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Research Paper

Treatment of Hyperbilirubinemia in Eisai Hyperbilirubinemic Rat by Transfecting Human MRP2/ABCC2 Gene

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Purpose. Multidrug resistance–associated protein 2 (MRP2/ABCC2) is predominantly expressed in the liver canalicular membrane and plays an important role in the biliary excretion of organic anions including glucuronide and glutathione conjugates. The purpose of this study is to construct a new evaluation system for human MRP2 by expressing human MRP2 in Eisai hyperbilirubinemic rat (EHBR) liver, the rat Mrp2 function of which is hereditarily defective.
Methods. In order to express human MRP2 in liver, we used the Tet-off adenovirus expression system. After 72 h infection, we evaluated the protein expression and localization in the liver and the transport activity of [^{3}H]$E_2$17ßG and [^{3}H]DNP-SG by preparing canalicular membrane vesicles (CMVs). We also evaluated the biliary excretion and plasma concentration of DBSP after bolus administration and the plasma concentration of endogenous direct and indirect bilirubin.
Results. The localization of human MRP2 in EHBR liver was found to be at the bile canalicular membrane. Clear ATP-dependent uptake of [^{3}H]$E_2$17ßG and [^{3}H]DNP-SG into CMVs was observed by using the CMVs prepared from the liver where human MRP2 was transfected. Furthermore, the blood to bile clearance of DBSP increased approximately 3-fold after expression of human MRP2. In addition, the plasma direct bilirubin level in EHBR was reduced by the expression of human MRP2.
Conclusion. These results suggest that this evaluation system for human MRP2 may be useful for evaluating the function of human MRP2.

KEY WORDS: biliary excretion; EHBR; liver; MRP2.

INTRODUCTION

Multidrug resistance–associated protein 2 (MRP2/ABCC2) is a glycosylated integral plasma membrane protein belonging to the ATP-binding cassette transporter family. Its function has been studied extensively by comparing the transport across the bile canalicular membrane between normal and Mrp2-deficient mutant rats such as Eisai hyperbilirubinemic rats (EHBRs). Now, it is established that Mrp2 is mainly located on the bile canalicular membrane and the apical membrane of enterocytes and plays a primary role in biliary excretion and in restricting the oral absorption of its substrates, including anionic drugs and glutathione and glucuronide conjugates of xenobiotics (1–7). It has also been established that mutations in the MRP2 gene are responsible for the pathogenesis of Dubin-Johnson syndrome (DJS), characterized by a defect in the biliary excretion of bilirubin glucuronides (7–9). We have recently focused on the SNPs variants/mutants of MRP2, because, due to the fact that some MRP2 mutations found in DJS patients are associated with a loss of transport activity and/or the ability to traffick to the apical membrane by the single amino acid alteration (10,11), it is possible that the MRP2 SNPs may also be associated with alterations in function and, consequently, may be responsible for the inter-individual differences in the disposition of substrate drugs. Previously, we have characterized the function of SNPs variants/mutants of MRP2 using LLC-PK1 cells (12). Although the wild-type MRP2 was exclusively localized at the apical membrane, S789F and A1450T MRP2 were located not only at the apical membrane but also in the intracellular compartment, suggesting that the *in vivo* function of these two kinds of variants may be lower than wild type MRP2 (12).

However, there are some reports which suggest that the localization of some membrane proteins in cultured cells does not necessarily reflect the localization in tissues. For example, rat Oat-K1 is localized at the apical membrane in the proximal tubules (13), whereas this transporter localizes at the basolateral membrane in LLC-PK1 cells, derived from porcine kidney (14). Moreover, rat serotonin transporter and norepinephrine transporter are known to be sorted to the apical compartment (axolemmal domain) of neuronal cells, whereas these transporters are sorted to the basolateral membrane in MDCK cells, derived from canine kidney (15). One

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ABBREVIATIONS: Ad, adenovirus; CMV, canalicular membrane vesicle; DBSP, dibromosulfophtalein; DJS, Dubin-Johnson syndrome; DNP-SG, 2,4-dinitrophenyl-*S* glutathione; $E_2$17ßG, 17β-estradiol 17β-D-glucuronide; EHBR, Eisai hyperbilirubinemic rats; MRP, multidrug resistance–associated protein; pfu, plaque-forming unit; SNPs, single nucleotide polymorphisms; TRE, tet-responsive element; tTA, tetracycline-responsive transcriptional activator.

of the hypotheses for these alterations of the cellular localization is that some proteins which are necessary for the apical targeting under physiologic conditions are deficient in cultured cells. Concerning this point, it is essential to determine the localization of SNPs variants/mutants of MRP2 in human liver, although such determination is quite difficult. Therefore, we have planned to construct an *in vitro* evaluation system which may reflect the cellular localization under physiologic conditions. Thus, we have focused on using the Eisai hyperbilirubinemic rat (EHBR), as the mutation in the Mrp2 gene leads to the absence of Mrp2 protein in this mutant strain (16,17).

