Functional Characterization of Multidrug Resistance-Associated Protein 3 (Mrp3/Abcc3) in the Basolateral Efflux of Glucuronide Conjugates in the Mouse Small Intestine

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ABSTRACT

The intestine expresses metabolic enzymes and transporters as functions as a barrier to orally administered xenobiotics. This study aimed to examine the importance of multidrug resistance-associated protein 3 (Mrp3/Abcc3) in the serosal efflux of glucuronide conjugates formed in the intestine using wild-type and Mrp3(−/−) mice. The everted sacs of the intestine were incubated with 4-methylumbelliferone (4MU), and the efflux rates of intracellularly formed glucuronide conjugate of 4MU (4MUG) into the mucosal and serosal sides were determined. The permeability-surface area product across the serosal membrane (PSserosal) of 4MUG in wild-type mice was greater in the duodenum followed by the jejunum, ileum, and colon. The corresponding parameters were significantly reduced in Mrp3(−/−) mice (approximately 33% of that in wild-type mice) except for the colon where the PSserosal of 4MUG was similar between wild-type and Mrp3(−/−) mice. There was no difference in the PSmucosal of 4MUG in whole segments of the intestine between wild-type and Mrp3(−/−) mice. In addition to 4MUG, the PSserosal of the glucuronide conjugates of 7-ethyl-10-hydroxycamptothecin (SN-38) and acetaminophen in the jejunal everted sacs were also significantly reduced in Mrp3(−/−) mice compared with wild-type mice. There was no difference in the mRNA and protein expression of efflux transporters between wild-type and Mrp3(−/−) mice. These results suggest that Mrp3 plays major roles in the efflux transport of various glucuronide conjugates from the enterocytes to the portal blood in the small intestine together with unknown transporter(s), but the contribution of Mrp3 to the basolateral efflux of 4MUG was negligible in the colon.

The intestine functions as a barrier to orally administered xenobiotics. Various metabolic enzymes, including cytochrome P450 isoenzymes (P450s), carboxylesterases, uridine diphosphate-glucuronosyltransferases (UGTs), and sulfotransferases, are expressed in the intestinal epithelial cells (Kaminsky and Zhang, 2003). UGT and sulfotransferase catalyze conjugation of xenobiotics with glucuronic acid and sulfate that are generally less active than their parent compounds, although there are notable exceptions, such as morphine-6-glucuronide, S-8921 glucuronide, and minoxidil sulfate, which are more active than their parent compounds (Shipkova and Wieland, 2005; Zamek-Gliszczynski et al., 2006; Sakamoto et al., 2007). Glucuronide conjugation is also known as a bioactivation pathway because some acyl glucuronides cause toxicity by covalent binding with proteins in plasma and tissues (Shipkova et al., 2003).

Importance of cooperation of phase II metabolism and efflux transport has been accentuated in the xenobiotic detoxification (Zamek-Gliszczyńska et al., 2006; Nies et al., 2008). ATP-binding cassette (ABC) transporters, such as multidrug resistance-associated protein 1 (Mrp1/Abcc1), Mrp2/Abcc2, Mrp3/Abcc3, and breast cancer resistance protein (Bcrp/Abcg2), mediate the cellular efflux of phase II metabolites, glutathione, glucuronide, and sulfate conjugates. In the small intestine, Mrp2 and Bcrp, apical ABC transporters, have been shown to mediate the efflux of intracellularly formed conjugated metabolites into the lumen. The efflux of

