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Biochemical Pharmacology 65 (2003) 1843–1852
Biochemical Pharmacology
www.elsevier.com/locate/biochempharm

Human MDR1 polymorphism: G2677T/A and C3435T have no effect on MDR1 transport activities

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Received 9 December 2002; accepted 12 March 2003

Abstract

The two most frequently observed single nucleotide polymorphisms (SNPs) of the human multidrug resistance 1 (*MDR1*) gene are 2677G/T/A (893Ala/Ser/Thr) and 3435C/T (no amino acid substitution). In this study, six forms of MDR1 cDNAs with the SNPs were expressed in LLC-PK1 cells and their transport activities were determined. Nearly identical amounts of the recombinant MDR1 proteins were expressed in the established cell lines using the Flp recombinase, which integrates a gene of interest at a specific genomic location. Four structurally diverse compounds: verapamil, digoxin, vinblastine and cyclosporin A, were examined for transcellular transport activities and intracellular accumulation. No significant differences were observed between cells expressing five polymorphic types of the MDR1 cDNAs (2677G/3435T, 2677A/3435C, 2677A/3435T, 2677T/3435C, 2677T/3435T) and cells expressing the wild-type (2677G/3435C). These results suggested that the two frequently observed MDR1 SNPs had no effect on the transport activities of MDR1 proteins expressed in LLC-PK1 cells *in vitro*, and other genetic or environmental factors might control the expression of MDR1 and the *in vivo* activity of MDR1.

Keywords: MDR1; SNP; Polymorphism; P-Glycoprotein; Transporter; Efflux

1. Introduction

Large interindividual pharmacokinetic differences observed among humans are partially due to genetic polymorphisms in drug-metabolizing enzymes such as cytochrome P450 and several conjugating enzymes [1]. Single nucleotide polymorphisms (SNPs) of these enzymes cause changes in their expressed amounts and activities, which result in alterations in *in vivo* drug disposition and drug effects [2]. In addition to these metabolizing enzymes, uptake and efflux transporters are also involved in the processes of drug absorption, elimination and tissue distribution [3]. Nucleotide substitutions in an efflux transporter multidrug resistance associated protein 2 (MRP2) cause a deficiency of efflux activity in bile canalicular membranes, which is also known as Dubin–Johnson's syndrome [4]. Mutations of other drug transporters may also affect the pharmacokinetics of drugs. While the expressed amounts and the function of some transporters *in vivo* have been characterized to some extent, little is known about the effects of SNPs in transporter genes on their functional characteristics.

P-Glycoprotein, multidrug resistance 1 (MDR1, ABCB1), pumps a large number of structurally diverse drugs out of cells using ATP hydrolysis [5]. MDR1 is expressed in the apical membranes of the intestine, liver and kidney, where drug elimination or absorption occurs [6,7]. In *mdr1a* knockout mice, the concentrations of administered drugs were increased in several tissues, and especially on the brain, indicating that MDR1 determines a large part of the drug disposition in these tissues [8–10]. Moreover, differences in the amounts of MDR1 in the intestine partially account for large interindividual differences in the disposition of the MDR1 substrate,

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Abbreviations: MDR, multidrug resistance; FRT, Flp recombinase target; SNP, single nucleotide polymorphism; PCR, polymerase chain reaction.

0006-2952/03/$ – see front matter
doi:10.1016/S0006-2952(03)00178-3

cyclosporin A [11,12]. Considering that MDR1 plays a key role in drug disposition, SNPs in the *MDR1* gene may affect the pharmacokinetics of MDR1 substrates.

Recently, Hoffmeyer *et al.* reported that a silent polymorphism at nucleotide 3435 in the *MDR1* gene correlated with MDR1 expression in the human duodenum and the plasma concentration of an orally administered digoxin, a typical MDR1 substrate [13]. Several reports demonstrated higher plasma levels of MDR1 substrates in subjects with 3435T than in those with 3435C [13], but other studies contradicted to these results [14,15]. In addition, ethnic differences in the allelic frequencies of 3435 have been observed [16]. The allele frequency of 3435C was over 80% in the African subjects, while the frequency of this allele in the Japanese or Caucasian was approximately 50% [16–18]. However, the molecular mechanism by which the SNP, 3435C/T, alters the expressed amounts of MDR1 has not yet been clarified.