The purpose of this study is to establish a new system for evaluating the function of human MRP2 by expressing this transporter on the canalicular membrane of EHBR liver. To express human MRP2 in EHBR liver, we used Tet-off recombinant adenovirus systems. The expression level, localization and the transport function of human MRP2 in EHBR liver were analyzed.

MATERIALS AND METHODS

Materials

[^{3}H]$E_2$17ßG (51.0 μCi/nmol) with a purity of 98.5% was purchased from New England Nuclear (Boston, MA, USA). Unlabeled and [^{3}H]labeled 2,4-dinitrophenyl-*S*-glutathione ([^{3}H]DNP-SG, 44.8 μCi/nmol) were synthesized as described previously (18). ATP, creatine phosphate, creatine phosphokinase, acivicin, and glutathione *S*-transferase were purchased from Sigma Chemical (St. Louis, MO, USA). All other chemicals used were commercially available and of reagent grade. Sprague-Dawley rats (normal rats) and EHBR weighing approximately 180 g were used throughout the experiments.

Construction of Recombinant Adenoviruses Containing MRP2

Human MRP2 cDNA located between the Not I and Nco I sites of pBluescript SK(-) (Stratagene, La Jolla, CA, USA) was provided by Dr. Kuwano (Kyushu University School of Medicine). The MRP2 cDNA was subcloned into the Not I and Nco I sites of the pTRE shuttle, resulting in the production of pTRE shuttle-MRP2. pTRE shuttle-MRP2 has I-CeuI and PI-SceI sites upstream and downstream of the MRP2 expression cassette, respectively. The I-CeuI / PI-SceI digested fragments of pTRE shuttle-MRP2 were ligated with I-CeuI / PI-SceI digested Adeno-X Viral DNA (BD Biosciences, Palo Alto, CA, USA), resulting in pAd-MRP2. pAd containing tetracycline-responsive transcriptional activator (tTA) (pAd-tTA) was purchased from Clontech (Palo Alto, CA, USA).

To generate the recombinant viruses, both pAd-MRP2 and pAd-tTA were digested with PacI. Linearized DNAs were transfected to HEK293 cells grown on a 12-well dish with Fugene 6 (Roche Diagnostics Corp., Tokyo, Japan) according to the manufacturer's instructions. Viruses (Ad-MRP2 and Ad-tTA) were prepared as described previously (19). Recombinant viruses were purified by CsCl gradient centrifugation, dialyzed with a solution containing 10 mM Tris (pH 7.5), 1 mM $MgCl_2$, and 10% glycerol, and stored in aliquots at −80°C. Then, the resulting virus titer was determined as described previously (20).

Infection of Recombinant Adenovirus and the Preparation of Canalicular Membrane Vesicles

The adenoviruses were injected to EHBR via the tail vein. EHBR were infected with both Ad-MRP2 and Ad-tTA to express human MRP2 (MRP2-EHBR). For the negative control, EHBR were infected only with Ad-tTA (control EHBR). CMVs were prepared from EHBR liver 72 h after adenovirus infection as described previously (18) and kept as a suspension in 50 mM Tris buffer (pH 7.4) containing 250 mM sucrose. The membrane vesicles were frozen in liquid N_2 and stored at −80°C until required. The activity of the CMVs was checked by measuring the ATP-dependent uptake of standard substrates, [^{3}H]$E_2$17ßG (400 nM) and [^{3}H]DNP-SG (1 μM) for a 5-min incubation at 37°C. Protein concentrations were determined by the Lowry method.

Immunoblots

For the Western blot analysis, CMVs were dissolved in 3x SDS sample buffer (New England BioLabs, Beverly, MA, USA) and subjected to electrophoresis on a 7% SDS-polyacrylamide gel with a 4.4% stacking gel. The molecular weight was determined using a prestained protein marker (New England BioLabs). Proteins were transferred electrophoretically to a nitrocellulose membrane (Millipore, Bedford, MA, USA) using a blotter (Bio-Rad Laboratories, Richmond, CA, USA) at 15 V for 1 h The membrane was blocked with 2.5% skimmed milk for 1 h at room temperature. Then, the membrane was incubated for 40 min at room temperature with anti-MRP2 rabbit serum, diluted with skimmed milk (1:500), which was raised against 12 amino acids at the carboxyl terminus of the deduced MRP2 sequence (EAGIENVNSTKF). For the detection of MRP2, the membrane was allowed to bind to Alexa Fluor 680 goat anti-rabbit IgG (H+L) (Molecular Probes, Inc., Eugene, OR, USA). The fluorescence was assessed in a densitometer (Odyssey, ALOKA, Tokyo, Japan).