ABBREVIATIONS: P450, cytochrome P450; UGT, uridine diphosphate-glucuronosyltransferase; S-8921, 1-(3,4-dimethoxyphenyl)-3-(3-ethyl-valeryl)-4-hydroxy-6,7,8-trimethoxy-2-naphthoate; ABC, ATP-binding cassette; MRP/Mrp, multidrug resistance-associated protein; Bcrp, breast cancer resistance protein; E3040, 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl) benzothiazole; 4MU, 4-methylumbelliferone; E17βG, estradiol 17β-glucuronide; BLMV, basolateral membrane vesicles; SN-38, 7-ethyl-10-hydroxycamptothecin; APAP, acetaminophen (N-acetyl-p-aminophenol); 4MUG, 4-methylumbelliferone glucuronide; APAPG, p-acetamidophenyl-β-d-glucuronid sodium salt; SN-38G, 7-ethyl-10-hydroxycamptothecin glucuronide; LC/MS, liquid chromatography/mass spectrometry; PS, permeability-surface area product; HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction; Gapdh, glyceraldehyde-3-phosphate dehydrogenase; Ost, organic solute transporter; PBS, phosphate-buffered saline; 4MUS, 4-methylumbelliferone sulfate; BQ123, cyclo(Trp-Asp-Pro-Val-Leu).
the glucuronide conjugates of E3040 and S-8921 into the lumen after a single pass perfusion of the lumen with the parent compounds was significantly reduced in mutant rats hereditarily lacking Mrp2 (Adachi et al., 2005; Sakamoto et al., 2007) and that of glucuronide and sulfate conjugates of 4-methylumbelliferone (4MU) and sulfate conjugate of minoxidil was significantly reduced in Bcrp1−/− mice compared with wild-type mice (Adachi et al., 2005; Enokizono et al., 2007). There have been reports of the serosal efflux of glucuronide conjugates in the intestine. Simultaneous vascular and luminal perfusion of the mouse small intestine demonstrated that administration of 1-naphthol to the lumen resulted in a release of 1-naphthol glucuronide into the serosal side (Wollenberg et al., 1983). 4MUG was also found in the portal venous outflow when 4MU was perfused from the superior mesenteric artery in rats (Chen and Pang, 1997). In vitro transport studies using rat intestine suggested that resveratrol and S-8921 were mainly absorbed as glucuronide into the portal vein (Kuhnle et al., 2000; Sakamoto et al., 2007). A primary active transporter has been considered to mediate the serosal efflux of glucuronide conjugates since Shoji et al. (2004) demonstrated ATP-dependent uptake of estradiol 17β-glucuronide (E17βG) in the basolateral membrane vesicles (BLMV) from rat jejenum, ileum, and colon. The present study focused on Mrp3/Abcc3 as a candidate transporter for the efflux transport of glucuronide conjugates in the basolateral membrane of enterocytes. The substrates of Mrp3 include a variety of glucuronide conjugates as well as bile acids and folates (Hirohashi et al., 1999, 2000; Zeng et al., 2000). Mrp3 is abundantly expressed in the liver and intestine where it is expressed on the sinusoidal membrane of hepatocytes, and basolateral membrane of enterocytes (Zelcer et al., 2006). Recently, Zelcer et al. (2005, 2006) produced Mrp3−/− mice that exhibited a significant reduction in the plasma concentrations of glucuronide conjugates after administration of their parent compounds due to reduced sinusoidal efflux clearance (Manautou et al., 2005). Considering the membrane localization, Mrp3 can mediate the serosal efflux of its substrate compounds into the portal blood. Indeed, we recently demonstrated that Mrp3 mediates the serosal efflux of methotrexate in the duodenum, facilitating intestinal absorption of methotrexate in conjunction with uptake transporters (Kitamura et al., 2007). It was also reported that the plasma concentration of resveratrol glucuronide was markedly decreased in Mrp3−/− mice after oral administration of resveratrol. This reduced plasma concentration may be accounted for by the absence of Mrp3 in the intestine because resveratrol seems to be mainly glucuronidated in the gut (van de Wetering et al., 2009). However, no direct evidence was provided for the basolateral efflux of resveratrol glucuronide in the intestine.

The present study was aimed at examining the role of Mrp3 in the serosal efflux of glucuronide conjugates along the intestine. As test substances, we chose 4MU, SN-38, and acetaminophen (N-acetyl-p-aminophenol; APAP). Intestinal transport of glucuronide conjugates was examined using the everted sac. The serosal side of the everted sacs was perfused to collect the glucuronide conjugates extruded into the serosal side. Efflux transport activities across the mucosal and serosal membranes were compared to determine the importance of Mrp3 in the serosal efflux transport.

