/mg of protein), respectively, were obtained also for the double-transfected cells (Fig. 4).

Furthermore, to quantitatively evaluate the transport activity across the apical membrane, the $P_{spec}$ for E217G was also determined. As shown in Fig. 5, the $P_{spec}$ in the double-transfected was significantly higher than that in the control monolayer. Moreover, the $P_{spec}$ for E217G in the double-transfected cells was not saturated even if the concentration of E217G in the basal compartment was increased to 150 μM (Fig. 5).

Transcellular Transport of Pravastatin, LTC₄, TLC-S, E₁S, and DHEAS Across an MDCK II Monolayer—Transcellular transport of pravastatin across the monolayer was saturable, with $K_{m}$, $V_{max}$ and $P_{diff}$ values of 24.3 ± 10.4 μM, 149 ± 56 pmol/min/mg of protein, and 0.99 ± 0.32 μl/min/mg of protein, respectively (Fig. 7). The cellular accumulation of pravastatin, determined at 2 min, in the double-transfected cells was significantly lower than that in OATP2-expressing cells (4.7 ± 0.6 pmol/mg of protein versus 12.0 ± 2.9 pmol/mg of protein, n = 3, p < 0.05).

For LTC₄, the basal-to-apical flux was two and four times higher than that in the opposite direction in an MDCK II monolayer expressing OATP2 and the double transfecant, respectively (Fig. 8). In the same manner, the basal-to-apical flux of TLC-S was 3.1 and 11.5 times higher than that in the opposite direction in an MDCK II monolayer expressing OATP2 and MRP2, respectively.

FIG. 1. Immunolocalization of recombinant OATP2 and MRP2 in stably transfected cells. MDCK II cells transfected with empty-vector (A), OATP2 (B), MRP2 (C), and both OATP2 and MRP2 (double transfecant, D) cDNA, were stained with the polyclonal antibody against the carboxyl terminus of human OATP2 (green fluorescence) and the monoclonal antibody against the linker region of human MRP2 (red fluorescence). Nuclei were stained with SYTO61 (blue fluorescence). A, B, C, and D: 0.8-μm optical sections in the x-y plane. E, F, G, and H: vertical sections in the x-z plane indicated by the green lines in A, B, C, and D.

FIG. 2. Western blot analysis for OATP2 and MRP2. Crude membrane fraction from MDCK II cells transfected with empty-vector (A), OATP2 (B), MRP2 (C), and both OATP2 and MRP2 (double transfecant, D) were loaded with 20 μg and separated by SDS-PAGE (8.5% separating gel). OATP2 and MRP2 were detected by polyclonal antibody against the carboxyl terminus of human OATP2 and polyclonal antibody against the carboxyl terminus of human MRP2, respectively.

FIG. 3. Time profiles for the transcellular transport of [3H]E₂17G across MDCK II monolayers. Transcellular transport of [3H]E₂17G (1 μM) across MDCK II monolayers expressing OATP (B), MRP2 (C), and both OATP2 and MRP2 (double transfecant, D) was compared with that across the control MDCK II monolayer (A). Open and closed circles represent the transcellular transport in the apical-to-basal and basal-to-apical directions, respectively. Each point and vertical bar represents the mean ± S.E. of three determinations. Where vertical bars are not shown, the S.E. was contained within the limits of the symbol.
Transcellular transport of E1S and DHEAS was also determined across the monolayers. As shown in Figs. 10 and 11, the basal-to-apical flux of E1S and DHEAS in OATP2-expressing MDCK II monolayer was about 2 and 1.5 times higher than that in the opposite direction, respectively. This vectorial flux of E1S and DHEAS was not further stimulated by expressing MRP2 (Figs. 10 and 11). The cellular accumulation of E1S, determined at 2 min, was not different between the OATP2-expressing monolayer and the double transfectant (37.7 ± 0.7 pmol/mg of protein versus 42.0 ± 3.4 pmol/mg of protein, n = 3, p > 0.05).

