Function and Expression of Multidrug Resistance-Associated Protein Family in Human Colon Adenocarcinoma Cells (Caco-2)\textsuperscript{1}

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Accepted for publication September 15, 1999 This paper is available online at http://www.jpet.org

ABSTRACT

Several organic anions are actively extruded from intestinal epithelial cells into the lumen and vascular sides. To examine the role of the multidrug resistance-associated protein (MRP) family in the intestinal efflux of organic anions, the function and expression of these proteins were investigated with Caco-2, a human adenocarcinoma cell line that retains many of the characteristics of normal enterocytes. \textsuperscript{1}[\textsuperscript{3}H]2,4-Dinitrophenyl-S-glutathione (DNP-SG) and \textsuperscript{17}E[\textsuperscript{3}H]17\beta-estradiol 17\beta-D-glucuronide (E\textsubscript{2}17\betaG), typical substrates for MRP1 and cMOAT (canalicular multispecific organic anion transporter)/MRP2, were taken up into brush-border membrane vesicles (BBMVs) from Caco-2 in an ATP-dependent manner, with $K_m$ values of 16.9 $\pm$ 7.2 and 9.4 $\pm$ 1.2 $\mu$M, respectively. The uptake of \textsuperscript{1}[\textsuperscript{3}H]DNP-SG into BBMVs was osmotically sensitive and stimulated to some extent by other nucleotide triphosphates (GTP, CTP, and UTP) but not by ADP or AMP. An ATPase inhibitor, vanadate, inhibited the ATP-dependent uptake of \textsuperscript{1}[\textsuperscript{3}H]DNP-SG to some extent. Reverse-transcriptase polymerase chain reaction resulted in the amplification of MRP1, MRP3, and MRP5. Northern blot analysis indicated extensive expression of cMOAT/MRP2 and MRP3 and only minimal expression of MRP1 and MRP5. Although cMOAT/MRP2 was continuously expressed throughout the culture period, MRP3 was not expressed immediately after the confluent state was reached. Collectively, the presence of ATP-dependent transport systems for DNP-SG and E\textsubscript{2}17\betaG was demonstrated in Caco-2 cells. Because cMOAT/ cMOAT/MRP2 and MRP3 may be expressed on brush-border and basolateral membranes in epithelial cells, respectively, the transport activity associated with BBMVs may result from the function of cMOAT/MRP2.

The intestinal mucosa is directly exposed to xenobiotics. To prevent the invasion of xenobiotics, intestinal epithelial cells are equipped with several enzymes such as cytochrome P-450 (Wacher et al., 1998), UDP-glucuronosyltransferase (Vargas and Franklin, 1997), and glutathione $\gamma$-transferase (Gibbs et al., 1998). In addition to such metabolic enzymes, it has been established that P-glycoprotein is located on the brush-border membrane of enterocytes to prevent the entry of xenobiotics (Tsuiji and Tamai, 1996; Wacher et al., 1998). However, the excretion of xenobiotics from intestinal epithelial cells cannot be completely accounted for by P-glycoprotein. It has been established that, in the perfused rat small intestine, there is intestinal excretion of the glucuronide conjugates of 1-naphthol (de Vries et al., 1989), 4-methylumbelliferone (4-MU) (Mulder et al., 1984), and ethynylestradiol (Schwenk et al., 1982) formed in the intestinal epithelium. In addition to these conjugated metabolites, calcine is also excreted into the jejunum mucosa in a concentration- and energy-dependent manner without any metabolic conversion (Fujita et al., 1997). Because P-glycoprotein primarily accepts amphipathic cationic and neutral compounds as substrates (Kusuhara et al., 1998), the presence of transporters other than P-glycoprotein responsible for the extrusion of anionic compounds has been postulated. Concerning the cellular extrusion of organic anions, it has been shown that the MRP family plays an important role (Deeley and Cole, 1997; Ishikawa et al., 1997; Keppler and König, 1997). In particular, the role of cMOAT/ cMOAT/MRP2 has been confirmed in the biliary excretion of organic anions by comparing transport across the bile canalicular membrane of normal rats and mutant rats whose cMOAT/ cMOAT/MRP2 function is hereditarily defective [e.g., transport-deficient (TR\textsuperscript{c}) rats and Eisai hyperbilirubinemic rats (EHB\textsuperscript{c})] (Oude Elferink et al., 1995; Kusuhara et al., 1998; Suzuki and Sugiyama, 1996; Higashi et al., 1998; Fujita et al., 1997). In addition, MRP1, MRP3, and MRP5. The expression and function of cMOAT/ cMOAT/MRP2 was demonstrated in Caco-2 cells. Because cMOAT/ cMOAT/MRP2 and MRP3 may be expressed on brush-border and basolateral membranes in epithelial cells, respectively, the transport activity associated with BBMVs may result from the function of cMOAT/ cMOAT/MRP2.