### Materials and Methods

#### Chemicals and Reagents.

4MU, 4MU glucuronide (4-methylumbelliferyl β-d-glucuronide; 4MUG), acetaminophen (N-acetyl-p-aminophenol, APAP), and APAP glucuronide (p-acetamidophenyl β-d-glucuronide; APAPG) were purchased from Sigma-Aldrich (St. Louis, MO). SN-38 was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada) and SN-38 glucuronide (SN-38G) was obtained from Yakult Honsha Co. Ltd. (Tokyo, Japan). The carboxylate form of SN-38 was produced by dissolving the compounds in alkaline solution (0.25 N NaOH). Virtually complete conversion of the lactone to the corresponding carboxylate was confirmed by liquid chromatography/mass spectrometry (LC/MS). All other chemicals were commercially available, reagent grade products.

#### Animals.

Male Mrp3−/− (Zelcer et al., 2005) and wild-type FVB mice (12–16 weeks old) were used. All animals were maintained under a 12-h light/dark cycle and were allowed free access to water and solid laboratory food. The studies were carried out in accordance with the guidelines provided by the Institutional Animal Care Committee (Graduate School of Pharmaceutical Sciences, The University of Tokyo, Tokyo, Japan).

#### Transport of Glucuronides in Everted Intestinal Sacs.

The mucosal and the serosal efflux of the glucuronides was determined in everted sacs from mouse intestine. Everted sac analysis was performed as described previously (Enokizono et al., 2007; Kitamura et al., 2009). Krebs-Ringer bicarbonate buffer (119 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM KH2PO4, 1.2 mM CaCl2, 25 mM NaHCO3, and 11 mM d-glucose, pH 6.8) was used in the everted sac studies. The duodenum (1–8 cm from the stomach), jejunum (10–18 cm from the stomach), ileum (8 cm above the cecum), and colon (7 cm below the cecum) of the wild-type and Mrp3−/− mice were excised, trimmed to 6 cm, turned inside out, and immersed in the mucosal buffer (50 ml; 37°C) that was aerated with 95% O2 + 5% CO2. Serosal buffer was perfused through the everted bowels at 100 μl/min. After 15 min of preincubation, one of the parent compounds was added to the mucosal buffer at a concentration of 5 μM (4MU and SN-38 carboxylate) or 30 μM (APAP). The total volume of the perfused serosal buffer was collected every 10 min, and aliquots (100 μl) of the mucosal buffer were collected at 10, 20, 30, 40, and 50 min after addition of a parent compound. At 50 min, the everted sac tissue was collected. Concentrations of the glucuronide in buffer and tissue samples were determined by LC/MS.

#### Kinetic Analysis.

The cumulative amount of glucuronide extruded into the serosal buffer was calculated as a sum of the amount in the fractions obtained every 10 min. The amount in the fractions was calculated by multiplying the concentration by the volume of the fraction (1 ml). The amount of glucuronide extruded to the mucosal buffer up to the designated time was calculated by multiplying the concentration in the mucosal buffer by its volume (50 ml). The efflux transport rate across the serosal and mucosal membrane was obtained from the slope at 30 to 50 min. The slope was calculated by linear regression from the graph where the amount of glucuronide extruded to the serosal and mucosal side was plotted against time. The permeability-surface area products for the intrinsic efflux transport across the serosal membrane (Pf(serosal)) and across the mucosal membrane (Pf(mucosal)) were calculated by dividing the efflux transport rate across the mucosal and serosal membrane, respectively, by the concentration of glucuronide in the everted sac tissue (50 min). The total production of glucuronide was calculated as a sum of the amount in the everted sac tissue (50 min) and amount extruded into mucosal and serosal buffer throughout the incubation period (0–50 min). The tissue concentration was obtained by dividing the amount associated with tissue specimens by the wet tissue weight assuming that the specific gravity of the tissue is one.