Another SNP with an amino acid substitution found in the human *MDR1* gene is 2677G/T/A (893Ala/Ser/Thr) [18]. It has been suggested that polymorphisms at 2677 and 3435 are strongly associated in the Caucasian and Japanese subjects [14,18]. Remarkable ethnic differences in the polymorphic allelic frequencies of the *MDR1* SNP, 2677G/T/A, have been observed (21.8% 2677A, 41.7% 2677T in the Japanese and 1.9% 2677A, 41.6% 2677T in Caucasians [17,18]). In Japanese subjects, allelic frequencies of 2677G and 2677T are almost identical [18]. The amino acid substitution of Ala to Ser at position 893 of MDR1 have been examined for its transport activities using drug resistance and intracellular accumulation assays [14,19]. Kioka *et al.* found that a slight difference in activity (resistance to a cytotoxic drug adriamycin) might exist between MDR1 cDNAs carrying 2677G (893Ala) and those carrying 2677T (893Ser) [19]. It was also shown that 2677G/T (893Ala/Ser) substitution resulted in a decrease in the intracellular accumulation of digoxin *in vitro* [14]. However, the other study showed that 2677G/T did not alter the intracellular accumulation of several MDR1 substrates in HeLa cells [20]. So far, no evidence has been found suggesting that 2677G/T itself causes any statistically significant changes in the disposition of MDR1 substrates *in vivo* [14]. More recently, Kurata *et al.* demonstrated that the activity of the intestinal and renal MDR1 is higher in subjects with G/G2677C/C3435 than in those with G/T2677C/T3435 or T/T2677T/T3435 [21]. This result indicates that combinations of SNPs at position 2677 and 3435 may have additional effects on MDR1 activities *in vivo*. To elucidate these controversial results, we investigated the transport activities of MDR1 cDNAs with all possible nucleotide combinations. Two SNPs at 2677 and 3435 were also linked to the SNP at 1236C/T in the Asian populations [22]. More recently, it is suggested that haplotypes rather than independent genotypes affect cyclosporin A and digoxin disposition [23,24]. To better understand the mechanism of functional alteration of MDR1 in detail, characterizing the effects of differences in the transport activities of various haplotypes is necessary.

Since conventional methods for establishing transfectants include the random introduction of a cDNA into the genome of a host cell, the position and copy number of cDNA differ among the transfectants. Consequently, the integrated cDNA is expressed at various levels in the cells. To provide a clear indication from *in vitro* experiments, using cell lines with identical amounts of the expressed MDR1 proteins is desirable. To obtain such transfectants, we used the Flp recombinase-mediated integration of MDR1 cDNA at a specific site in the genome of LLC-PK1 [25,26]. This method is supposed to reduce variations in the amount of expressed MDR1 proteins and such a method allows us to compare MDR1 activities accurately. We also selected the epithelial cell line, LLC-PK1, as a host cell line to reflect the physiological situation because MDR1 is expressed in polarized cells *in vivo* [6,7,27].

In this report, we established cell lines in which human MDR1 cDNA carrying six haplotypes (2677A/3435C, 2677A/3435T, 2677G/3435C, 2677G/3435T, 2677T/3435C and 2677T/3435T) were expressed. Transcellular and intracellular transport assays were performed to clarify the effects of the *MDR1* SNPs 2677G/T/A and/or 3435C/T on MDR1-mediated transport activities.

2. Materials and methods

2.1. Materials

[^{3}H]Digoxin (15.8 Ci/mmol), [*N*-methyl-^{3}H]verapamil hydrochloride (85 Ci/mmol) and [carboxyl-^{14}C]inulin-carboxyl (2.4 mCi/g) were obtained from Perkin-Elmer Life Sciences. [^{3}H]Vinblastine sulfate (10.9 Ci/mmol) and [*membt*-β-^{3}H]cyclosporin A (8 Ci/mmol) were obtained from Amersham Biosciences. Unlabeled digoxin, verapamil, vinblastine and cyclosporin A were purchased from Sigma. The Flp-In™ System, Zeocin and hygromycin were obtained from Invitrogen. The porcine renal epithelial cell line, LLC-PK1 (JCRB0060), was purchased from the Health Science Research Resources Bank. Human MDR1 cDNA in pMDRA1 was obtained from the Riken BRC DNA bank (RDB No. 1372 [19]). Restriction enzymes were purchased from New England Biolabs. All cell culture media and reagents were of analytical grade and were available from commercial sources.

2.2. Construction of expression plasmids

The *Pme* I site was introduced just downstream of the termination codon of the MDR1 cDNA in the pMDRA1 [19] by a polymerase chain reaction (PCR) with the 5′-primer, 2729-S, and the 3′-primer containing the site, 5′-AAACGTTTAAACTCACTGGCGCTTTGTTCCAGC-3′

Table 1
Oligonucleotides used for generating MDR1 mutants and sequencing

Oligonucleotides	Oligonucleotide sequence
2677A-S	5′-GAAAGAACTAGAAGGTACTGGGAAGATCGCTAC-3′
2677A-AS	5′-GTAGCGATCTTCCCAGTACCTTCTAGTTCTTTC-3′
2677G-S	5′-GAAAGAACTAGAAGGTGCTGGGAAGATCGCTAC-3′
2677G-AS	5′-GTAGCGATCTTCCCAGCACCTTCTAGTTCTTTC-3′
3435C-S	5′-GTCACAGGAAGAGATCGTGAGGGCAGCAAAG-3′
3435C-AS	5′-CTTTGCTGCCCTCACGATCTCTTCCTGTGAC-3′
1995-S	5′-AAGATCAACTCGTAGGAGTG-3′
2729-S	5′-TGACTCAGGAGCAGAAGTTTG-3′
2888-AS	5′-TCATGAGTTTATGTGCCAC-3′
3278-S	5′-AGTGCTGCTTGATGGCAAAG-3′

(Table 1). The PCR products were digested with *Nde* I and *Pme* I and then subcloned into the pMDRA1 previously digested with the same restriction enzymes. The modified pMDRA1 was digested with *Sac* II and *Pme* I and blunt-ended with T4 DNA polymerase (Takara Bio). The *Sac* II/*Pme* I fragments, including the entire coding sequence of the MDR1 cDNA, were subcloned into pcDNA5/FRT (Invitrogen), which was previously digested by *Pme* I (pMDR1/FRT). The correct sequences of the PCR-amplified fragments and the junction regions have been confirmed by DNA sequencing. Primers used for sequencing are described in Table 1.