ABBREVIATIONS: BBMVs, brush-border membrane vesicles; ABC, ATP-binding cassette; MRP, multidrug resistance-associated protein; cMOAT, canalicular multispecific organic anion transporter; EHB, Eisai hyperbilirubinemic rat; DNP-SG, 2,4-dinitrophenyl-S-glutathione; E\textsubscript{2}17\betaG, 17\beta-estradiol 17\beta-D-glucuronide; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; 4-MU, 4-methylumbelliferone.
1998). Such comparison, along with the functional analysis of cloned cMOAT/ MRP2 product (Evers et al., 1998; Ito et al., 1998; Kinosita et al., 1998; König et al., 1998; van Aubel et al., 1998), has revealed that the substrates for cMOAT/ MRP2 include glutathione conjugates [e.g., leukotriene C4, 2,4-dinitrophenyl-S-glutathione (DNPSG), and glutathione disulfide], glucuronide conjugates [e.g., 17β-estradiol 17(β-glucuronide) (E217βG)], bilirubin glucuronides, and glucuronide conjugates of xenobiotics [e.g., 6-hydroxy-5,7-dimethyl-2-methylamino-4-(3-pyridylmethyl)benzothiazole (E3040) glucuronide and SN-38 glucuronide], and nonconjugated organic anions (e.g., methotrexate, pravastatin, and carbonylfoxides of CPT-11 and its active metabolite) (Oude Elferink et al., 1995; Keppler and König, 1997; Kusuhara et al., 1998; Suzuki and Sugiyama, 1998), and its substrates are similar to those of Mrp1. Moreover, Northern blot analysis has indicated the expression of cMOAT/MRP2 in tissues from small intestine (Pau1usma et al., 1996; Ito et al., 1997; Kool et al., 1997). In addition to cMOAT/MRP2, we recently found that rat mrp3 can extrude organic anions (Hirohashi et al., 1999). Although mrp3 has been cloned as an inducible transporter in rat liver, it is extensively expressed in the small intestine and colon of rat and human (Kool et al., 1997; Hirohashi et al., 1998; Kiuchi et al., 1998). It has been shown that mrp3 accepts glucuronide conjugates (e.g., E217βG and E3040 glucuronide) and nonconjugated organic anions (e.g., methotrexate) as suitable substrates. The transport properties of mrp3, however, are different from those of Mrp1 and cMOAT/MRP2 in that glutathione conjugates are poor substrates for mrp3 (Hirohashi et al., 1999).

In this study, we examined the possibility that the MRP family may be responsible for the intestinal extrusion of organic anions. As an in vitro model, we used Caco-2, a human colon adenocarcinoma cell line, that retains many of the characteristics of normal enterocytes (Meunier et al., 1995). We examined the ATP-dependent transport of DNPSG and E217βG, typical substrates for the MRP family, into the brush-border membrane vesicles (BBMVs) from Caco-2 cells. The membrane vesicles from Caco-2 cells are particularly useful because 1) it is impossible to obtain inside-out membrane vesicles from the small intestine of experimental animals (Hsing et al., 1992), and 2) Caco-2 cells retain the ability to excrete DNPSG (Oude Elferink et al., 1993).