#### LCMS Analysis.

The concentration of glucuronides was determined by LC/MS. For 4MUG, buffer samples were diluted 10-fold with water and centrifuged. The supernatant (10 μl) was applied to the LC/MS system. Tissue samples were homogenized with 9 vol-
umes of methanol and the supernatant of the homogenate was diluted 10-fold with water, and a 10-μl aliquot was injected into the LC/MS system. For SN-38G, buffer samples were diluted with an equal volume of 1% formic acid and centrifuged. The supernatant (10 μl) was injected into the LC/MS system. Tissue samples were homogenized with 9 volumes of methanol and the supernatant of the homogenate was diluted 5-fold with 1% formic acid, and a 10-μl aliquot was injected into the LC/MS system. For the determination of APAPG concentration, buffer samples were centrifuged and the supernatant (10 μl) was injected into the LC/MS system. Tissue samples were homogenized with 9 volumes of methanol and the supernatant of the homogenate was diluted 10-fold with water, and a 10-μl aliquot was subjected to LC/MS analysis.

Separation by high-performance liquid chromatography (HPLC) was performed on a Waters 1525 U Binary HPLC Pump system (Waters, Milford, MA). The HPLC column was an L-column ODS (2.1 × 150 mm, 5 μm; Chemicals Evaluation and Research Institute, Tokyo, Japan). The column temperature was 40°C, and the composition of the mobile phase was acetonitrile/0.05% formic acid (17:83, 7:93, and 20:80 for 4MUG, SN-38G, and APAPG, respectively). The flow rate was 0.3 ml/min (4MUG and APAPG) or 0.4 ml/min (SN-38G). The mass spectrometer was a Micromass ZQ2000 instrument (Waters), with an electrospray ionization interface. Data were acquired in the selected ion-recording mode by use of a positive ion m/z 569.0 for SN-38G and negative ions m/z 351.0 and 326.0 for 4MUG and APAPG, respectively.

Quantification of mRNA for the Efflux Transport along the Intestine. Total RNA was prepared from the duodenum, jejunum, ileum, and colon of wild-type and Mrp3−/− mice using ISOGEN (Wako Pure Chemicals, Osaka, Japan). After DNaseI treatment (Takara Bio Inc., Otsu, Japan), the RNA was reverse-transcribed to single-stranded cDNA using a TaKaRa RNA PCR kit (avian myeloblastosis virus) version 3.0 (Takara Bio Inc.). Applied Biosystems (Foster City, CA) inventoried TaqMan gene expression assays were used to quantify the mRNA levels of Mrp1 (GenBank accession number NM_008576, assay ID Mm00456516_m1), Mrp2 (GenBank accession number NM_013806, Mm00496899_m1), Bcrp (GenBank accession number NM_011920, Mm00496364_m1), and glyceraldehyde-3-phosphate dehydrogenase (Gapdh, GenBank accession number NM_008084, Mm99999915_g1). The mRNA levels of organic solute transporter α (Osto, GenBank accession number NM_145932) and Ostβ (GenBank accession number NM_178933) were quantified using the following TaqMan probes and primers: Ostα, 5′-ACCCACAGGAAATTTT-3′ (probe), 5′-CTCATCCCGTACCCGATCTATG-3′ (forward primer), and 5′-GCAAGGAGATTGTTGATCCAGAGA-3′ (reverse primer); and Ostβ, 5′-TCGTCGAGAAAGATGCG-3′ (probe), 5′-GCAAGGAGATTGTTGATCCAGAGA-3′ (forward primer), and 5′-GCCAGGACCAGGATCTGAAATTC-3′ (reverse primer).

For the real-time-polymerase chain reaction (PCR), the TaqMan One-Step RT-PCR Master Mix Reagents kit (Applied Biosystems) containing 900 nM forward primer, 900 nM reverse primer, and 250 mM TaqMan probe was used at 15 μl/well. The real-time PCR assay was performed with the 7500 Real-Time PCR System (Applied Biosystems), with the following profile: 50°C for 2 min, 95°C for 10 min, and 40 cycles each at 95°C for 15 s and 60°C for 1 min. An external standard curve was generated by dilution of the target PCR product, which was purified by agarose gel electrophoresis (NuSieve GTG Agarose; Lonza Rockland, Inc., Rockland, ME). All gene expression was normalized with regard to the expression of Gapdh mRNA.