2.3. Site-directed mutagenesis

The QuickChange™ Site-Directed Mutagenesis Kit (Stratagene) was used to introduce point mutations (T2677G, T2677A, T3435C) into the MDR1 cDNA in the expression vector according to the instructions. A *Hin*d III fragment from the modified pMDRA1 was subcloned into pBluescript II SK (−) (Stratagene), which was used as a template. Complementary oligonucleotides used for mutagenesis are described in Table 1. The correct generation of the desired mutations and the absence of undesired mutations were confirmed by the sequencing of the cDNA region upstream of the *Hin*d III site to beyond the *Pme* I site. The *Hin*d III/*Pme* I fragments from the obtained plasmids with the correct mutations were then ligated into the *Hin*d III/*Pme* I digested expression plasmid, pMDR1/FRT. All the final sequences and junction regions were confirmed by DNA sequencing.

2.4. Establishment of cell lines

LLC-PK1 cells were cultured in Medium 199 supplemented with 10% fetal bovine serum at 37°, 95% humidity and 5% CO_2. LLC-PK1 cell lines expressing wild and mutant MDR1 were established. To create Flp-In™ host cell lines carrying a single integrated Flp recombinase target (FRT) site, the Flp-In™ target site vector, pFRT/*lac*Zeo, was transfected into LLC-PK1 cells and transformants were selected with 100 mg/mL Zeocin. Subsequently, pMDR1/FRT containing six kinds of MDR1 cDNAs and pOG44 (the Flp recombinase expression plasmid) were co-transfected into Flp-In™ host cell lines harboring an single integrated FRT site which was confirmed by Southern blot analysis (data not shown). Stable cell lines carrying the wild and mutant types of MDR1 cDNAs (Fig. 1) were selected on the basis of hygromycin resistance. The expression of MDR1 proteins was confirmed by immunoblot analysis and the sequences of the integrated MDR1 cDNAs were confirmed by the sequencing of genomic DNA from the transfected cells.

2.5. Immunoblot analysis

Apical membrane fractions were prepared from cultured LLC-PK1 cells as previously described [28]. Proteins were separated by SDS–polyacrylamide gel (7.5%) electrophoresis and transferred onto Immobilon polyvinylidene difluoride membranes (Millipore) using a Transblotter™ (Bio-Rad). The membranes were cut into two pieces at the position where each piece would have a molecular weight of 60 kDa. The membrane containing proteins over 60 kDa was incubated with the anti-MDR1 antibody C219 (ALEXIS Biochemicals) at 1:500 dilution in Tris-buffered saline supplemented with 0.1% Tween 20 (T-TBS) and 3% ECL blocking agent (Amersham Biosciences). The other membrane was incubated with the anti-actin antibody ACTN05 (NEOMARKERS) in T-TBS. The proteins were visualized using ECL Plus (Amersham Biosciences), and the signal density was analyzed by LAS-1000plus and ScienceLab (FUJIFILM). The experiments were repeated five times.

2.6. Transcellular transport study

A transcellular transport study was carried out as previously described [9]. Transfected LLC-PK1 cells were plated at a density of 4×10^5 cells/12-mm well on porous

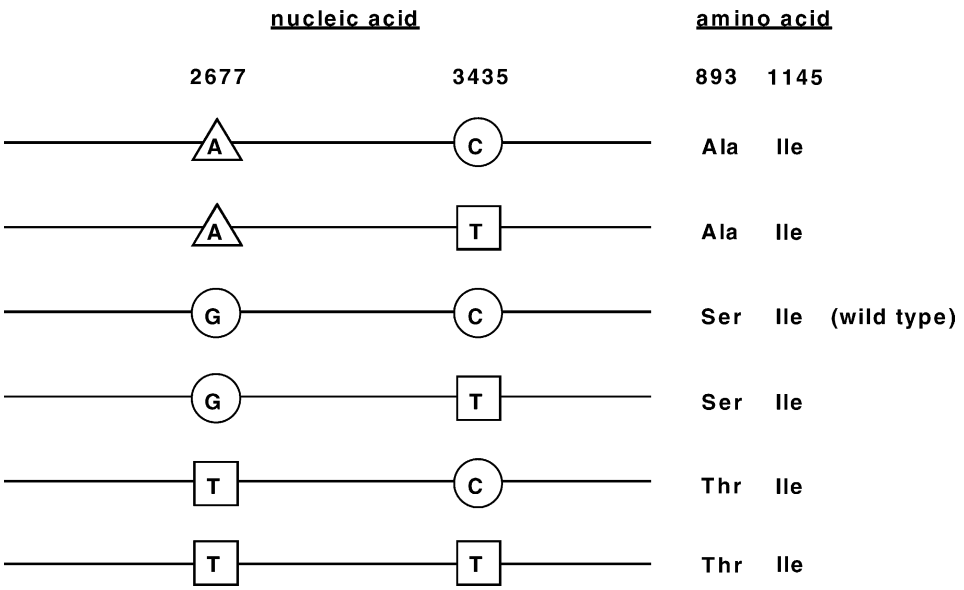

Fig. 1. Schematic diagrams of MDR1 cDNAs. The wild-type MDR1 possessed 2677G and 3435C, which are shown in the circles. Nucleotides different from the wild-type MDR1 cDNA are shown in boxes and triangles.