### Experimental Procedures

**Materials.** Unlabeled and [3H]DNP-SG (50 µCi/nmol) were synthesized enzymatically with glycine-2-[3H]glutathione (NEN Life Science Products, Boston, MA), 1-chloro-2,4-dinitrobenzene, and glutathione S-transferase (Sigma Chemical Co., St. Louis, MO) as described previously (Kobayashi et al., 1990). Unlabeled and [3H]E217βG (51.0 µCi/nmol) were purchased from Sigma and NEN Life Science Products, respectively. ATP, ADP, AMP, GTP, CTP, UTP, creatine phosphate, creatine phosphokinase, sodium orthovanadate, and acivicin were purchased from Sigma. All other chemicals used were commercially available and of reagent grade.

**Cell Culture.** Caco-2 cells (passage number 46–55) were cultured in Dulbecco’s modified Eagle’s medium (25 mM glucose) supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 2 mM L-glutamine, penicillin (100 IU/ml), streptomycin (100 µg/ml), and amphotericin B (250 µg/ml) in a humidified incubator (5% CO₂, 37°C). For P-glycoprotein-mediated transport studies, Caco-2 cells with several passage numbers have been used previously: 30 to 50 passages (Hosoya et al., 1996) and 85 to 105 passages (Hunter et al., 1993). In each passage, P-glycoprotein was functionally expressed.

**Preparation of BBMVs.** For the preparation of BBMVs, Caco-2 cells were seeded in 20 incubation flasks (175 cm²) for 5 days after a confluent state was reached. BBMVs were isolated by the CaCl₂ precipitation method as described previously (Muranushi et al., 1994) and then frozen in liquid nitrogen and stored at −100°C until required. Protein concentrations were determined by the Lowry method. To determine the enrichment and orientation of BBMVs, the activity of alkaline phosphatase, a marker enzyme for BBMVs, was measured in the presence and absence of 0.1% Triton X-100 as described previously (Ninuma et al., 1997). Briefly, after the incubation of BBMVs (1 µg protein) in the presence or absence of 0.1% Triton X-100 at room temperature for 30 min, BBMVs were incubated in 1 ml of the mixture containing 5 mM p-nitrophenyl phosphate, 5 mM MgCl₂, 50 mM 2-amino-2-methylpropanol-HCl (pH 10.0) for 30 min. The reaction was terminated by the addition of 1 ml 10% trichloroacetic acid. The denatured protein was removed by centrifugation, and the clear supernatant (1 ml) was neutralized by addition of 2 ml of 1 N NaOH, and the amount of p-nitrophenol released was determined colorimetrically (A₄₀₅).

**Uptake of Ligands by BBMVs.** The transport study was performed with the rapid-filtration technique (Ito et al., 1998). Transport medium (250 mM mannitol, 10 mM MgCl₂, and 10 mM Tris-HCl, pH 7.4) containing the radiolabeled ligands (15 µl), with or without unlabeled ligands, was preincubated at 37°C for 3 min and then mixed with 5 µl membrane vesicle suspension (20 µg protein) with ATP (or AMP) and ATP-regenerating system (10 mM creatine phosphate and 100 µg/ml creatine phosphokinase). To avoid the degradation of DNPSG by γ-glutamyltranspeptidase (γ-GTP), BBMVs were incubated in 1 mM a-acivicin, an inhibitor of γ-GTP, for 30 min at 25°C before starting the uptake of [3H]DNPSG. In some instances, ATP was replaced by AMP, ADP, GTP, UTP, or CTP. The effect of vanadate (100 µM) on ATP-dependent transport without ATP-regenerating system was also examined. The transport reaction was stopped by the addition of 1 ml ice-cold stop solution containing 250 mM mannitol, 0.1 M NaCl, and 10 mM Tris-HCl, pH 7.4. The stopped reaction mixture was filtered through a 0.45-µm mixed cellulose ester filter (Millipore Corp., Bedford, MA) and then washed twice with 5 ml of stop solution. Radioactivity retained on the filter was determined with a liquid scintillation counter (LSC-3500; Aloka Co., Tokyo, Japan). The ATP-dependent uptake of ligands was calculated by subtracting the ligand uptake in the absence of ATP from that in the presence of ATP.