Western Blot Analysis. Wild-type and Mrp3−/− mice were sacrificed and the duodenum, jejunum, ileum, and colon were excised. Each intestinal segment was cut longitudinally, and the mucosal side was exposed. The mucosa was scraped with the flat end of a spatula to obtain an epithelial cell-enriched fraction. This epithelial cell-enriched fraction was suspended in phosphate-buffered saline (PBS) containing a 0.5% protease inhibitor cocktail (product P8340; Sigma-Aldrich, Saint Louis, MO). The suspension was sonicated and centrifuged at 3000g for 15 min at 4°C, and the supernatant was centrifuged at 100,000g for 15 min at 4°C. After centrifugation, the pellet was suspended in PBS containing 0.5% protease inhibitor cocktail. The protein concentration was determined by bichinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL), and the crude membrane fractions were stored at −80°C.

The crude membrane fractions (50 μg of protein) from the duodenum, jejunum, ileum, and colon of wild-type and Mrp3−/− mice were subjected to electrophoresis using 7% SDS-polyacrylamide gels (140 V; 70 min) and electrophoretically to a polyvinylidene difluoride membrane (15 V; 80 min). Then, the polyvinylidene difluoride membrane was incubated with 125 times diluted anti-Mrp2 monoclonal antibody (M2II-6; Alexis Laboratories, San Diego, CA). 200 times diluted anti-Bcrp monoclonal antibody (BXP-53; SOLVO Biotechnol- ogy, Szeged, Hungary), or 1000 times diluted anti-actin antibody (C4; MP Biomedicals, Solon, OH) overnight at 4°C. Then, the membrane was incubated with 5000 times diluted horseradish peroxi- dase-labeled anti-rat (Bcrp) or anti-mouse (Mrp2 and actin) IgG (GE Healthcare, Little Chalfont, Buckinghamshire, UK) for 1 h at room temperature. An ECL Plus system (GE Healthcare) was used for detection, and the luminescence image was taken by an image analy- zer (LAS-3000 mini; Fuji Film, Tokyo, Japan).

Statistical Analysis. All data represent at least three independent experiments and are expressed as the means ± S.D. In the assessment of the significant differences in the amount of glucuro- nide conjugates extruded to the serosal and mucosal side, analysis of variance (ANOVA) with repeated observations was performed. In the case of intrinsic efflux activity, concentration in the sac, and glucuronide production, statistically significant differences were assessed using a two-tailed Student’s t test. P < 0.05 was considered statistically significant.

Results

Serosal and Mucosal Efflux of 4MUG in Everted Sacs from the Duodenum, Jejunum, Ileum, and Colon. The efflux transport of 4MUG to the serosal and mucosal sides was determined in vitro using everted sacs from the intesti- nal segments (duodenum, jejunum, ileum, and colon) of wild- type and Mrp3−/− mice. Incubating the everted sacs with 4MU resulted in the appearance of 4MUG in the serosal perfusate and mucosal buffer. The amount of 4MUG extruded to the serosal side was greatest in the intestine in wild-type mice. The amounts of 4MUG in the serosal perfusate were lower in the small intestine of Mrp3−/− mice compared with wild-type mice (Fig. 1, A–D). The difference reached statistical signifi- cance in the duodenum, jejunum, and ileum. Mrp3−/− mice also exhibited lower 4MUG concentrations in the serosal perfusate in the colon, but the difference was not statistically significant. The amount of 4MUG in the mucosal buffer was higher in the proximal intestine and gradually decreased to the distal segment of wild-type mice. The amounts of 4MUG in the mucosal perfusate were lower in the small intestine of Mrp3−/− mice compared with wild-type mice (Fig. 1, A–D). The difference reached statistical signifi- cance in the duodenum, jejunum, and ileum. Mrp3−/− mice also exhibited lower 4MUG concentrations in the serosal perfusate in the colon, but the difference was not statistically significant. The amount of 4MUG in the mucosal buffer was higher in the proximal intestine and gradually decreased to the distal segment (Fig. 1, E–H). There was no difference in the amount of 4MUG in the mucosal buffer between wild- type and Mrp3−/− mice except for the jejunum where the amount was slightly but significantly higher in Mrp3−/− mice than in wild-type mice. Neither the concentration of 4MUG in the everted sac tissue (Csac) nor the total produc- tion of 4MUG showed any differences between wild-type and Mrp3−/− mice (Fig. 2). The Csac of 4MUG was almost identical throughout the intestine, whereas the 4MUG production exhibited apparent regional differences.