(3.0 mm) polycarbonate membrane filters (Transwell™; Costar Corp.). Two days after plating, cells were supplemented with fresh medium and cultured for two more days. On the fifth day, the cells were washed by Hanks' balanced salt solution (HBSS) supplemented with 10 mM HEPES (pH 7.4) for 1 hr. This buffer is called 'transport medium'. At the start time of the incubation (time 0), each compartment (apical and basal) was replaced with the fresh transport medium with or without radiolabeled substrates. At the appropriate time, aliquots were taken from the one compartment to which fresh transport medium had been added at time 0 and these aliquots were replaced with an equal volume of the fresh transport medium. Radioactivity of the samples was analyzed by a liquid scintillation counter. The amount of transported ligands was presented as a percentage of the radioactivity in the opposite compartment to the total radioactivity added at the beginning of the experiment. The paracellular efflux monitored by the transcellular transport of [^{14}C]inulin in both directions was less than 1% in 3 hr. The experiments were repeated twice for cyclosporin A and three times for the other compounds.

2.7. Intracellular accumulation assay

Intracellular accumulation experiments were carried out as previously described with a slight modification [9]. Transfected LLC-PK1 cells were plated at a density of 4×10^5 cells/24-well plates and cultured for 4 days. On the fourth day, cells were preincubated with the transport medium for 1 hr and then incubated with labeled substrates for a desired period at 37°. After being washed three times with ice-cold phosphate-buffered saline, the cells were dissolved with 0.2 M sodium hydroxide and neutralized by hydrochloric acid. Radioactivity of the samples was analyzed by a liquid scintillation counter. Protein content of the samples was determined by a BCA assay (Pierce). The amounts of ligands in the cells were normalized by the amounts of protein. The experiments were repeated twice for cyclosporin A and three times for the others.

2.8. Statistical analysis

All data are presented as the mean ± SD of the three independent cell lines with the same haplotype (N = 3). MDR1 cDNA with 2677G/3435C (GC) was used as the wild type throughout this study, as given in the study by Chen *et al.* [29]. Differences between the parameters of the wild-type MDR1 and those of the other types of MDR1 were analyzed by an unpaired Student's *t*-test (N = 3).

3. Results

3.1. Expression of MDR1 in established cell lines

The expression of recombinant human MDR1 proteins in the established cell lines was confirmed by the quantitative immunoblot analysis with the anti-MDR1 antibody, C219. A single band was detected at the expected position (apparent molecular weight of ~170 kDa, Fig. 2A) in each MDR1-transfected cell line but the signals were barely detected in the vector-transfected cell lines. To compare the expressed amounts of the wild and mutant type MDR1 protein, the band intensities of MDR1 and actin in the same sample were quantified by densitometric readings (Fig. 2B). The expressed amount of MDR1 protein was determined by the ratio of the amount of MDR1 relative to the amount of actin. No statistically significant differences were observed in the amount of the recombinant MDR1 proteins among

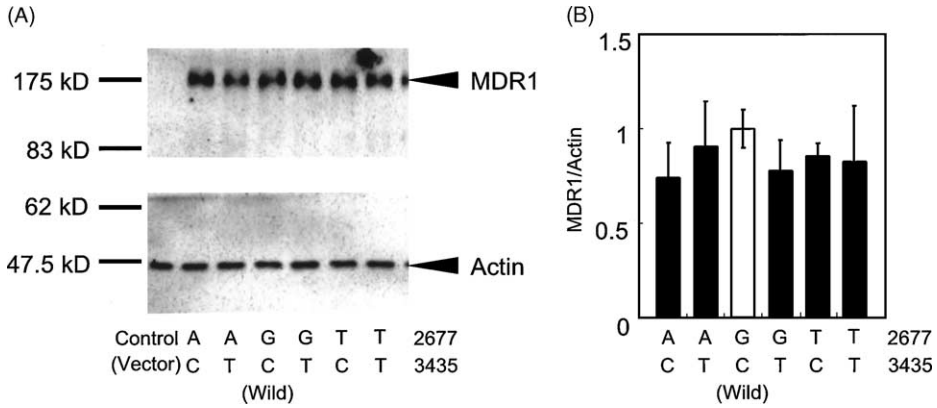

Fig. 2. Immunoblot analysis of the recombinant MDR1 proteins. (A) Typical image of immunoblotting (1 μg protein/lane). Position and size of the marker proteins are presented on the left. The nucleotides corresponding to the SNPs introduced to the cell lines are presented below the image. (B) Expressed amounts of MDR1 proteins quantified from the band intensity of the immunoblot analysis. The open column represents the contents of the wild-type MDR1 (GC) protein expressed in cells. The amounts of MDR1 proteins in cells with the mutant type MDR1 are shown as the ratios relative to those of the wild type (closed columns). Data are the mean ± SD of three independent cell lines.

the cells with the five mutant types (AC, AT, GT, TC, TT) and the wild-type MDR1 (GC) ($P > 0.05$).