**Amplification of cDNA Fragments.** Total RNA was prepared by a single-step guanidium thiocyanate procedure. Subsequently, poly(A)⁺ RNA was purified with oligotex-dT30 (Takara Shuzo, Kyoto, Japan). The cDNA fragments encoding the COOH-terminal ABC region of MRP1 (424 bp), MRP3 (422 bp), and MRP5 (423 bp) were amplified from Caco-2 cell polyA⁺ RNA by reverse-transcription polymerase chain reaction (RT-PCR) with degenerate PCR primers as described previously (Ito et al., 1997). The sequences of the forward and reverse primers were 5’-dGAGAAGTCGGCATCGTGGGAGTCG/CGAGTCAGCG/GG-3’ and 5’-dGTCCAG-GTCTGA(GT)GTAGTCGCT/TC/TAGC/TC-3’, respectively. The cDNA fragment encoding the COOH-terminal ABC region of cMOAT/MRP2 (603 bp) was amplified with a rigid primer for human cMOAT/MRP2 as a reverse primer (5’-dAAAAAGTCGACCGGATTT- GTAGACGTTGCTTC-3’). RT was performed with random primer at 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 5°C for 5 min. Then PCR was carried out at 94°C for 30 s, 37°C for 30 s and 72°C for 1 min for 40 cycles with Taq polymerase. The amplified PCR products were subcloned into the EcoR V site of pBluescript II SK(−) (Stratagene, La Jolla, CA), and the sequence was determined. PCR products were excised from the vector by digestion with EcoRI and HindIII and were used as probes to detect their expression.

**Northern Blot Analysis.** Poly(A)⁺ RNA was separated on 0.7% agarose gel containing 3.7% formaldehyde and transferred to a nylon
membrane (Biodyne; Pall Co., Glen Cove, NY), before fixation by baking for 2 h at 80°C. The membranes were prehybridized in hybridization buffer containing 4 times standard saline citrate (SSC), five times Denhardt’s solution, 0.2% SDS, 0.1 mg/ml of sonicated salmon sperm DNA, and 50% formamide for 2 h at 42°C and hybridized for 10 h at 42°C in the same buffer with a 32P-labeled cDNA probe that was prepared by a random primed labeling method (Replig, Amersham Pharmacia Biotech, Uppsala, Sweden). The hybridized membrane was washed in 2 SSC and 0.1% SDS at room temperature for 20 min, followed by washing in 2 times SSC and 0.1% SDS at 55°C for 20 min and then in 0.1 times SSC and 0.1% SDS at 55°C for 20 min. The filters were exposed to an imaging plate, which was followed by analysis with a Fujix BAS 2000 image analyzer (Fuji Photo Film, Co., Ltd., Tokyo, Japan).

Results

Uptake of [3H]DNP-SG and [3H]E217βG into BBMVs from Caco-2 Cells. The enrichment of alkaline phosphatase in BBMVs from 5-day-old confluent Caco-2 cells was 8.94 ± 1.92-fold. Determination of alkaline phosphatase in the presence and absence of 0.1% Triton X-100 revealed that 27.3 ± 3.6% of the membrane vesicles were inside out.

Figure 1 shows the time profiles for the uptake of [3H]DNP-SG and [3H]E217βG into BBMVs in the presence or absence of ATP. ATP significantly stimulates the uptake of both ligands (Fig. 1). In accordance with the reproducibility in the enrichment of alkaline phosphatase and inside-out membrane fractions, variation in the uptake of [3H]DNP-SG was minimal; uptake of [3H]DNP-SG at 10 min was 4.46 ± 0.45 ml · min⁻¹ · mg protein⁻¹.

To determine the kinetic parameters, saturation of the uptake of DNP-SG and E217βG was determined (Fig. 2). Nonlinear regression analysis showed that the Kₘ and Vₘₐₓ for the ATP-dependent uptake of DNP-SG by BBMVs were 16.9 ± 7.2 μM and 5.0 ± 1.3 pmol · min⁻¹ · mg protein⁻¹, respectively (Fig. 2A). The transport of E217βG was also saturable and could be described by a Kₘ of 9.4 ± 1.1 μM and Vₘₐₓ of 6.5 ± 0.6 pmol · min⁻¹ · mg protein⁻¹ (Fig. 2B).