**DISCUSSION**

In the present study, we have established the double-transfected MDCK II cells, which express both OATP2 and MRP2. Immunohistochemical analysis with confocal laser microscopy suggested the basal and apical expression of OATP2 and MRP2, respectively (Fig. 1), which is consistent with the previously reported localization of these transporters (9, 16, 17). Because the antibodies against OATP2 and MRP2 were raised against their intracellular domains and the MDCK II cells were permeabilized for the immunohistochemical studies, it is possible that these transporters are also expressed in the intracellular vesicles near to the plasma membrane. However, the fact that Evers et al. (16) could demonstrate the apical preferential efflux of 2,4-dinitrophenyl-S-glutathione, an MRP2 substrate, from MDCK II monolayer after preloading the cells with its precursor (1-chloro-2,4-dinitrobenzene), along with the fact that we ourselves could find the vectorial transport of several ligands across the cell monolayers in the present study, suggests that these transporters may also be located on the
plasma membrane to a significant level. The Western blot analysis revealed that the expression levels of OATP2 and MRP2 in the single-transfected MDCK II cells were about the same as those in the double-transfected cells (Fig. 2). We have examined the transcellular transport of several kinds of ligands using this characterized MDCK II monolayer.

The basal-to-apical flux of E217G/H9252G across OATP2 expressing an MDCK II monolayer was approximately two times higher than in the opposite direction (Fig. 3). This result may be accounted for by assuming the presence of one or more endogenous transporters on the apical membrane, which is able to extrude E217G/H9252G from the cells; E217G molecules in the basal compartment are taken up into the monolayer via OATP2 and then excreted into the apical compartment with the aid of such an endogenous transporter. If OATP2 is not expressed on the basolateral membrane, E217G molecules are not significantly taken up by MDCK II monolayers, resulting in the observed symmetrical transport in control and MRP2-expressing MDCK II monolayers.
II cells (Fig. 3). The basal-to-apical flux of E$_{17}$G was stimulated to a greater extent by co-expressing MRP2 with OATP2 (Fig. 3), suggesting that E$_{17}$G molecules taken up into the monolayer are much more efficiently extruded into the apical compartment with the aid of MRP2. This suggestion is further supported by the fact that the cellular accumulation of E$_{17}$G was significantly lower in the double-transfected cells compared with OATP2 expressing cells (see “Results”).

Kinetic analysis was also performed for the transcellular transport of E$_{17}$G. The $K_{m}$ value for the transcellular transport of E$_{17}$G across MDCK II cells expressing OATP2 (single transfectant) and those expressing both OATP2 and MRP2 (double transfectant) was 23.8 ± 6.1 and 27.9 ± 4.1 μM, respectively (Fig. 4). These $K_{m}$ values are comparable with those reported for OATP2-mediated transport of E$_{17}$G in cRNA-injected Xenopus laevis oocytes (9.7 μM) (23) and in cDNA-transfected MDCK II cells (8.2 μM) (9). Moreover, the $P_{app}$ value was not saturated even if the concentration of E$_{17}$G in the basal compartment was increased up to 150 μM (Fig. 5). Taking all these observations into consideration, the saturation observed in the transcellular transport of E$_{17}$G may be ascribed to the saturation of OATP2-mediated uptake, suggesting that the uptake may be the rate-determining step in the transcellular transport of E$_{17}$G. In other words, E$_{17}$G molecules taken up into the MDCK II monolayer may be efficiently extruded into the apical compartment with the aid of MRP2. The reason for the high $P_{app}$ value for E$_{17}$G across the double transfectant (Fig. 4) remains unknown.