3.2. Transcellular transport activities of expressed MDR1

To ascertain the transport activities of the expressed wild-type MDR1, the transcellular transport assays of verapamil, digoxin, vinblastine and cyclosporin A were determined (Fig. 3). Transcellularly detected radioactivities of the four compounds in both directions increased proportionally up to 2 or 3 hr (Fig. 3). Transported amounts of the four drugs from the basal to apical direction in the wild-type MDR1-expressed cell lines were higher than those in the control vector-transfected cells (Fig. 3), indicating that the efflux activity of the recombinant MDR1 was appropriately observed in the established cell lines. The transport activity

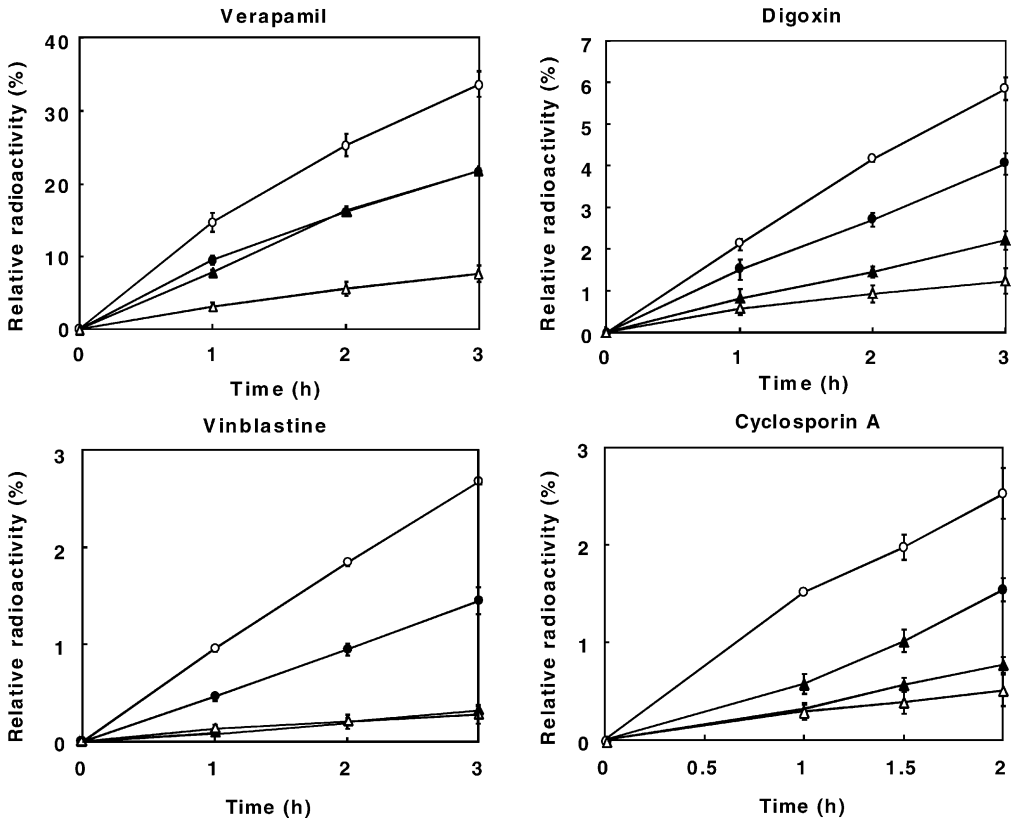

Fig. 3. Time course of transcellular transport activities of verapamil (1 μM, 0.05 μCi/mL), digoxin (1 μM, 0.15 μCi/mL), vinblastine (1 μM, 1 μCi/mL) and cyclosporin A (1 μM, 0.1 μCi/mL) in the wild-type MDR1-transfected LLC-PK1 (○) and the vector-transfected LLC-PK1 (△). Circles (●) represent the radioactivities detected in the apical side from the basal side relative to the initial radioactivity added. Triangles (▲) represent the opposites. Each symbol represents the mean ± SD (N = 3).