Osmotic Sensitivity and Nucleotide Specificity of [3H]DNP-SG Uptake into BBMVs from Caco-2 Cells. To confirm that the vesicle-associated uptake of [3H]DNP-SG reflects transport into a vesicular space rather than binding to the vesicle surface, the uptake of [3H]DNP-SG was measured in the presence of several concentrations of sucrose in the transport medium. The uptake of [3H]DNP-SG into BBMVs was reduced by increasing the sucrose concentration in the medium (Fig. 3). The y-intercept for the relationship between the amount of DNP-SG associated with the vesicles and the reciprocal of the sucrose concentration in the medium was 0.73 μl/mg of protein, indicating that the binding of DNP-SG to the surface of vesicles was less than 15% when the transport experiment was performed in isotonic medium (Fig. 3).

The nucleotide specificity for the uptake of [3H]DNP-SG into BBMVs was examined by replacing ATP by other nucleotides (Table 1). GTP, CTP, and UTP weakly stimulated the uptake of [3H]DNP-SG into BBMVs, whereas ADP or AMP had no effect on the uptake of DNP-SG (Table 1). The ATP-dependent uptake of [3H]DNP-SG was reduced to approximately 40% in the presence of 100 μM vanadate in the uptake medium (Table 1).

Northern Blot Analysis of the MRP Family in Caco-2 Cells. To identify the MRP family of molecules expressed in Caco-2 cells, RT-PCR was performed with the degenerated primers designed for the highly conserved COOH-terminal ABC region of human MRPs. Three MRP members (MRP1, 424 bp; MRP3, 422 bp; and MRP5, 423 bp) were amplified. The expression of MRP3 was markedly increased in 5-, 7-, 10-, and 16-day-old confluent Caco-2 cells. MRP5 was also highly expressed throughout the culture period, whereas the expression of MRP1 and MRP5 was minimal throughout this period (Fig. 4). The expression of MRP3 was markedly increased in 5-, 7-, 10-, and 16-day-old confluent cells compared with cells immediately after becoming confluent.

Discussion

It has been established that organic anions are excreted from small intestinal epithelial cells via specific mechanisms (Schwenk et al., 1982; Mulder et al., 1984; de Vries et al., 1989; Fujita et al., 1997). However, little is known about the molecular mechanism by which intestinal epithelial cells secrete these organic anions. Because MRP family proteins, such as MRP1 and cMOAT/MRP2, are well known to accept many organic anions including glutathione and glucuronide conjugates as substrates (Oude Elferink et al., 1995; Kusuhara et al., 1998; Suzuki and Sugiyama, 1998), we hypothesized that the MRPs that are expressed in the intestine are involved in the extrusion of these organic anions.

In this study, the transport of organic anions was examined in BBMVs from Caco-2 cells, which are equipped with the characteristics of normal enterocytes (Meunier et al., 1995). In our preliminary experiments, we had difficulty in demonstrating the ATP-dependent uptake of [3H]DNP-SG or [3H]E217βG into rat intestinal BBMVs (T.H., H.S., and Y.S., unpublished observations) because these membrane vesicles...
are mostly composed of right-side-out vesicles (Hsing et al., 1992). Because 30% of BBMVs from Caco-2 cells used in this study consisted of inside-out membrane vesicles (see Results), BBMVs from this cultured cell line are useful for the study of active efflux transporters. Although Oude Elferink et al. (1993) found the energy-dependent efflux of DNP-SG across the apical and basal membrane of Caco-2 cells after preloading its precursor (1-chloro-2,4-dinitrobenzene), little is known about the molecular mechanism by which intestinal epithelial cells secrete organic anions.