Immunofluorescence

For the immunofluorescence, liver from MRP2-EHBR and control-EHBR was removed 72 h after infection. Cryostat sections (5 μm in thickness) were fixed in methanol at −20°C for 10 min, washed three times with PBS, and blocked with 1% BSA/PBS at room temperature for 15 min. Then, slices were incubated with the monoclonal antibody against MRP2 (M2-6 (Molecular Probes Inc., Eugene, OR, USA), diluted 40-fold in PBS) for 1 h at room temperature. Following this, slices were washed three times with PBS and incubated with goat anti-mouse IgG Alexa 488 (Molecular Probes, Inc.) diluted 250-fold in PBS for 1 h at room temperature, and mounted in VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA). The localization of the MRP2 protein was visualized by confocal laser microscopy (Zeiss LSM-510; Carl Zeiss Inc., Thornwood, NY, USA).

Vesicle Transport Assays

The uptake study of [^{3}H]$E_2$17ßG (400 nM) and [^{3}H]DNP-SG (1 μM) was performed as reported previously (21). Briefly, the transport medium (10 mM Tris, 250 mM sucrose and 10 mM $MgCl_2 \cdot 6H_2O$, pH 7.4) contained the ligands, 5 mM ATP, and an ATP-regenerating system (10 mM creatine phosphate and 100 μg/ml of creatine phosphokinase). An aliquot of transport medium (17–18 μl) was mixed rapidly with the vesicle suspension (5 μg protein in 2–3 μl). The transport reaction was stopped by the addition of 1 ml ice-cold buffer containing 250 mM sucrose, 0.1 M NaCl and 10 mM Tris-HCl (pH 7.4). The stopped reaction mixture was filtered through 0.45-μm HA filters (Millipore Corp.) and then washed twice with 5 ml of the stop solution. The radioactivity retained on the filter was measured in a liquid scintillation counter (LS 6000SE, Beckman Instruments, Fullerton, CA, USA) after the addition of scintillation cocktail (Clear-sol I, Nacarai Tesque, Tokyo, Japan). Ligand uptake was normalized in terms of the amount of membrane protein.

Blood and Bile Sampling

Sprague-Dawley rats (normal rats) and EHBR were kept under ether anesthesia and then the femoral artery and vein were cannulated with polyethylene tubing (PE-50) for ligand administration and blood sampling, respectively. The common bile duct was also cannulated with PE-10. DBSP (10 μmol/kg) in saline was administered intravenously through the femoral vein cannula. Blood samples were obtained at given times. The rectal temperature was maintained at 37°C ± 0.5°C with a heat lamp.

Analysis of Specimens Obtained from *in Vivo* Experiments

To determine the DBSP concentrations, plasma and bile specimens were diluted with 50 mM Tris-HCl buffer (pH 7.4) free from bovine serum albumin, and then made alkaline by the addition of NaOH. Concentrations of DBSP in the diluted specimens were determined in a dual-wave length spectrophotometer (Hitachi Ltd., Tokyo, Japan) at a wavelength of 575 nm/620 nm. Direct and indirect bilirubin concentrations of some specimens were also determined using a test kit (Bilirubin BII-Test Wako, Wako Pure Chemical Industries, Ltd., Tokyo, Japan). Bile volume was measured gravimetrically, assuming a density of 1.0 for bile specimens.

RESULTS

Expression Level of MRP2 Protein in EHBR Liver

To determine the appropriate amount of adenoviruses for the protein expression of human MRP2 in EHBR liver, 1×10^9, 7.5×10^9, 1×10^{10}, and 2.5×10^{10} plaque-forming units (pfu) of both Ad-MRP2 and Ad-tTA were injected to EHBR through the tail vein. Presumably due to the liver toxicity of the viruses, EHBR infected with 1×10^{10} and 2.5×10^{10} pfu died soon after injection. The expression level of human MRP2 in the canalicular membrane fraction isolated from EHBR liver was examined by Western blot analysis. It was found that the human MRP2 antibody recognized the band with an approximate molecular weight of 190 kDa (Fig. 1), which is consistent with a previous report (22). When infected with 7.5×10^9 pfu, the band density of MRP2 was approximately 5-fold higher compared with that in EHBR infected with 1×10^9 pfu (Fig. 1).