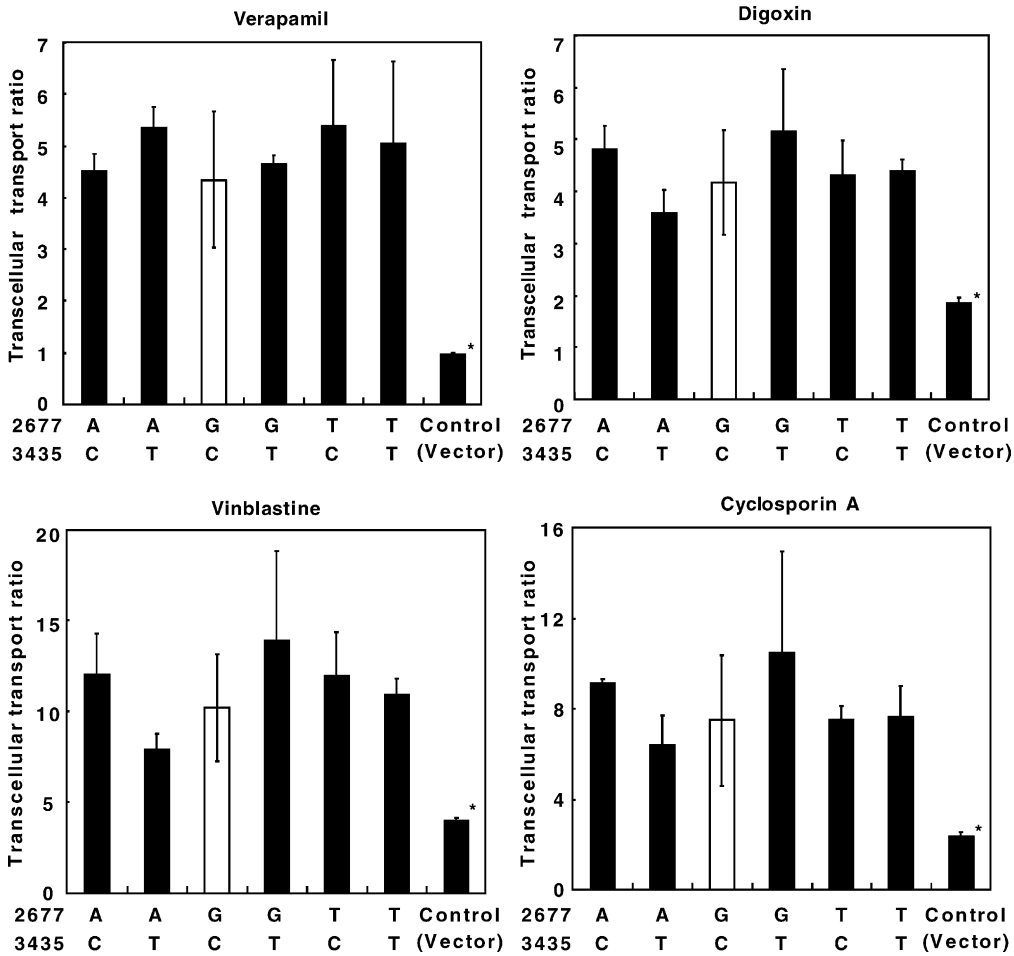

Fig. 4. Transcellular transport ratios of verapamil, digoxin, vinblastine and cyclosporin A. The columns represent the ratios of radioactivities detected in the apical side to those in the basal side at 2 hr, except for the radioactivity of cyclosporin A, which was detected at 1 hr. Open and closed columns represent the transport ratios of the wild and the mutant types of MDR1 cDNA or the control vector-introduced cell lines. Each column represents the mean ± SD (N = 3). $^{*}P < 0.05$.

of the recombinant MDR1 was presented as a transcellular transport ratio (a transported amount from basal to apical: a transported amount from apical to basal at a time point within the linear range). The transcellular transport ratios of the four drugs tested in the wild-type MDR1-expressed cell lines were significantly higher than those in the vector-transfected cell lines (Fig. 4). The same assays were also performed in the cell lines expressing the other five mutant types of MDR1 proteins. Although the transcellular transport ratios for the four compounds across cell monolayers expressing the six types of MDR1 were significantly higher compared with the vector-transfected cells (Fig. 4) ($P < 0.05$), there was no significant difference between transcellular transport ratios of the mutant and the wild-type MDR1-expressed cells (Fig. 4).

3.3. Efflux activities of recombinant MDR1

To confirm the result of transcellular transport assay, another transport assay was examined. As shown in Fig. 5, the intracellularly accumulated amounts of verapamil, digoxin, vinblastine and cyclosporin A in LLC-PK1 cells transfected with the MDR1 cDNAs containing 2677A/3435C, 2677A/3435T, 2677G/3435C, 2677G/3435T, 2677T/3435C and 2677T/3435T were significantly lower than those in the vector-transfected cells ($P < 0.05$). No significant difference in the intracellularly accumulated amounts of the four compounds was observed between the wild-type MDR1-expressed cells and the other five mutant types of MDR1-expressed cells ($P > 0.05$).

4. Discussion

The relationship between MDR1 polymorphisms and the *in vivo* pharmacokinetics of MDR1 substrates has been widely investigated [13,14,30,31]. Several results from *in vivo* and *in vitro* studies have already been reported. However, how MDR1 SNPs alter the expressed amount of MDR1 protein and MDR1 transport activity remains unclear. In this study, we chose a new approach using Flp recombinase-mediated integration to obtain cell lines with identical amounts of the expressed MDR1 proteins. This approach allowed us to compare transport activities more