As shown in Fig. 1, [3H]DNP-SG and [3H]E217βG were taken up into BBMVs from Caco-2 cells in an ATP-dependent manner. No overshoot, however, was observed for the uptake of these ligands up to 45 and 15 min, respectively (Fig. 1). The absence of overshoot for such a relatively long incubation time has also been observed commonly in membrane vesicles from several cultured cell lines (e.g., MRP1-overexpressing tumor cells or MRP1-transfected cells; Loe et al., 1996). These results are in contrast to the presence of marked overshoot for the ATP-dependent uptake of [3H]DNP-SG into isolated canalicular membrane vesicles (CMVs) prepared from Sprague-Dawley rats (Niinuma et al., 1997). The difference may be because the ATP consumption is much less in membrane vesicles from cultured cells than in CMVs. Indeed, in the transport studies with CMVs, we have found rapid consumption of ATP in the medium, resulting from the high ATPase activity associated with these membrane vesicles (Watanabe et al., 1995).

The ATP-dependent uptake of [3H]DNP-SG was osmotically sensitive (Fig. 3), suggesting that the major part of the uptake of [3H]DNP-SG is actually due to uptake and not adsorption to the surface of BBMVs. As shown in Table 1,

![Fig. 2](image-url)

**Fig. 2.** Concentration dependence for the ATP-dependent uptake of [3H]DNP-SG (A) and [3H]E217βG (B) by BBMVs from Caco-2 cells. BBMVs (20 μg protein) were incubated with different concentrations of [3H]DNP-SG for 10 min (A) or [3H]E217βG for 5 min (B) at 37°C. Before starting the uptake of [3H]DNP-SG, BBMVs were preincubated with 1 mM acivicin at 25°C for 30 min (A). ○ and ■ represent the ATP-dependent uptake obtained by subtracting the uptake in the absence of 5 mM ATP from that in its presence. Each point and vertical bar represents the mean ± S.E. of three determinations.

![Fig. 3](image-url)

**Fig. 3.** Osmotic sensitivity of [3H]DNP-SG uptake into BBMVs. BBMVs (20 μg of protein) were incubated at 25°C for 30 min with 1 mM acivicin and different concentrations of sucrose (0.25, 0.33, 0.50, 0.66, and 1.0 M). Then, vesicle suspensions were incubated with [3H]DNP-SG at 37°C for 10 min in the presence of ATP (○) or AMP (■). Each point and vertical bar represents the mean ± S.E. of three determinations.

### Table 1

| Nucleotide Specificity for the Uptake of [3H]DNP-SG into BBMV from Caco-2 Cells |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| Nucleotide | Uptake μl/mg protein | Relative Uptake |
| ATP | 5.94 ± 0.41 | 100 |
| ATP + vanadate* | 3.20 ± 0.16 |
| ATP | 4.35 ± 0.19 | 100 |
| ATP + vanadate* | 2.70 ± 0.38 | 62 |
| ADP | 1.24 ± 0.43 | 28 |
| AMP | 1.32 ± 0.10 | 30 |
| GTP | 2.17 ± 0.19 | 50 |
| CTP | 2.39 ± 0.37 | 55 |
| UTP | 2.16 ± 0.15 | 50 |
| None | 1.48 ± 0.31 | 34 |

* The concentration of vanadate in the incubation medium was 100 μM.
and antiluminal (blood) sides (absorption) across the plasma membranes, respectively. Indeed, the role of cMOAT/MRP2 in the small intestinal excretion of DNP-SG has been demonstrated by comparing the behavior between Sprague-Dawley rats and EHBRs in vivo and in vitro experiments (Ussing chamber and everted sacs studies; Goto et al., in press).

The direction of the efflux can be studied further in intact Caco-2 monolayers. Our preliminary experiments indicated that glutathione-bimane, a substrate for cMOAT/MRP2 but not for MRP3, is excreted predominantly in the apical direction after preloading Caco-2 cells with its precursor (monochlorobimane). In contrast, 4-MU glucuronide was excreted almost equally to the apical and basal sides after preloading Caco-2 monolayers with its precursor (4-MU) (A. Seta, H.S., and S.Y., unpublished observations). Because 4-MU glucuronide may be recognized by both cMOAT/MRP2 and MRP3, these data can be accounted for by assuming that both the apical and basal efflux of 4-MU glucuronide is mediated by cMOAT/MRP2 and MRP3, respectively.