To investigate the role of Mrp3, P8340, an intrinsic para- meter representing the serosal efflux, was compared between wild-type and Mrp3−/− mice (Fig. 3A). The P8340 was highest in the duodenum and lowest in the colon in
wild-type mice. The $P_{\text{serosal}}$ of 4MUG in the duodenum, jejunum, and ileum of Mrp3($-/-$) mice was 23, 46, and 30% of that of wild-type mice, respectively. In contrast, the $P_{\text{serosal}}$ in the colon was almost identical in both strains. The $P_{\text{serosal}}$ remaining in Mrp3($-/-$) mice was approximately the same over the whole length of the intestine, whereas $P_{\text{mucosal}}$ was highest in the duodenum and was reduced to the colon (Fig. 3B). There was no difference in the $P_{\text{mucosal}}$ of 4MUG throughout the whole segments between wild-type and Mrp3($-/-$) mice.

**Serosal and Mucosal Efflux of SN-38G and APAPG in the Everted Sacs.** In addition to 4MUG, the efflux transport activities of SN-38G and APAPG were also determined in vitro using everted sacs prepared from the jejunum of wild-type and Mrp3($-/-$) mice. The amounts of the glucuronide conjugates in the serosal perfusate were considerably lower in Mrp3($-/-$) mice than in wild-type mice ($P < 0.05$), whereas the amounts in the mucosal buffer were identical in wild-type and Mrp3($-/-$) mice (Fig. 4). No difference was observed in the SN-38G concentrations in everted sac tissue ($C_{\text{tissue}}$) in the two strains, whereas the $C_{\text{tissue}}$ value of APAPG was 5 times higher in Mrp3($-/-$) mice ($P < 0.05$). The total production of glucuronide conjugates during incubation was similar in both strains (Table 1). Mrp3($-/-$) mice exhibited a marked reduction in $P_{\text{serosal}}$ ($P < 0.05$) and the $P_{\text{serosal}}$ values of...
SN-38G and APAPG in Mrp3−/− mice were reduced to 8.5 and 5.4%, respectively, compared with wild-type mice. The PSmucosal of SN-38G in Mrp3−/− mice was nearly equal to that in wild-type mice. With regard to APAPG, Mrp3−/− mice had a significantly lower PSmucosal than wild-type mice. The PSmucosal value of APAPG was reduced to 22% in Mrp3−/− mice (Table 2).

**Distribution of mRNA of Efflux Transporters along the Intestine.** Intestinal transcripts of Mrp1, Mrp2, Bcrp, Ostα, and Ostβ in wild-type and Mrp3−/− mice were quantified by RT-PCR (Fig. 5). There were no obvious differences between wild-type and Mrp3−/− mice. Mrp1 was most highly expressed in the colon, and the expression was almost similar in other segments. Expression of Mrp2 was abundant in the small intestine but was hardly detectable in the colon. The highest expression of Bcrp was observed in the ileum and the expression in other three segments was lower. The expression of Ostα and Ostβ was predominantly detected in the ileum.

**Expression of Mrp2 and Bcrp along the Intestine.** Intestinal expressions of Mrp2 and Bcrp in wild-type and Mrp3−/− mice were analyzed by Western blotting (Fig. 6). There were no differences between the strains. The expression of Mrp2 was higher in the distal segment of the intestine. The expression of Bcrp was the highest in the ileum followed by the jejunum.
TABLE 1
Tissue concentration and total production of SN-38G and APAPG in the everted sac study

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>Mrp3(-/-)</th>
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<tbody>
<tr>
<td>Cmax (μM)</td>
<td>0.236 ± 0.091</td>
<td>0.221 ± 0.048</td>
</tr>
<tr>
<td>SN-38G</td>
<td>4.61 ± 1.93</td>
<td>24.6 ± 3.0*</td>
</tr>
<tr>
<td>APAPG</td>
<td>1.67 ± 0.42</td>
<td>1.82 ± 0.19</td>
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* P < 0.05, significantly different from wild-type mice.