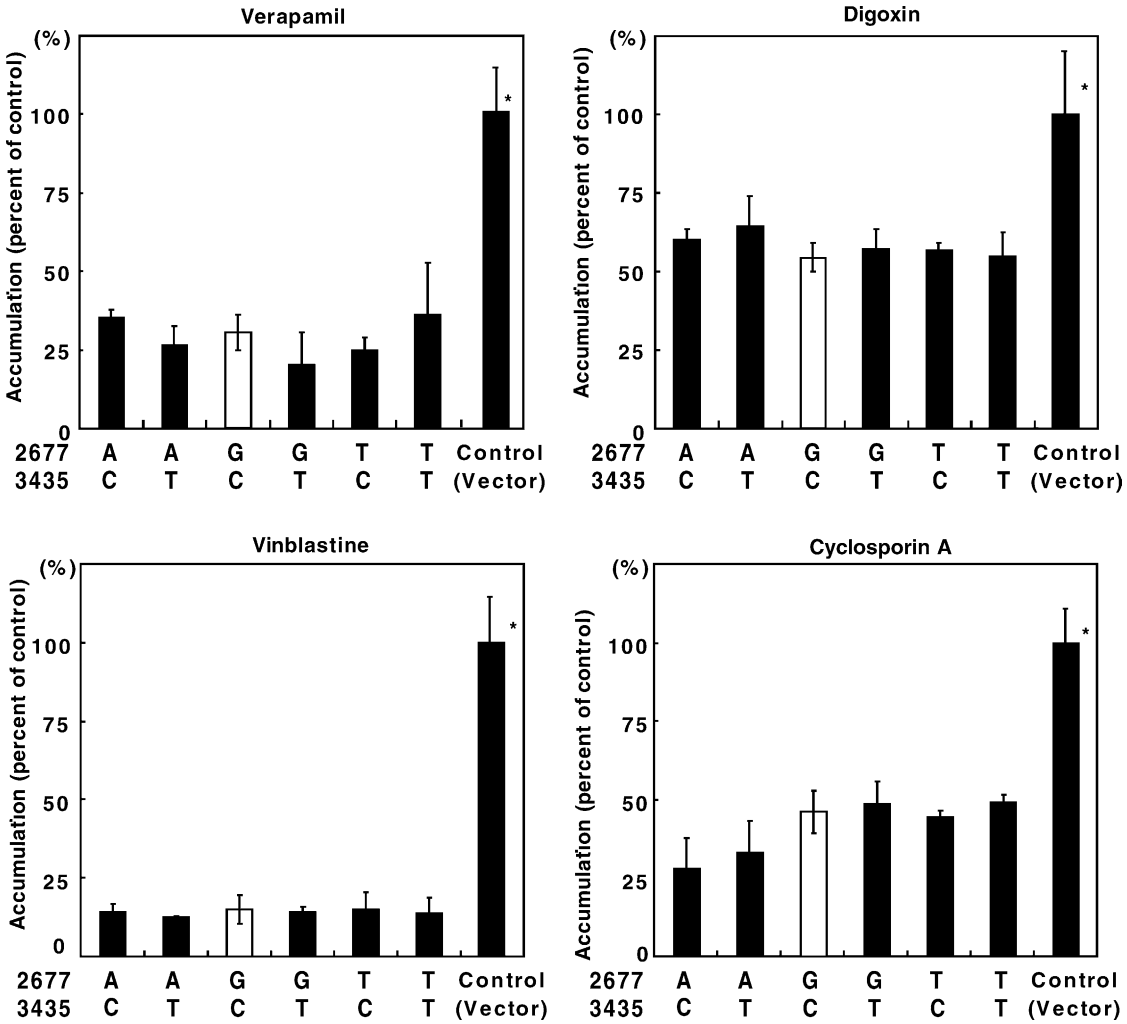

Fig. 5. The intracellular accumulation of verapamil (0.1 μM, 0.1 μCi/mL), digoxin (0.1 μM, 1 μCi/mL), vinblastine (0.1 μM, 1 μCi/mL) and cyclosporin A (0.1 μM, 1 μCi/mL). The intracellular contents of the compounds are shown in the radioactivity in the cells relative to that in the vector-transfected cells after 1 hr (verapamil, vinblastine and cyclosporin A) or 2 hr (digoxin) of incubation. Open and closed columns represent the percentages of the accumulated amounts in the cell lines with the wild and the mutant types of MDR1 cDNA relative to the mean of the accumulated amounts in the vector-transfected cell lines. Each column represents the mean ± SD (N = 3). $^{*}P < 0.05$.

accurately (Fig. 2). We conclude that the transport activities of four structurally diverse drugs in epithelial cells, where MDR1 proteins are expressed *in vivo*, were not changed by two *MDR1* SNPs (2677G/T/A and 3435C/T) (Figs. 4 and 5).

Several SNPs have been detected in the human *MDR1* gene [13,18]. In this study, we focused on the two SNPs at positions 2677 and 3435, which are most frequently observed in the Japanese (2677T; 41.7%, 2677A; 21.8% and 3435T; 49.0%) [18]. Although the allelic frequencies of 2677G/T/A and 3435C/T show the ethnic differences [16–18], this study demonstrated no alterations in MDR1 activity by the SNPs. Therefore, results of this study will help to bridge the pharmacokinetics data between the different ethnic groups. Furthermore, it was necessary to analyze the effect of the SNPs on the transport activities of MDR1 using haplotypes as a combination of 2677G/T/A and 3435C/T because the nucleotides at these positions are strongly associated [14,18]. We used the MDR1 cDNA with 2677G/3435C as the wild type (Fig. 1) [29], and we compared the characteristics of the other polymorphic proteins with the wild-type proteins.