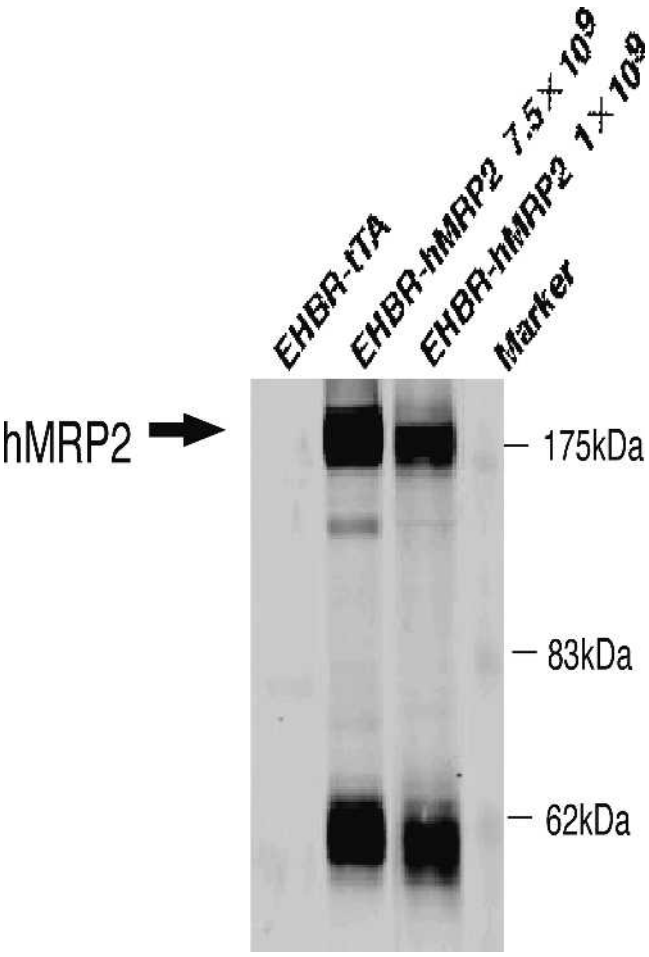

Fig. 1. Expression of human MRP2 in CMVs. Expression of the human MRP2 protein was determined in CMVs (5 μg) isolated from control-EHBR and MRP2-EHBR by Western blot analysis.

Localization of Human MRP2 in EHBR Liver

The localization of human MRP2 in EHBR liver was determined by immunofluorescence. Using an antibody against MRP2, the fluorescence signal was detected exclusively at the bile canalicular membrane of EHBR liver after simultaneous infection of Ad-tTA and Ad-MRP2 (Fig. 2). No

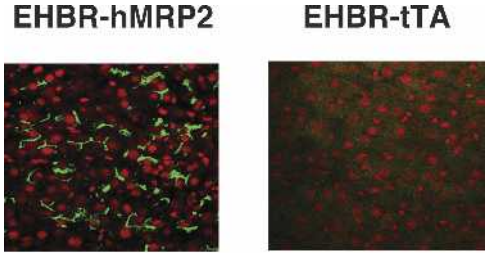

Fig. 2. Localization of human MRP2 in EHBR liver. The immunolocalization of MRP2 molecules in MRP2-EHBR was determined using a monoclonal antibody for human MRP2 (green). Nuclei were stained with PI (red).

specific fluorescence signal of MRP2 was observed in the EHBR liver after infection of only Ad-tTA (Fig. 2).

Transport Activity Associated with the Isolated CMVs

After confirming the protein expression level of MRP2, the uptake of MRP2 substrates was examined using thr isolated CMVs. Typical substrates of MRP2, [^{3}H]$E_2$17ßG and [^{3}H]DNP-SG, were clearly taken up into CMVs prepared from MRP2-EHBR in an ATP-dependent manner (Fig. 3). In particular, the ATP-dependent uptake of CMVs was higher when infected with 7.5×10^9 pfu than with 1.0×10^9 pfu, which is consistent with the result of the Western blot analysis (Fig. 1). Moreover, the extent of the uptake of [^{3}H]$E_2$17ßG and [^{3}H]DNP-SG was almost identical to that of the CMVs prepared from normal SD rats when infected with 7.5×10^9 pfu of Ad-MRP2 (Fig. 3).

Biliary Excretion of DBSP

The plasma disappearance of DBSP was compared between control-EHBR and MRP2-EHBR. Figure 4A shows that the plasma disappearance curves for DBSP after *i.v.* administration were similar for control-EHBR and MRP2-EHBR up to 21 min. In contrast, the maximum biliary excretion rate in MRP2-EHBR was approximately 3-fold higher than that in control-EHBR (Fig. 4B). The cumulative amount of DBSP excreted into the bile up to 2 h in MRP2-EHBR was greater than that in control-EHBR (Fig. 4C). The biliary excretion clearance value, defined by dividing the total amount excreted into bile by AUC of DBSP, in MRP2-EHBR in creased 3-fold compared with that in control-EHBR (Table I).