TABLE 2
Intrinsic efflux transport activity of SN-38G and APAPG across the serosal and mucosal membrane

<table>
<thead>
<tr>
<th></th>
<th>Wild Type</th>
<th>Mrp3(-/-)</th>
</tr>
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<tbody>
<tr>
<td>PSserosal (μl/min/sac)</td>
<td>17.0 ± 5.7</td>
<td>1.45 ± 0.86*</td>
</tr>
<tr>
<td>SN-38G</td>
<td>16.6 ± 1.1</td>
<td>0.806 ± 0.047*</td>
</tr>
<tr>
<td>APAPG</td>
<td>195 ± 34</td>
<td>252 ± 67</td>
</tr>
<tr>
<td>PSmucosal (μl/min/sac)</td>
<td>15.6 ± 7.2</td>
<td>3.41 ± 0.53*</td>
</tr>
</tbody>
</table>

* P < 0.05, significantly different from wild-type mice.

Discussion

Cooperation of phase II metabolism and efflux transport forms an efficient xenobiotic detoxification system in the small intestine as well as the liver. The present study investigated the transporter required for the serosal efflux of glucuronide conjugates in the small intestine by comparing the intrinsic efflux activity along the intestine between wild-type and Mrp3(-/-) mice.

The efflux transport activities of glucuronide conjugates formed intracellularly from the corresponding parent compounds via the serosal and mucosal membranes were determined using the everted sac. There was a clear regional difference in the glucuronidation activity (Fig. 2), which is consistent with the distribution of total UGT1 mRNA along the intestine (Grams et al., 2000). The efflux activities (PSserosal and PSmucosal) of 4MUG exhibited a marked regional difference, being highest in the duodenum and gradually decreased to the colon (Fig. 3). The pattern of efflux activity along the intestine is reasonable to prevent accumulation of xenobiotic metabolites inside the cells, considering the highest glucuronidation activity in the duodenum (Fig. 2B). Indeed, the concentration of 4MUG associated with everted sacs kept similar irrespective to the segment of the intestine in spite of large regional differences in the glucuronidation activity (Fig. 2). It was also found that PSmucosal of 4MUG exhibited a sharper reduction from the duodenum to the colon than PSserosal. PSmucosal of 4MUG was greater than PSserosal in the duodenum, but the difference was decreased in the jejunum and ileum, and finally reversed in the colon. The rationale of this reversed efflux in the colon is unknown.

The serosal efflux rate of 4MUG was significantly reduced in the entire small intestine of Mrp3(-/-) mice compared with wild-type mice, whereas the mucosal efflux rate of 4MUG was slightly greater in Mrp3(-/-) mice (Fig. 1). This is ascribed to the reduction in the intrinsic efflux activity in the serosal membrane (PSserosal) (Fig. 3) because there was no significant change in the concentration of 4MUG associated with everted sacs between wild-type and Mrp3(-/-) mice (Table 1). The Mrp3 expression along the small intestine, highest in the duodenum (Kitamura et al., 2008), is consistent with the pattern of PSserosal along the intestine (Fig. 3), and quantification of the mRNA of other serosal efflux transporters, such as Mrp1, Ostα, and Ostβ, excluded the occurrence of adaptive regulation at least at the mRNA level (Fig. 3). Therefore, it is most likely that reduced serosal efflux is ascribed to the lack of Mrp3. Previously, we explained the discrepancy between Mrp3 distribution and the effect of Mrp3 dysfunction on the mucosal-to-serosal transport of methotrexate by the regional distribution of uptake transporters (Kitamura et al., 2008). Unlike methotrexate, which requires the uptake transporter for the mucosal-to-serosal transport, 4MUG is formed inside the cells and subjected to the serosal efflux by Mrp3, allowing direct measurement of Mrp3 activity in the small intestine. Neither the amount of 4MUG that was produced during the experiment nor PSmucosal changed in Mrp3(-/-) mice compared with wild-type mice (Figs. 2B and 3B), indicating that the UGT and efflux activities across the mucosal membrane are unchanged in Mrp3(-/-) mice. Actually, mRNA and protein expression of Bcrp, which makes a significant contribution to the mucosal efflux of 4MUG in the small intestine (Adachi et al., 2005), was unchanged in Mrp3(-/-) mice (Figs. 5 and 6). It is worth mentioning that the regional difference in the PSmucosal of 4MU sulfate (4MUS), which represents the Bcrp activity, was not so marked as that of the PSmucosal of 4MUG. PSmucosal of 4MUS was at most 3-fold greater in the jejunum than in the colon but similar between in the jejunum and ileum (Enokizono et al., 2007). Thus, another transporter, the expression of which shows large regional differences from the proximal to the distal intestine, must be involved in the luminal efflux of 4MUG. The mechanisms underlying the reversed efflux of 4MUG in the colon is partly due to the regional difference in this unidentified transporter. Although Mrp2 expression shows such regional difference, in situ study using Mrp2-deficient mutant rats has excluded its involvement (Adachi et al., 2005).