The results of the kinetic analysis of the transport studies also suggest the expression of cMOAT/MRP2 in BBMVs. The $K_m$ value for DNP-SG and $E_{17\beta G}$ were determined to be 16.9 ± 7.2 and 9.4 ± 1.1 $\mu$M, respectively (Fig. 2). These values are in good agreement with the previously determined $K_m$ values; with membrane vesicles isolated from Hek 293 cells transfected with human cMOAT cDNA, the $K_m$ value for $E_{17\beta G}$ was determined to be 7.2 ± 0.7 $\mu$M (König et al., 1998). In the same manner, the $K_m$ for DNP-SG was determined to be 5.6 $\mu$M in membrane vesicles from MDCK cells transfected with the same cDNA (Evers et al., 1998). In contrast, the transport characteristics of rat mrp3 are different from those of MRP1 and CMOAT/MRP2 in that the glucuronide conjugates, but not glutathione conjugates, are good substrates for MRP3 (Hirohashi et al., 1999). Moreover, the $K_m$ value for $E_{17\beta G}$ by mrp3 was 110 and 67 $\mu$M in mrp3-transfected LLC-PK1 and HeLa cells, respectively (Hirohashi et al., 1999), which is much higher than that reported for cMOAT/MRP2 (König et al., 1998). Considering these results, it is possible that the ATP-dependent uptake observed in BBMVs from Caco-2 cells is mainly mediated by cMOAT/MRP2 rather than MRP3.

In culture, Caco-2 differentiated into polarized cell monolayers with the characteristics of intestinal epithelial cells. Because some reports have been published showing that the function and expression of P-glycoprotein is increased to some extent during culture (Hosoya et al., 1996), the expression of MRPs was determined as a function of the culture period in this study (Fig. 4). Moreover, we normalized the expression levels of cMOAT/MRP2 and MRP3 by GAPDH with a densitometer. The relative expression level of these transporters, obtained by dividing by that of GAPDH, is summarized in Table 2. Although the relative expression of cMOAT/MRP2 increased in 5-day-confluent Caco-2 cells (2-fold) compared with that in 0-day-confluent cells, it was not significantly different from the cells at 7 (1.9-fold) and 10 (1.8-fold) days after becoming confluent (Table 2). However, a reduction in the relative expression level of cMOAT/MRP2 was observed in 16-day-confluent cells (only 1.2-fold higher than that of 0-day-confluent cells) (Table 2). The relative expression level of MRP3 markedly increased during the first 5 days after becoming confluent (5.8-fold) and exhibited

![Fig. 4. Northern blot analysis of MRP1, cMOAT/MRP2, MRP3, and MRP5 in Caco-2 cells. PolyA⁺ RNA was prepared from Caco-2 cells that were cultured for 0, 5, 7, 10, and 16 days after confluence was reached. Five micrograms of polyA⁺ RNA was loaded in each lane. The filters, hybridized with 32P-labeled cDNA fragments of MRP1 (424 bp), cMOAT/MRP2 (603 bp), MRP3 (422 bp), and MRP5 (423 bp), had an 8-h exposure to an imaging plate followed by analysis with a Fujix BAS 2000 image analyzer. Rehybridization was performed with 32P-labeled GAPDH cDNA as a loading control, and the filters had a 2-h exposure in the same way.](image-url)
small changes in 7- (6.2-fold), 10- (10-fold), and 16-day (4.8-fold) cultures. Because the purpose of this study was to demonstrate the presence of primary active transporter(s) for organic anions in Caco-2 cells, we have isolated BBMVs from Caco-2 cells at 5 days confluent, when the expression level of MRP family is greatest. Currently, we cannot exclude the possibility that the transport properties of the MRP family are different depending on the culture days.

In conclusion, we have demonstrated the presence of ATP-dependent transporters in Caco-2 cells by use of BBMVs. Because both MRPs and MRP3 are also expressed in Caco-2 and in human intestinal tissues, membrane vesicles from Caco-2 cells represent a good tool for investigating the functions of MRPs as in vitro models of the human intestine.

References