Bilirubin Concentration in Serum and Bile

The concentration of both direct and indirect bilirubin in the serum of normal rats, EHBR, control-EHBR, and MRP2-EHBR was also measured. The concentration of both direct and indirect bilirubin in the serum was significantly lower in MRP2-EHBR compared with control-EHBR (7.1 mg/ml vs. 97.4 mg/ml) (Table I). The decrease in direct bilirubin after human MRP2 expression was much greater than that in indirect bilirubin (from 97.4 mg/ml to 7.1 mg/ml, vs. 10.8 mg/ml to 4.9 mg/ml) (Table I), presumably because the direct bilirubin can be excreted into bile via human MRP2 (23). However, the concentration of direct bilirubin in control-EHBR plasma was much greater than that in untreated EHBR (97.4 mg/ml vs. 30.1 mg/ml) (Table I). In our preliminary results, infection of adenoviruses induced the expression of Mrp3, which is ex-

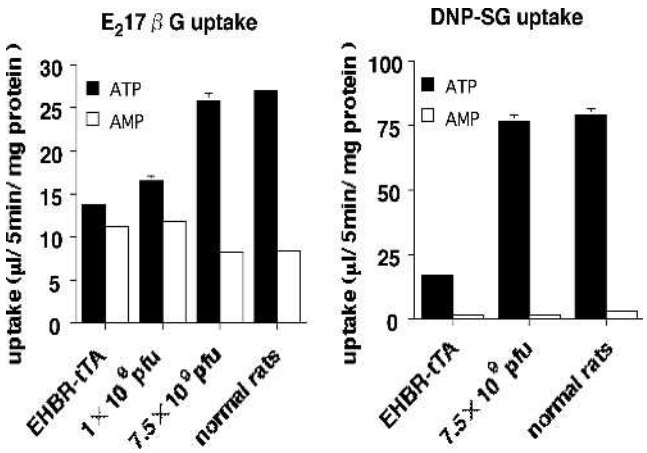

Fig. 3. Transport activity of human MRP2 in CMVs. [^{3}H]$E_2$17ßG and [^{3}H]DNP-SG uptake was measured in CMVs from MRP2-EHBR and control-EHBR. CMVs were incubated at 37°C with [^{3}H]$E_2$17ßG (400 nM) and [^{3}H]DNP-SG (500 nM) in the medium containing ATP (solid bar) and AMP (open bar) for 5 min. The results are shown as the mean ± SE of triplicate determinations.

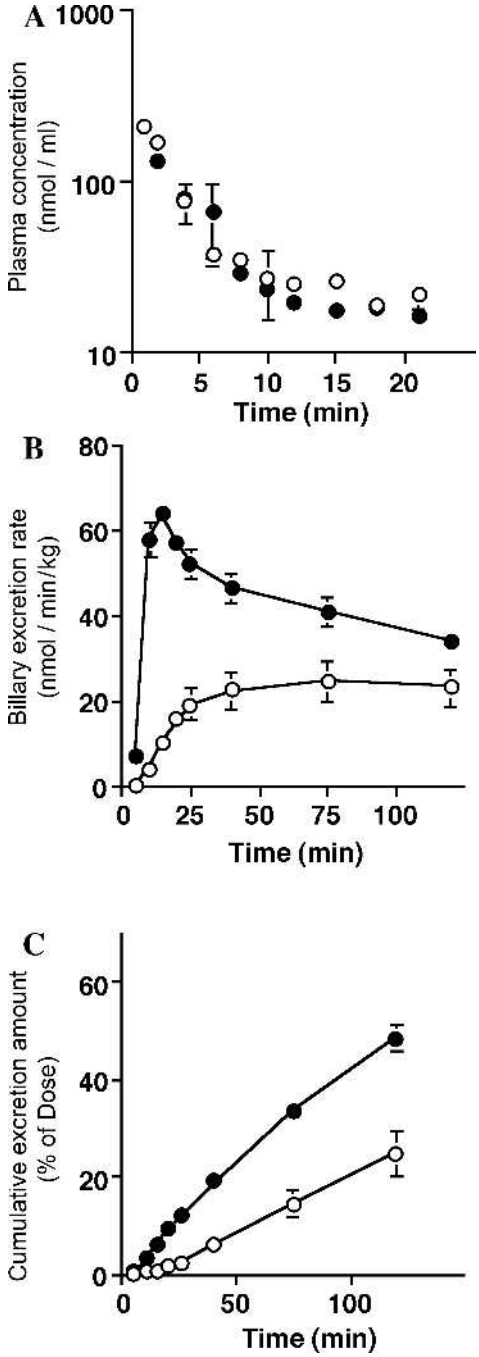

Fig. 4. Biliary excretion of DBSP. DBSP (10 µmol/kg) was administered intravenously as a bolus to MRP2-EHBR (●) and control-EHBR (○). (A) shows the plasma disappearance curves for DBSP. (B) and (C) show the time-course for the biliary excretion rate and that for cumulative biliary excretion, respectively. Each point and bar represents the mean ± SE of 3 rats.