In the same manner, it was found that the vectorial transport of TLC-S, which is transported by rat oatp1 and Mrp2 (18,24), was also stimulated by OATP2 and MRP2 expression (Fig. 9), suggesting that this sulfated bile acid is transported by human OATP2 and MRP2. Moreover, we have examined the transcellular transport of pravastatin and LTC$_{4}$, which are substrates of both OATP2 (5, 6) and MRP2 (4), to perform kinetic analysis. As shown in Fig. 6, the vectorial transport of pravastatin from the basal to apical compartment was stimulated by expressing both OATP2 and MRP2. The $K_{m}$ value for the transcellular transport of pravastatin (24.3 ± 10.4 μM; Fig. 7) also agrees with the OATP2-mediated uptake of this drug determined in cRNA-injected oocytes (33.7 μM) (6). Because pravastatin is a low affinity substrate of MRP2 (220 μM) (25), the rate-determining step in the transcellular transport of this compound may also be the uptake mediated by OATP2. This hypothesis is also consistent with our previous observations that uptake is the rate-determining step for the biliary excretion of pravastatin in rats (25).

Moreover, transcellular transport of LTC$_{4}$ was examined for the purpose of comparing its transport properties with those of E$_{17}$G. An analysis of the data shown in Fig. 8 indicated that the transport activity for the basal to apical flux of LTC$_{4}$ was $-4 \mu$mol/mg protein, which is significantly lower than that for E$_{17}$G ($-12 \mu$mol/mg protein; Fig. 3). These results should be discussed in the light of the transport properties of these compounds mediated by OATP2 and MRP2. In OATP2-expressing oocytes, Abe et al. (5) and Kullak-Ublick et al. (11) demonstrated that the transport activity for LTC$_{4}$ is $-4.8$ and 2.6 times lower than that for E$_{17}$G, respectively. In contrast, using membrane vesicles isolated from MRP2-transfected HEK-293 cells, Cui et al. (26) demonstrated that the transport activity for LTC$_{4}$ is much higher than that for E$_{17}$G (351 versus $20 \mu$mol/mg protein). Collectively, our finding that E$_{17}$G, rather than LTC$_{4}$, is efficiently transported across the double-transfected MDCK II monolayer expressing both OATP2 and MRP2 may also be accounted for by the fact that the transcellular transport rate of ligands is governed by the cellular uptake rate.

In addition, transcellular transport of E$_{S}$ and DHEAS was also examined. As shown in Figs. 10 and 11, basal-to-apical flux of E$_{S}$ and DHEAS was stimulated by OATP2 transfection, which is consistent with the previous finding that E$_{S}$ and DHEAS are substrates of OATP2 (5). In contrast, additional expression of MRP2 did not further stimulate the transcellular transport of these sulfated conjugates (Figs. 10 and 11). Moreover, the cellular accumulation of E$_{S}$ was not significantly different between OATP2-expressing cells and the double transfecant (see “Results”). These results are consistent with the previous finding that E$_{S}$ may not be a good substrate of MRP2. Indeed, no ATP-dependent transport of E$_{S}$ or DHEAS has been observed in isolated bile canaliculal membrane vesicles, which expresses MRP2, and E$_{S}$ actually stimulated MRP2-mediated transport of substrates such as 2,4-dinitrophenyl-S-glutathione (27). Molecular cloning of a transporter, which is responsible for the canaliculal efflux of E$_{S}$, is required to establish an in vitro model to predict the hepatobiliary excretion of sulfated conjugates.

In conclusion, we have been able to establish an MDCK II cell line that expresses both OATP2 and MRP2 on the basal and apical membranes, respectively. Kinetic analysis revealed the rate-determining step in the transcellular transport of OATP2 and MRP2 bisubstrates across this cell monolayer. By normalizing the level of expression of these transporters in human liver, it may be possible to quantitatively predict the in vivo human biliary excretion of substrates of these transporters.

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Addendum—In the 2nd International Research Conference (PharmaConference 2001) “Membrane Transporters: From Identification to Drug Discovery” held August 5–10, 2001, in Interlaken, Switzerland, Cui et al. (28) presented their data on vectorial transport across MDCK II cell monolayer expressing both OATP2/SLC21A6 and MRP2/ABC1C2, which was performed independently from the present study.

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