Unexpectedly, Mrp3 dysfunction had a negligible impact on the serosal efflux of 4MUG in the colon (Fig. 3A), although Mrp3 mRNA is abundant in the colon compared with the small intestine (Kitamura et al., 2008). Other efflux transporter(s) will make a greater contribution than Mrp3 to the serosal efflux of 4MUG in the colon. Indeed, Shoji et al. (2004) reported the involvement of two saturable components in the ATP-dependent uptake of E17βG by BLMV from rat colon, with K_m values of 0.82 and 35 μM, which make an equal contribution based on the V_max divided by K_m, whereas that by BLMV from the jejunum and ileum consists of only one saturable component with K_m values of 24 and 8.3 μM, respectively. The recombinant human MRP3 showed one saturable component for the transport of E17βG, with a K_m value from 17 to 42 μM (Zeng et al., 2000; Zelcer et al., 2001;
Akita et al., 2002), similar to the $K_m$ value of the low-affinity component in the BMLV from the colon. Real-time PCR analysis revealed that mRNA expression of Mrp1 is more abundant in the colon than in the small intestine (Fig. 5), consistent with the protein expression level (Peng et al., 1999). Furthermore, the $K_m$ value of $E217$/$H9252$ transport by human MRP1 (1.5–4.8 $M$) is lower than that by MRP3 (Jedlitschky et al., 1996; Loe et al., 1996; Stride et al., 1997). Mrp1 may serve as the high-affinity component in the colon. Because the serosal efflux of 4MUG did not completely vanish away in Mrp3($−/−$) mice, it is also possible that Mrp1 is involved in the serosal efflux of 4MUG in the small intestine as well as the colon.

In addition to 4MUG, the PS$_{serosal}$ of other glucuronide conjugates, SN-38G and APAPG, was also significantly reduced in the jejunum of Mrp3($−/−$) mice compared with wild-type mice (Table 2). Thus, Mrp3 probably plays a major role in the serosal efflux of a variety of glucuronide conjugates in the small intestine. Unlike 4MUG and SN-38G,
tissue concentration (Csac) of APAPG was 5-fold higher in Mrp3(−/−) mice, although the total amount formed during the experiment was similar (Table 1). Because Psac was similar to Psms in wild-type mice, reduction in Psac in Mrp3(−/−) mice, which is distinct from that for 4MUG and SN-38G. Further studies are necessary to identify it.

The present study demonstrated the significance of Mrp3 in the ileal efflux of glucuronide conjugates in the entire small intestine. Together with previous findings in the liver, the Mrp3 activity is closely associated with the systemic exposure of the glucuronide conjugates particularly when the parent compounds are given orally. In addition, Mrp3 may mediate the ileal efflux in the mucosal-to-ileal transport of its substrate drugs, such as fexofenadine (Matsushima et al., 2008), pravastatin, temocapril, and BQ123 (Suzuki and Sugiyama, 2000), in conjunction with the uptake transporters such as PEPT1, organic anion-transporting polypeptide 2b1, and organic anion-transporting polypeptide 1a5. Further studies are required to elucidate the role of Mrp3 in drug absorption.

In conclusion, Mrp3 plays a major role in the ileal efflux of glucuronide conjugates in the small intestine. In spite of abundant expression in the colon, its contribution to the ileal efflux of 4MUG is limited because of involvement of other efflux transporters.

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References


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