Table I. Bile Flow Rate, Concentration of Bilirubin in Serum, and the Biliary Excretion Clearance of DBSP

	SD rat	MRP2-EHBR	Control-EHBR	EHBR
Bile flow rate ($\mu l\ min^{-1}\ g\ liver^{-1}$)	1.14 ± 0.10	1.08 ± 0.13	0.79 ± 0.10	0.79 ± 0.08
Serum bilirubin (μM)				
Direct	1.0 ± 0.5	7.1 ± 0.9**	97.4 ± 8.2	30.1 ± 4.5
Indirect	1.1 ± 0.5	4.9 ± 2.3	10.8 ± 1.7	6.2 ± 3.2
CL_H ($ml\ min^{-1}\ kg^{-1}$)	32.1 ± 7.8	3.2 ± 0.5*	1.1 ± 0.2	2.4 ± 0.2

The biliary excretion clearance of DBSP are analyzed from the data of Fig. 4.
* $p < 0.05$ vs. control-EHBR; ** $p < 0.01$ vs. control-EHBR by student's t-test.

pressed at the sinusoidal membrane of the liver. Since direct bilirubin can be excreted into blood via Mrp3 (24), the increase in the plasma direct bilirubin levels in control-EHBR may be accounted for by the increase of sinusoidal Mrp3 expression induced by adenovirus infection.

DISCUSSION

The function of Mrp2/MRP2 has been studied extensively by comparing the transport across the bile canalicular membrane between normal and Mrp2-deficient rats (such as Groningen Yellow (GY), transport deficient (TR-), and Eisai hyperbilirubinemic rats (EHBRs)) (16,17,25,26). Furthermore, MRP2 function has been characterized by using the cDNA-transfected cells (1,3,6,22). The canalicular localization of MRP2 (23) has been reflected by the apical localization in polarized mammalian cell lines (22). It is also reported that the pathogenesis of some DJS is accounted for by the lack in the normal apical sorting of MRP2 molecules (10,11). The localization of MRP2 have been characterized by the polarized mammalian cell lines (22), and by the liver sample from DJS patients (10,11). Moreover, we have analyzed the function of MRP2 SNPs variants/mutants using the LLC-PK1 cell line, and found that some variants were localized not only at the apical membrane but also in the intracellular compartment of this polarized cell line (12). Since the SNPs/mutations of MRP2 may have an effect on drug disposition, identification of the *in vivo* localization of MRP2 variants is quite important. In the current study, we have constructed a model for evaluating the function of human MRP2 using EHBR.

We first investigated whether the localization of human MRP2 in liver is preserved in EHBR or not. The human MRP2 was expressed on the canalicular membrane after the adenovirus infection (Fig. 2), which is consistent with the physiologic localization of rat and human MRP2 (23), suggesting that the sorting mechanism of human MRP2 is also present in rat liver. Although we were unable to compare the absolute value of MRP2 protein expression between normal rats (rat MRP2) and MRP2-EHBR (human MRP2), the transport activity of CMVs from normal rats and MRP2-EHBR infected with 7.5×10^9 pfu was almost identical after the normalization of the membrane protein amount of CMVs (Fig. 3), suggesting that the expression level of human MPR2 may be enough for performing the *in vivo* experiments.