Kim *et al.* reported lower intracellular accumulation of digoxin in cells with 2677T than in those with 2677G [14]. However, another study by Kimchi-Sarfaty *et al.* regarding 2677G/T in the vaccinia virus-mediated transient expression system supported our results and showed that the SNP, 2677G/T, had no effect on MDR1 activities [20]. Difference among cell lines (NIH3T3, HeLa and LLC-PK1) and assay procedures might have caused this discrepancy. We can exclude the possibility that the disparity in the test compounds led to the conflicts, since we used both digoxin and verapamil, which have been also investigated in the two other studies [17,20]. We used the four structurally unrelated substrates: verapamil (Ca^{+}-channel inhibitor), digoxin (cardiovascular drug), vinblastine (anticancer drug) and cyclosporin A (immunodepressant drug) to cover a wide range of molecular weight and hydrophobicity.

The quantification of the amount of expressed proteins might account for this discrepancy. Tanaka *et al.* reported that the degree of resistance to the cytotoxic agents and the transport activity of MDR1 were dependent on the expression levels of the MDR1 protein [32]. The large variability in the expressed amount of recombinant proteins among transfectants used to be a critical problem when the transport activities of the MDR1 proteins produced from the introduced cDNA were compared using the conventional method. In fact, the expressed amounts of MDR1 proteins differed greatly among cell lines when we established LLC-PK1 cell lines with the wild and the mutant MDR1 cDNA by a conventional method (data not shown). To establish the stable cell lines with the identical amounts of MDR1 proteins, we chose the Flp recombinase-mediated integration of interested cDNAs into the same genomic location [25,26]. Using this system, we were able to establish the three LLC-PK1 cell lines with nearly identical amounts of the expressed MDR1 proteins for all haplotypes of MDR1 cDNAs (Fig. 2). Using these transfectants, we directly compared the transport activities of the wild MDR1 proteins with those of the mutants. Control vector-introduced cell lines expressed a marginal amounts of porcine P-gp (Fig. 2) which is consistent with previous observations showing no transport activities in parental cell lines [9,27,33–35].

MDR1 protein is localized at the apical membrane of LLC-PK1 cells [33] as well as in the intestine, liver and kidney [6,7]. Membrane fractions used in the immunoblot analysis of this study were rich in apical membranes because the basolateral marker sodium–potassium ATPase (Na^+–K^+ ATPase) was not detected in all the membrane fractions used (data not shown) [36]. In the immunoblot analysis by the anti-MDR1 antibody, the amount of the recombinant MDR1 proteins in the apical membranes in cells was expected to be almost identical among all haplotypes of the MDR1 cDNAs (Fig. 2). Since the MDR1 proteins were expressed in the apical membrane-enriched fractions, it is likely that the MDR1 proteins were localized in the apical membrane of LLC-PK1 cells as shown in the previous and present studies [33]. Considering that the expressed amounts and activities of the MDR1 proteins were almost identical in the apical membranes of LLC-PK1 cells, the intrinsic activities of the wild and mutant MDR1 proteins are expected to be identical in the established epithelial cells. This identicalness could eliminate the possibility that the SNPs, 2677G/T/A and 3435C/T, affect the transport activity itself. By using the FRT system, we could not detect any differences in the expressed amounts of MDR1 proteins showing that a single nucleotide substitution at 3435 has no effect on the expression of MDR1 (Fig. 2). However, the results did not yet clarify the mechanism of the reduced expression of MDR1 by the SNP. Other factors responsible for the reduced expression of MDR1 in some subjects *in vivo* remains to be investigated [13,15].

A loss of or reduction in the transport activities of other transporters with SNPs has previously been reported [4,37,38]. A deletion mutation in the human *MRP2* gene results in the mis-sorting of proteins, which can lead to the reduction of efflux activities [39]. Uptake transporters, organic anion transporting polypeptides-C (OATP-C) and organic cation transporter 2 (OCT2) also possess naturally occurring SNPs that can change their localization or decrease their activity [37,38]. In LLC-PK1 cells, 2677G/T/A and 3435C/T are not expected to change the sorting of MDR1 proteins, because the expressed amount of MDR1 in the apical membranes is equivalent among the cells carrying six types of cDNAs (Fig. 2).

Contradictory results have also been reported for the relationship between the nucleotide at 3435 and MDR1 amounts or the pharmacokinetics of MDR1 substrates [13–15]. Our results showed that nucleotides at 2677 and 3435 do not change the transport activities of MDR1 *in vitro*; this may also be the case for the *in vivo* situation. The discrepancies might be due to the existence of other SNPs that are associated with 3435C/T and are responsible for the transcription or translation of MDR1. MDR1 is induced by many agents [40,41], therefore, the differences in food intake or environmental conditions make it difficult to quantify the expressed MDR1 amount. In addition, other SNPs in genes that regulate MDR1 expression might also be linked to the 3435C/T SNP.

In conclusion, we demonstrated that the SNPs at 2677G/T/A and 3435C/T do not alter the transport activities of the MDR1 proteins in epithelial cells *in vitro*. Our *in vitro* finding suggests that the naturally occurring MDR1 SNPs 2677G/T/A and 3435C/T do not affect the MDR1 activity *in vivo*. It would be necessary to investigate the expression and activities of the MDR1 and its related proteins both *in vivo* and *in vitro* to obtain a better understanding of what causes the large differences in the disposition of MDR1 substrates in humans.

Acknowledgments

We are indebted to Dr. Kazumitsu Ueda (Kyoto University) for providing us with human MDR1 cDNA.

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