From the uptake study with CMVs, we are only able to estimate the drug transport from the liver across the bile canalicular membrane, whereas by performing the *in vivo* study, we are also able to evaluate the vectorical transport of drugs from blood to bile, which is essential for estimating the *in vivo* human biliary excretion. We have previously suggested that the biliary excretion of DBSP is reduced in EHBR compared with that in normal rats *in vivo* (27). Moreover, from the kinetic analysis, the reduction in the biliary excretion of organic anions may be due to a defect in the transport carriers on the bile canalicular membrane (27), suggesting that DBSP is predominantly excreted into bile via MRP2. Therefore, we selected DBSP as a model compound for examining its biliary excretion in a bolus infusion study. After iv injection of DBSP, the plasma concentration was similar between control-EHBR and MRP2-EHBR (Fig. 4A). Because the initial elimination of DBSP from plasma represents the hepatic uptake of this dye (27), this result suggests that the hepatic uptake activity is comparable between control-EHBR and MRP2-EHBR. However, a significant difference in the biliary excretion rate and that in the cumulative excretion were observed (Figs. 4B and 4C), suggesting that human MRP2 introduced by adenovirus recognizes DBSP as a substrate, and excretes DBSP from liver to bile via human MRP2. Moreover, the serum concentration of direct bilirubin was greatly reduced in MRP2-EHBR compared with that in control EHBR (Table I). This result suggests that human MRP2 excretes direct bilirubin into bile, resulting in an improvement of the symptoms of hyperbilirubinemia. However, the biliary excretion clearance in DBSP by MRP2-EHBR was only 3-fold of that of control-EHBR (Table I). It was in contrast to the fact that the biliary excretion clearance of DBSP was 10-fold higher in normal rats than that in EHBR, which is consistent with our previous result (27). Because the result of the *in vitro* transport activity for $E_2$17ßG and DNP-SG indicated that the expression level of human MRP2 is high enough (Fig. 3), we expected that the biliary excretion clearance of DBSP would be comparable between normal rats and MRP2-EHBR. This unexpected finding can be explained by considering the induction of sinusoidal Mrp3 by the adenovirus infection. It is possible that DBSP is excreted into blood via Mrp3, as a similar substrate specificity of Mrp2 and Mrp3 has been reported (28–30). The fact that the serum concentration of direct bilirubin was much greater in control-EHBR than that in untreated EHBR (97.4 mg/ml vs. 30.1 mg/ml) (Table I), may also be explained by Mrp3 induction by infection with the adenoviruses. Alternatively, the transport activity of DBSP by human MRP2 is lower than that by rat Mrp2, resulting the lower biliary excretion clearance of DBSP by MRP2-EHBR compared with normal rats.

In conclusion, we have been able to express human MRP2 at the canalicular membrane in EHBR liver. By comparing the expression level of MRP2 between MRP2-EHBR

and in human liver, this system may be useful in quantatively predicting the *in vivo* human biliary excretion of human MRP2 substrates. Moreover, because the wild-type human MRP2 was expressed at the canalicular membrane of EHBR liver, it may be possible to predict the localization and function of SNPs variants/mutants of human MRP2 in the intact liver.

REFERENCES

1. H. Suzuki and Y. Sugiyama. Transporters for bile acids and organic anions. *Pharm. Biotechnol.* **12**:387–439 (1999).
2. Y. Gotoh, H. Suzuki, S. Kinoshita, T. Hirohashi, Y. Kato, and Y. Sugiyama. Involvement of an organic anion transporter (canalicular multispecific organic anion transporter/multidrug resistance-associated protein 2) in gastrointestinal secretion of glutathione conjugates in rats. *J. Pharmacol. Exp. Ther.* **292**:433–439 (2000).
3. D. Keppler and J. Konig. Hepatic secretion of conjugated drugs and endogenous substances. *Semin. Liver Dis.* **20**:265–272 (2000).
4. H. Suzuki and Y. Sugiyama. Role of metabolic enzymes and efflux transporters in the absorption of drugs from the small intestine. *Eur. J. Pharm. Sci.* **12**:3–12 (2000).
5. C. G. Dietrich, D. R. de Waart, R. Ottenhoff, I. G. Schoots, and R. P. Elferink. Increased bioavailability of the food-derived carcinogen 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine in MRP2-deficient rats. *Mol. Pharmacol.* **59**:974–980 (2001).
6. L. Payen, L. Sparfel, A. Courtois, L. Vernhet, A. Guillouzo, and O. Fardel. The drug efflux pump MRP2: regulation of expression in physiopathological situations and by endogenous and exogenous compounds. *Cell Biol. Toxicol.* **18**:221–233 (2002).
7. H. Suzuki and Y. Sugiyama. Single nucleotide polymorphisms in multidrug resistance associated protein 2 (MRP2/ABCC2): its impact on drug disposition. *Adv. Drug Deliv. Rev.* **54**:1311–1331 (2002).
8. P. Borst and R. O. Elferink. Mammalian ABC transporters in health and disease. *Annu. Rev. Biochem.* **71**:537–592 (2002).
9. R. O. Elferink and A. K. Groen. Genetic defects in hepatobiliary transport. *Biochim. Biophys. Acta* **1586**:129–145 (2002).
10. V. Keitel, J. Kartenbeck, A. T. Nies, H. Spring, M. Brom, and D. Keppler. Impaired protein maturation of the conjugate export pump multidrug resistance protein 2 as a consequence of a deletion mutation in Dubin-Johnson syndrome. *Hepatology* **32**:1317–1328 (2000).
11. K. Hashimoto, T. Uchiumi, T. Konno, T. Ebihara, T. Nakamura, M. Wada, S. Sakisaka, F. Maniwa, T. Amachi, K. Ueda, and M. Kuwano. Trafficking and functional defects by mutations of the ATP-binding domains in MRP2 in patients with Dubin-Johnson syndrome. *Hepatology* **36**:1236–1245 (2002).
12. M. Hirouchi, H. Suzuki, and Y. Sugiyama. Characterization of the cellular localization, expression level, and function of SNP variants of MRP2/ABCC2. *Pharm. Res.* **12**:387–439 (2004).
13. S. Masuda, K. Ibaramoto, A. Takeuchi, H. Saito, Y. Hashimoto, and K. I. Inui. Cloning and functional characterization of a new multispecific organic anion transporter, OAT-K2, in rat kidney. *Mol. Pharmacol.* **55**:743–752 (1999).
14. S. Masuda, H. Saito, and K. I. Inui. Interactions of nonsteroidal anti-inflammatory drugs with rat renal organic anion transporter, OAT-K1. *J. Pharmacol. Exp. Ther.* **283**:1039–1042 (1997).
15. H. H. Gu, J. Ahn, M. J. Caplan, R. D. Blakely, A. I. Levey, and G. Rudnick. Cell-specific sorting of biogenic amine transporters expressed in epithelial cells. *J. Biol. Chem.* **271**:18100–18106 (1996).
16. M. Buchler, J. Konig, M. Brom, J. Kartenbeck, H. Spring, T. Horie, and D. Keppler. cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. *J. Biol. Chem.* **271**:15091–15098 (1996).
17. K. Ito, H. Suzuki, T. Hirohashi, K. Kume, T. Shimizu, and Y. Sugiyama. Molecular cloning of canalicular multispecific organic anion transporter defective in EHBR. *Am. J. Physiol.* **272**: G16–G22 (1997).
18. K. Kobayashi, Y. Sogame, H. Hara, and K. Hayashi. Mechanism of glutathione S-conjugate transport in canalicular and basolateral rat liver plasma membranes. *J. Biol. Chem.* **265**:7737–7741 (1990).
19. H. Mizuguchi and M. A. Kay. Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum. Gene Ther.* **9**:2577–2583 (1998).
20. H. Mizuguchi and M. A. Kay. A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum. Gene Ther.* **10**:2013–2017 (1999).
21. T. Hirohashi, H. Suzuki, X. Y. Chu, I. Tamai, A. Tsuji, and Y. Sugiyama. Function and expression of multidrug resistance-associated protein family in human colon adenocarcinoma cells (Caco-2). *J. Pharmacol. Exp. Ther.* **292**:265–270 (2000).
22. R. Evers, M. Kool, L. van Deemter, H. Janssen, J. Calafat, L. C. Oomen, C. C. Paulusma, R. P. Oude Elferink, F. Baas, A. H. Schinkel, and P. Borst. Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing cMOAT (MRP2) cDNA. *J. Clin. Invest.* **101**:1310–1319 (1998).
23. D. Keppler, J. Konig, and M. Buchler. The canalicular multidrug resistance protein, cMRP/MRP2, a novel conjugate export pump expressed in the apical membrane of hepatocytes. *Adv. Enzyme Regul.* **37**:321–333 (1997).
24. T. Kamisako, Y. Kobayashi, K. Takeuchi, T. Ishihara, K. Higuchi, Y. Tanaka, E. C. Gabazza, and Y. Adachi. Recent advances in bilirubin metabolism research: the molecular mechanism of hepatocyte bilirubin transport and its clinical relevance. *J. Gastroenterol.* **35**:659–664 (2000).
25. R. Mayer, J. Kartenbeck, M. Buchler, G. Jedlitschky, I. Leier, and D. Keppler. Expression of the MRP gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. *J. Cell Biol.* **131**:137–150 (1995).
26. C. C. Paulusma, P. J. Bosma, G. J. Zaman, C. T. Bakker, M. Otter, G. L. Scheffer, R. J. Scheper, P. Borst, and R. P. Oude Elferink. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. *Science* **271**:1126–1128 (1996).
27. K. Sathirakul, H. Suzuki, K. Yasuda, M. Hanano, O. Tagaya, T. Horie, and Y. Sugiyama. Kinetic analysis of hepatobiliary transport of organic anions in Eisai hyperbilirubinemic mutant rats. *J. Pharmacol. Exp. Ther.* **265**:1301–1312 (1993).
28. H. Akita, H. Suzuki, T. Hirohashi, H. Takikawa, and Y. Sugiyama. Transport activity of human MRP3 expressed in Sf9 cells: comparative studies with rat MRP3. *Pharm. Res.* **19**:34–41 (2002).
29. T. Hirohashi, H. Suzuki, H. Takikawa, and Y. Sugiyama. ATP-dependent transport of bile salts by rat multidrug resistance-associated protein 3 (Mrp3). *J. Biol. Chem.* **275**:2905–2910 (2000).
30. J. Konig, A. T. Nies, Y. Cui, I. Leier, and D. Keppler. Conjugate export pumps of the multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-mediated drug resistance. *Biochim. Biophys. Acta* **1461**:377–394 (1999).